THE INFLUENCE OF MATERNAL NICOTINE EXPOSURE ON NEONATAL LUNG DEVELOPMENT: AN ENZYMATIC AND METABOLIC STUDY

by

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In memory of "Goffer"

DECLARATION

I, the undersigned, declare that ***The influence of maternal nicotine exposure on neonatal lung development: An enzymatic and metabolic study*** is my own work and has not previously in its entirety, or in part, been submitted at any university. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

C. Kordom **Date**

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ABSTRACT

Chapter I, the literature review, gives the reader a sound background knowledge of the following: normal lung structural development in humans and rats, metabolic development with reference to structural development, tobacco smoke and nicotine and the effect of nicotine and smoking on metabolism. This will enable the reader to understand the motivation for this study and to give insight to the following chapters.

Chapter II comprises the influence of maternal nicotine exposure on the enzymes, hexokinase and phosphofructokinase. The aim of this study was to determine the influence of maternal nicotine exposure during pregnancy and lactation on the enzymes involved in the control of energy metabolism of developing lungs of rats at postnatal days 1, 7, 14, 21 and 49. The objectives were to determine the effect of maternal nicotine exposure during gestation and lactation on, (a) the activity of selected allosteric enzymes, hexokinase (HK) and phosphofructokinase (PFK), (b) the influence of various ATP concentrations and ATP/ADP ratios within the reaction medium on the activity of these enzymes and (c) to express the activity of these enzymes as a function of age and (d) to correlate the findings with the stage of neonatal lung structural development as explained in the literature. Female Wistar rats were used in this study. The pregnant rats were randomly assigned as a control and an experimental (nicotine) group. The experimental dams received a single dosage of 1mg nicotine/kg body weight/day subcutaneously and the controls received the same volume of saline. The enzymatic activity was determine by means of spectrophotometery. It was found that HK and PFK activity are age dependent. The structural development of the neonatal lung correlates

with the activities of HK and PFK for both control and nicotine exposed animals. Thus exposure of the rats to nicotine via the placenta and mothers milk had no effect on the developmental pattern of these enzymes. The variation in ATP concentrations and ATP/ADP ratios did influence the activity of the HK and PFK by either increasing or decreasing the activity of these enzymes. Due to the fact that most of the alveoli are being formed between postnatal days 4 and 13, it was interesting to find that nicotine increased HK activity between postnatal days 7 and 14 whereas it suppressed PFK activity between postnatal days 7 and 14, thereby suppressing the flux of glucose through the glycolytic pathway. Even though nicotine exposure via the placenta and mothers milk had no effect on the enzyme's developmental pattern, it had an adverse effect on the glycolytic pathway. Inhibition of the PFK activity contributed to the inhibition of glycolysis and therefore could lead to the slower formation of alveoli in lungs of neonates who were exposed to maternal nicotine.

Chapter III entails the influence of maternal nicotine exposure on the adenine nucleotides (ATP, ADP and AMP) and adenosine. The aim of this study was to determine the influence of maternal nicotine exposure during pregnancy and lactation on the adenine nucleotide metabolism of developing neonatal lungs of rats at postnatal days 1, 7, 14, 21 and 49. The objectives were to determine, (a) the effect of age on the nucleotide pool and adenosine of the neonatal lung and the impact of maternal nicotine exposure, (b) to determine the effect of age on the adenine nucleotide pool and adenosine, (c) to propose a mechanism whereby nicotine induce an increase in lung ATP content and (d) to determine whether the increased ATP/ADP ratio indeed result in inhibition of glycolysis. In order to ensure that all the experimental conditions were

the same, the sample selection and management was exactly as described in chapter II. The determination of the nucleotides and adenosine was done by means of high performance liquid chromatography (HPLC). It was found that the nucleotide pool (ATP, ADP and AMP) and adenosine were age dependent. However, maternal nicotine exposure interfered with the developmental pattern of these nucleotides and adenosine. Results showed the energy stores to be age dependant and this corresponds with the pattern of lung development. However, maternal nicotine exposure influenced the energy stores of the lungs, by suppressing the energy stores during lung development especially the phase of alveolarization (tissue proliferation). This could therefore relate to the adverse effects of maternal nicotine exposure on the inhibition of glycolysis and the decrease in alveoli formation in the offsprings of those animals exposed to maternal nicotine.

CHAPTER I

Literature Review

CHAPTER I

Literature Review

1.1 Introduction

Before the onset of normal respiratory function in the newborn, several maturational events must take place (Thibeault and Gregory, 1986). During the last part of gestation, the fetal lung undergoes marked changes that include the processes of growth, epithelial cellular differentiation, and morphological and biochemical maturation, including formation and release of pulmonary surfactant (Joe *et al*, 1997).

The lung is more than just an organ for passive gas exchange. Lung tissue is composed of metabolically active cells that require energy for their synthetic and other life-sustaining reactions (Fisher *et al*, 1974). Carbohydrates play an important role in the structural (Tierney and Levy, 1976) and functional development and maturation of fetal and neonatal lung tissue (Bourbon and Jost, 1982; Gilden *et al*, 1977; Maniscalco *et al*, 1978). Adenosine triphosphate (ATP) plays a key role in cell growth and development (Maritz and Najaar, 1995). Like most other organs, the lung produces energy in the form of ATP via glycolysis and further oxidation of substrates through the tricarboxylic acid cycle and the electron transport chain (Sayeed and Murthy, 1981). However, the transformation of energy is carried out by enzyme molecules that are integral parts of highly organized assemblies (Stryer, 1975). The ability of the tissues to utilize glucose and generate the stored energy depends on the availability of the enzymes involved in the biochemical pathways (Bhavnani and Wallace, 1990). Any alterations or absence

of a specific enzyme could therefore, result either in death or a serious disease (Dressler and Potter, 1991).

Interference with energy metabolism may therefore also interfere with lung growth and maturation. Environmental factors during pregnancy can modify the process of lung development, thereby leading to defective and insufficient functionality, thus decreasing its resistance to disease. Cigarette smoking by the pregnant mother, and fetal exposure to the cigarette smoke of others, are environmental factors that might affect lung maturation (Lieberman *et al*, 1992).

Since 1957, evidence has been accumulating that has established that maternal smoking in pregnancy adversely affects the growth of the fetus and it is associated with increased risk of miscarriages, premature birth, perinatal death, and low birth weight (Charlton, 1994; Moessinger, 1989). Smoking during pregnancy appears to increase the risk of a child to develop respiratory disease (Stein *et al*, 1999; Young, 1992) which reduce the respiratory function (Stick *et al*, 1996). Furthermore, children of smokers are twice as likely to suffer from serious respiratory infections and asthma. Active smoking has even more serious consequences such as laying the foundation for susceptibility to lung cancer, cardiovascular disease, respiratory disease, infertility (in women), and even human immunodeficiency virus-1 infection through decreased immune function (Young, 1992).

Since this project deals with the effect of maternal nicotine exposure on the metabolism of the developing lung in rats, a sound knowledge is required of the following: normal

lung structural development in humans and rats, and metabolic development with reference to structural development, tobacco smoke and nicotine. The effect of nicotine and smoking on metabolism will also be discussed briefly in this chapter.

1.2 Normal Lung Development

In many mammalian species, including humans, the formation of pulmonary alveoli occurs to a substantial extent after birth (Massaro *et al*, 1985).

1.2.1 Normal Human Lung Development

In the human, there are five well-recognized stages of intrauterine lung development: embryonal, pseudoglandular, canalicular, saccular, and alveolar (Thurlbeck, 1992).

The *embryonic period* covers the first 5 to 6 weeks of embryonic life, including the early phases of creation and development of the lung (Slonim and Hamilton, 1981). First, there is a ventral out pouching from the laryngotracheal groove at 28 days gestation. Two bronchial buds appear almost immediately, and these form the right and left mainstem bronchi. These bronchi then subdivide by asymmetric dichotomy to form the segmental bronchi (Thurlbeck, 1992), with ten principal branches of the bronchial tree developing on the left and eight on the right (Slonim and Hamilton, 1981). At the end of the embryonal phase, the major airways are completely developed (Thurlbeck, 1992).

The *pseudoglandular period* lasts until the 15th or 16th week of development and includes the period of bronchial development (Slonim and Hamilton, 1981). During

this stage, there is an extensive subdivision of the airway system as well as the development of all the conducting airways (Bucher and Reid, 1961). The potential gas-exchanging part of the lung (the acinus, or part of the lung distal to the terminal bronchiole) may also be developed completely. This phase has been termed pseudoglandular because random histologic sections show multiple, round structures resembling glands that are separated from each other by mesenchyme and its derivatives. The cells lining the spaces are columnar and contain glycogen (Thurlbeck, 1992).

The *canalicular period* lasts from the 16th to the 24th or 26th week (Slonim and Hamilton, 1981). During this time, further growth and subdivision of the distal airways probably occurs, and development and maturation of the acinar epithelium becomes apparent (Thurlbeck, 1992). There is a relative decrease in the amount of connective tissue and the lung becomes lobular and more vascular. The epithelium that lines the airways flattens and thins. Toward the end of this period type I and type II alveolar lining epithelia appear and capillaries protrude into the epithelium, sometimes forming potential areas of thin capillary-airway interface. As this process continues, there is proliferation of areas that could maintain gas-exchange if required (Slonim and Hamilton, 1981).

The *terminal sac* or *saccular period* lasts until the 32nd week (Thurlbeck, 1992). During this period dramatic changes takes place in the gas-exchange part of the lung. At the beginning of this phase, the terminal structures are termed saccules and are relatively smooth-walled cylindrical structures. They are then subdivided by ridges

known as secondary crests. As the crests protrude into the saccules, part of the capillary net is drawn in with them, forming a double capillary layer (Thurlbeck, 1992). Further subdivision between the crests or out pouchings result in smaller spaces, which have been termed "subsaccules". Whether these structures can be called alveoli is still controversial. In this period, the extracellular matrix molecules continue to regulate pulmonary branching, morphogenesis, cytodifferentiation and alveolarization (Merkus *et al*, 1996). It is also during this phase that the alveolar type II cell, which is the source of pulmonary surfactant production, differentiates. Topographically, this pattern progresses in an ordered sequence beginning at the hilus and extending outward, in the direction of cellular growth toward the periphery in what has been termed centrifugal development (Torday, 1992). Alveoli can be seen as early as 32 weeks and are always present at 36 weeks (Merkus *et al*, 1996).

The *final* or *alveolar period* lasts from 32 weeks to term (Thurlbeck, 1992). During this phase, immature alveoli with a double capillary network forms (Dornan and Meban, 1985). About two days before birth, the rate of tracheal fluid production progressively decreases and apparently ceases by the time of birth (Kitterman, 1984). At birth up to 65% of the alveoli are formed (Dornan and Meban, 1985).

Investigations of the lungs of various mammals have since brought growing evidence for the general concept that the development of the mammalian lung is not yet completed at birth and that new alveoli are formed postnatally (Burri, 1974). After birth, each of the structural components, airways, alveoli and blood vessels has different growth patterns with respect to increases in cell number as well as size (Merkus *et al*,

1996). In man, approximately 15 to 25 million alveoli are present at birth. Most alveoli develop during the early months after birth. Regardless of the exact time of their appearance, alveoli continue to develop during infancy and childhood. The postnatal growth of the lung has been a matter of controversy, since some researchers believe that the adult number of alveoli (about 300 million) are present by the end of the first year of life, whereas others believe that alveolar multiplication continues at a gradually decreasing rate until somatic growth stops. The most common estimate is that alveolar multiplication ceases at about 8 years of age, after which the number of alveoli remains constant for many years (Slonim and Hamilton, 1981).

Figures 1.1 and 1.2 show the alveoli which are the functional units of the lungs (Jacob and Francone, 1970). Gaseous exchange between blood and air occurs only in the alveoli (Jacob and Francone, 1970).

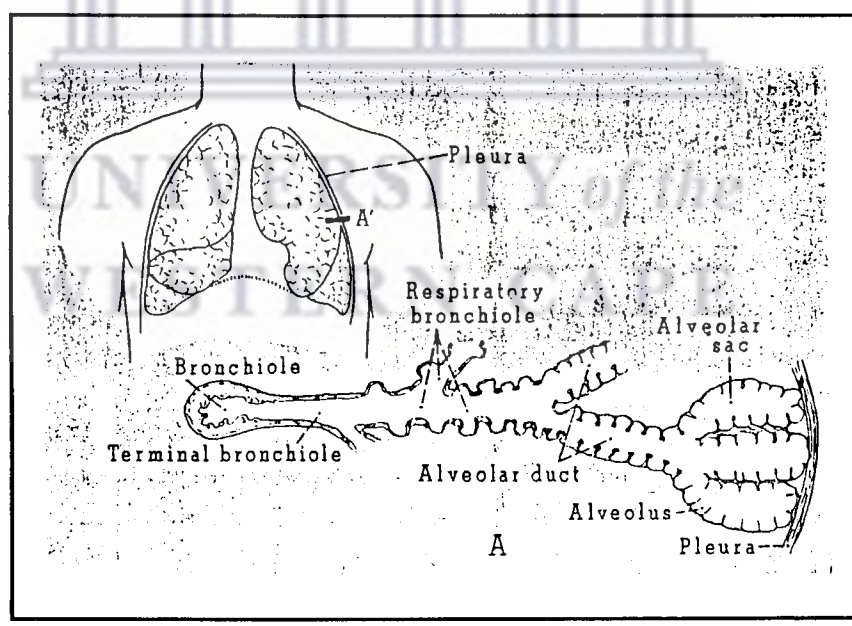

Figure 1.1: Branching of the bronchiole into the functional unit, the alveolus (Jacob and Francone, 1970).

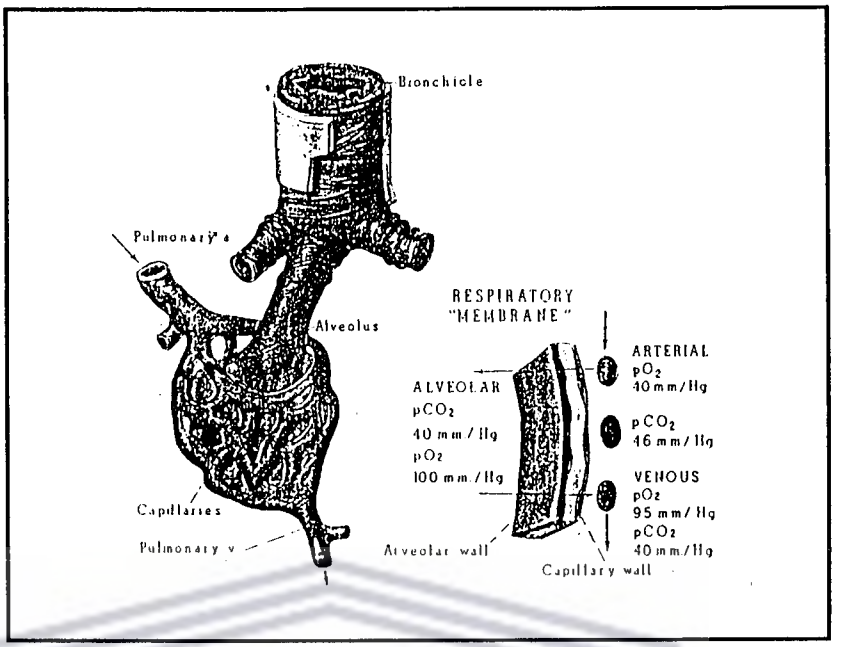

Figure 1.2: The basic microscopic functional unit of the lung (Jacob and Francone, 1970).

1.2.2 Normal Rat Lung Development

Since this project deals with postnatal lung development and the rat was used as an animal model, prenatal lung development of the rat will be discussed briefly. The phases of lung development are the same as for humans.

The growth and the development of the fetal lung of rat occur over a much shorter time than that of humans, due to a gestation period of only 22 days. Therefore, profound changes could occur in its morphology over periods as short as 24 hours. Prenatal lung development can be divided into three stages: the pseudoglandular stage (0-18 days), the canalicular stage (19-20 days), and the saccular stage (day 21- term) (Meyrick and Reid, 1977). At birth, the lungs of rats have no alveoli (Engel, 1953) and gas exchange

occurs in smooth walled saccules (alveolar sacs), the prospective alveoli (Burri, 1974).

Throughout postnatal lung development, the lung increases steadily in size and weight. This is due to the fact that the newborn rat lung is not simply the adult lung in miniature, its growth also includes changes in structure with shifts in balance between tissue components that remodel the lung to the adult architecture (Meyrick and Reid, 1982). Burri and colleagues (1974), divided the postnatal development of the alveolar region in rats into three phases: lung expansion (1 to 4 days), tissue proliferation (4 to 13 days), and equilibrated growth from postnatal day 13.

a) Phase of lung expansion: The lung undergoes an expansion of its air spaces (Burri *et al*, 1974) and an enlargement of air space volume by 87% and only moderate tissue volume increase (Kauffman *et al*, 1974). At birth, the conducting airways occupy a relatively large portion of the lung volume when compared to the adult lung. The main stems have a large calibre and extend far to the periphery of the lung. Peripherally the epithelium of the airways flatten, forming straight, smooth walled channels. These channels divide further and then terminate into small air chambers, which are called "saccules" rather than alveoli. The septa of a newborn lung are rather thick and in contrast to mature septa, they show little folding or branching, and mostly possess capillaries on either side. In lungs of 4 day old rats, air space seem to be slightly more irregular in shape with short ridges appearing on the surface of certain septa (Burri, 1974).

b) Phase of tissue proliferation: This phase is characterised by a marked increase in

tissue mass and surface area (Burri *et al*, 1974). This period entails intense cell production and outgrowth of secondary alveolar septa characterized by differential cell proliferation on septal buds and concomitant increase in alveolar and capillary surface areas (Kauffman *et al*, 1974). This period could therefore be seen as the period where most of the alveoli are formed (Burri *et al*, 1974).

c) Phase of equilibrated growth: During this period cell differentiation with rapid decline in cell production occur, while the alveolar and capillary surface area continue to increase. Thinning of interstitium and mean alveolar blood air barrier thickness also occur to improve the efficiency of gas exchange (Kauffman *et al*, 1974).

Over the first week, the number of type I epithelial cells steadily increases while the number of type II cells remains constant. Subsequently the number of type II cells increases rapidly, reaches a peak on day 13 and then decreases, whereas type I cells continued to increase in number. These facts may indicate that type II epithelial cells represent the stem cell population of alveolar epithelium. However, the type II cell height of proliferative activity on day 7 coincides with the outgrowth of septal crests and is followed by the steepest increase in number of type I and II cells (Kauffman *et al*, 1974).

Burri and colleagues (1974) found that after day 21 tissue mass increases further but to a lesser degree than air space volume. They have also found that the volume of the blood compartment (capillaries and larger blood vessels) show the greatest increase between days 21 and 131 after birth. This increase coincides with the enlargement of

the alveolar surface area as well as the alveolar air compartment. However, further research has to be done on this aspect of continued alveoli formation in adult rats.

1.3 Metabolic Development of Lung: with reference to structural development

It is important to link metabolic development to structural development, since carbohydrate metabolism plays an important role in the structural (Tierney and Levy, 1976) and functional development and maturation of fetal and neonatal lung tissue (Bourbon and Jost, 1982; Gilden *et al*, 1977; Maniscalco *et al*, 1978). Therefore, any interference with the metabolic development or glycolysis of lung cells will have an effect on the structural and functional development of the lung.

During fetal life, the lungs serve no respiratory function. They produce fluid which fills the potential airways and airspaces and flows out of the trachea. For the lungs to function efficient immediately after birth as the organ of respiratory gas exchange, profound changes in fluid metabolism, including cessation of production of tracheal fluid, removal of excess lung water and surfactant production, must occur (Kitterman, 1984).

Based on studies of fetal sheep, beginning about 2 days before birth, the rate of tracheal fluid production progressively decreases and probably ceases before birth. Pulmonary extra-vascular water, most of which is in the potential airspaces, decreases during labour so that 69% of fluid which leaves the lung has done so by the time of birth (Kitterman, 1984). Some evidence suggests that these changes in fluid balance are stimulated by cortisol and β-adrenergic agents. The decrease in tracheal fluid

production begins about 48 hours after the pre-partum rise in fetal plasma cortisol concentration (Kitterman *et al*, 1979). However, Bland and colleagues (1982) found no change in the fetal plasma epinephrine concentration with the onset of labour which was accompanied by a marked decrease in pulmonary extra-vascular water. Fetal plasma epinephrine concentrations do not rise until late in labour, about 3 hours before birth (Eliot *et al*, 1981). The pre-partum surge in fetal cortisol may cause an increase in pulmonary β-adrenergic receptors and thus makes the lungs more responsive to epinephrine. This could account for the pre-partum changes in pulmonary fluid balance (the decrease in tracheal fluid production and pulmonary extra-vascular water) without associated changes in fetal plasma concentrations of epinephrine (Kitterman, 1984). Conformation of this speculation must however, await further studies. However, production of fluid by the lungs are important for the control of development of lung structure and metabolism (Harding and Hooper, 1993).

The development of the surfactant closely parallels the morphological maturation of the respiratory epithelium. On day 17 of gestation, which coincides with the pseudoglandular phase, the respiratory epithelium of the fetal rat lung is composed of undifferentiated columnar cells that contain small amounts of glycogen. By day 19 of gestation, (canalicular phase) the epithelium consists of cuboidal cells that contain large pools of glycogen and a few lamellar bodies. Progressive differentiation of the epithelium leads on day 21 of gestation to the appearance of thin type I cells and cuboidal type II cells, which are devoid of glycogen and which contain numerous lamellar bodies (Rannels *et al*, 1997). The inverse relationship between glycogen and lamellar bodies has been noted by a number of investigators and has led to the

speculation that glycogenolysis during the canalicular phase might be associated with surfactant synthesis (Schellhase *et al*, 1989). During the last part of gestation in fetal sheep, the concentration of surfactant increases in lung tissue and lung lavage fluid and there is a similar increase in the flux of surfactant in fetal tracheal fluid. These changes are particularly marked during the last few days before birth (Kitterman, 1984). The production of surfactant in the fetal lung is initiated during the terminal part of gestation which is associated with alveolar sac or alveolar formation (Heesbeen *et al*, 1989). Pulmonary surfactant is important because it prevents alveolar collapse by lowering the surface tension at the alveolar surface of the lung (Tortora and Grabowski, 1993).

The alveolar type II cells synthesize and secrete pulmonary surfactant. The composition of surfactant lipoprotein aggregate, as recovered from lungs of various mammalian species by alveolar wash procedures, contains 70 to 80% phospholipids, 10 to 20% protein and 10 to 20% neutral lipids, primarily cholesterol (Thibeault and Gregory, 1986). Glycogen and glucose are associated with surfactant synthesis during late gestation (Hamosh *et al*, 1978) and in the adult (Salisbury-Murphy *et al*, 1966). A direct link between glycogen catabolism and surfactant synthesis in late gestation fetal lung was demonstrated by Farrell and Bourbon (1986) who showed that glucose originating from glycogen, provided substrate for fatty acids and glycerol synthesis which in turn, were incorporated into surfactant dipalmitoyl phosphatidylcholine. Glycogen and glucose also supply sufficient amounts of precursors such as dihydroxyacetone phosphate [an intermediary product of glycolysis which is the first acceptor of acyls in the synthesis of phospholipids in lung (Agranoff and Hajra, 1971)] for phospholipid synthesis, provided that the glycolytic pathway is optimally active (Maritz, 1986).

Glycogen is stored in the alveolar type II cells prior to the initiation of surfactant synthesis (Heesbeen *et al*, 1989). Based on studies carried out on fetal rat lung, it was found that glycogen disappeared from the type II pneumocytes of fetal rat lung from gestational day 19 till term and is associated with surfactant synthesis (Bourbon and Jost, 1982). Maritz (1986) also showed that the glycogen content in the lung decreases gradually from gestational day 19 to 21 days after birth. However, the glycogen content seems to increase on day 7 after birth, just to decrease again till day 21 after birth. The sudden increase and decrease correspond with the phase of rapid alveolarization and cell proliferation between postnatal day 4 to 13 and the phase of equilibrated growth from postnatal day 13 (Burri *et al*, 1974). As mentioned previously, type II cells reach a height of proliferation activity on postnatal day 7 (Kauffman *et al*, 1974). This corresponds to the increase of glycogen on day 7 after birth, since it is known that the type II cells store glycogen (Rannels *et al*, 1997). Early studies of Williams and Mason (1977) suggested an inverse temporal correlation between the glycogen and phospholipid content of the developing rat lung. Between days 19 and 21 of the gestation period, disaturated phosphatidylcholine (the major component of surfactant) levels doubled, whereas tissue glycogen levels fell and lamellar inclusions became abundant (Maniscalco *et al*, 1978). Maniscalco and colleagues (1978) also noted a similar decrease in glycogen content coincident with increased synthesis of phosphatidylcholine and its disaturated species. It is therefore conceivable that glycogen supply the glucose required for the glycolytic pathway, since surfactant synthesis depends on glycolytic activity (Rinaudo *et al*, 1976).

With respect to enzymatic development, the changes in enzyme levels occur in three

clusters: the late fetal, the neonatal and the late suckling groups (Henning, 1981). Phosphofructokinase (PFK) is one of the key enzymes at the onset of surfactant synthesis in the fetal rat lung during the period of glycogen breakdown. In contrast to the other allosteric enzymes hexokinase (HK) and pyruvatekinase (PK), the activity of PFK was the highest during the period of fetal glycogen breakdown (gestation days 17 to 22). The developmental pattern of PFK activity in type II pneumocytes suggests a regulatory role for this enzyme during the period preceding birth (Heesbeen *et al*, 1989) and HK is reported to be the rate - limiting enzyme for glucose utilization in lung tissue (Rijksen *et al*, 1985).

In a study done by Maritz (unpublished) it was found that the activity of HK gradually decreased from postnatal day 1 to day 7, however, no change in activity was observed between postnatal day 7 to day 14. According to Salotra and Singh (1982), HK catalyse the rate limiting step in the utilization of exogenous glucose by the rat lung. Maritz also found that between postnatal days 14 and 21, the activity of PFK was constant. In contrast to PFK the activity of HK increased from day 14 to day 21 after birth. The decrease in HK activity up to day 14 implies the importance of glucose in lung growth and maturation. This decrease in HK activity corresponds with the phases of cell proliferation and differentiation and lung expansion. He furthermore found that the activity of PK increased between postnatal days 1 and 7 however, no change in PK activity occurred from day 7 to 21 after birth, which indicates that a plateau was formed. The high HK activity after birth probably ensure a sufficient supply of precursors for the synthesis of surfactant. It is well known that before birth, the rate of surfactant synthesis increases and a large tissue store of surfactant is rapidly released

into the alveoli. This alveolar pool continues to increase at least 24 hours after birth. Regulation of the pool size of surfactant within the alveoli induces a burst of secretion immediately after birth and allows term newborns to have much more alveolar surfactant per kilogram lung tissue than adults. However, the mechanisms by which the alveolar pool of surfactant is regulated, remains unknown (Thibeault and Gregory, 1986).

1.4 Tobacco Smoke

1.4.1 The History of Tobacco Smoke

Although humans probably began sampling tobacco during the first millennium, based on Mayan stone carvings dated at about A.D. 600 to 900, physicians did not begin to suspect in earnest that the plant could produce ill effects until around the 19th century (Bartecchi *et al*, 1995).

With the invention of the cigarette-rolling machine in 1881 and the development of safety matches smoking was significantly encouraged and by 1945, cigarettes had largely replaced other forms of tobacco consumption such as pipe tobacco, cigars, chewing tobacco or snuff. Smokers increased their average of 40 cigarettes a year in 1880 to an average of 12 854 cigarettes in 1977, the peak of American consumption per individual smoker (Bartecchi *et al*, 1995). Cigarette smoking remains a major health problem in not only the United States (USA) but the world (Franklin, 1992). Today cigarette smoking is the leading cause of pulmonary illness and death in the USA (Bartecchi *et al*, 1995). In the USA alone, over 400 000 deaths and $50 billion in medical costs annually are directly attributed to smoking (Epping-Jordan *et al*, 1998).

Data from a National Household Survey in the USA show that 90% males and 70% females smoke at least 20 cigarettes per day, and 19% of men and 11% of women smoke 40 or more cigarettes per day. Over the period from 1978-80, among both men and women, the percentages of current smokers smoking 20 or more cigarettes has increased, as has the percentage smoking 40 or more cigarettes per day (Wonnacott *et al*, 1990). An overwhelming number of American women, estimated to be 22 million, were smokers in 1993 according to the Center for Disease Control. According to the 1995 Advance Report of Final Natality Statistics, 15.8% of women in the USA continue to smoke throughout their pregnancy, even though smoking has long been associated with adverse pregnancy outcomes, for both the mother, her fetus, and her newborn (Lambers and Clark, 1996).

1.4.2 The Composition of Tobacco Smoke

An unburned cigarette is comprised of many organic (tobacco leaves, paper products, sugars, nicotine) and inorganic (water, radioactive elements, metals) materials (Henningfield, 1985). A burning cigarette has been described by many as "a miniature chemical factory", which produces multitudes of new components from its basic raw materials (Huber, 1989). Figure 1.3 shows what happens during a puff. Cigarette smoke consist out of side stream and mainstream smoke. The mainstream smoke is collected from the stream of air passing through the centre of the cigarette. It is filtered by the tobacco itself and perhaps further by a filter. It is also diluted by air passing through the paper (most modern cigarettes also have tiny ventilation holes which further dilute the smoke). Side stream smoke is that which escapes from the tip of the cigarette. It is not filtered by the cigarette and results from a slightly cooler burning process at the edge

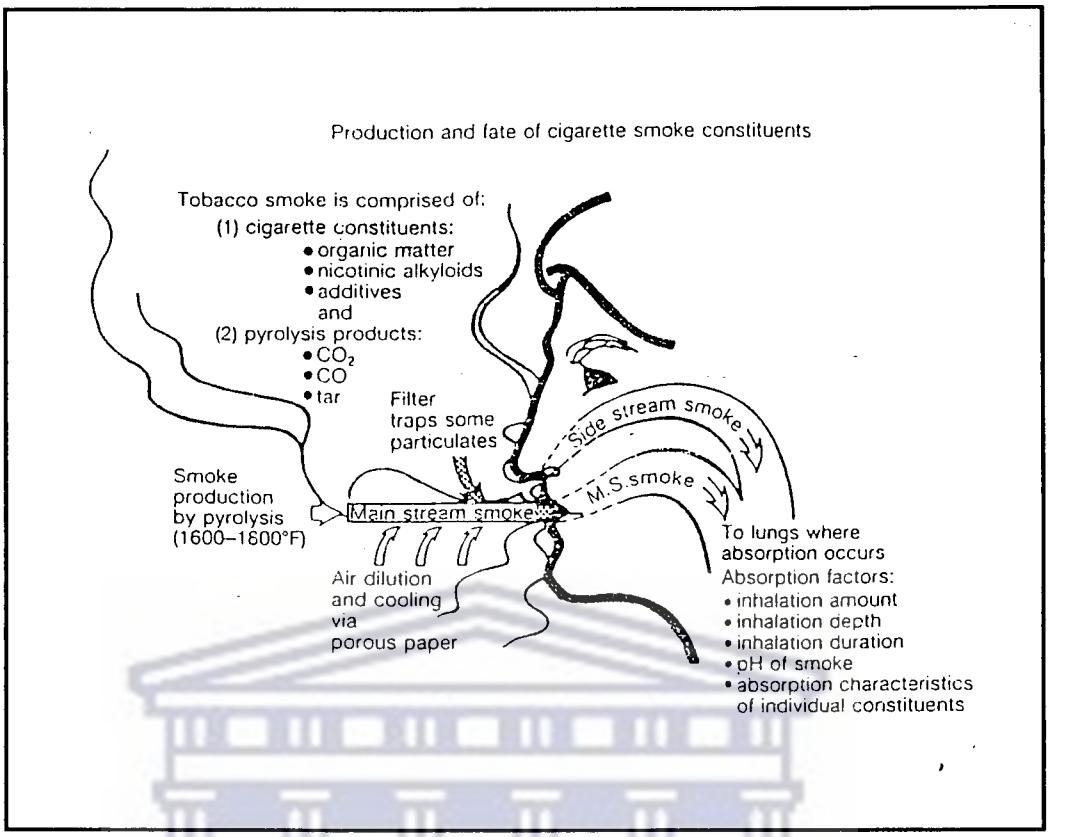

Figure 1.3: The diagram illustrates what happens when a cigarette is smoked. The burning of the cigarette creates various substances (*i.e* carbon dioxide, carbon monoxide, and tar) in addition to the cigarette's constituents prior to burning; M.S = main stream (Henningfield, 1985).

of the burning cone. Since the tobacco is therefore burned less completely, the side stream smoke has more particulate (unburned) material in it (Henningfield, 1985).

Cigarette smoke is made up of both a gas phase and a particulate (solid) phase (Henningfield, 1985) and contains more than 4 700 chemical compounds (Bartecchi *et al*, 1995). Most are delivered in such minute amounts that they are not usually considered in discussions of the medical effects of cigarette smoking. Three compounds

of undisputed importance are tar, carbon monoxide and nicotine (Henningfield, 1985). Tar is expressed as the total particulate matter (dry weight of the collected condensate) minus water and nicotine, which is trapped by the Cambridge filter used in smoke collection machines. Tar, not present in unburned tobacco, is a product of organic matter being burned in the presence of air and water at a sufficiently high temperature (during puffing 860 to 900^{0}C and between puffs 500 to 600^{0}C) (Henningfield, 1985; Huber, 1989). Carbon monoxide (CO) is a gas that results when materials are burned. CO production is increased by restricting the oxygen supply, as is the case inside a cigarette (Henningfield, 1985). Nicotine, the primary active ingredient in tobacco, occurs naturally in the leaves of *Nicotiana tabacum* (Henningfield, 1985; Huber, 1989). Nicotine is a drug (Henningfield, 1985) and is the primary reason why people consume tobacco products and it may contribute to causation of tobacco-related disease (Benowitz, 1986).

1.5 Nicotine

1.5.1 Nicotine as a Tobacco Product

Discovered in the early 1800's and named *nicotianne*, the oily essence now called nicotine is the main active ingredient of tobacco (Bartecchi *et al*, 1995). Nicotine is important in human biology for two reasons; (1) it appears to be the primary reason why people consume tobacco products, and (2) it may contribute to causation of some tobacco-related diseases (Benowitz, 1986). Tobacco addiction had lead to extensive research (Benowitz, 1986; Wonnacott *et al*, 1990; Editorials, 1991) of nicotine as a therapeutic agent in the form of nicotine gum, skin patches, vapour inhalers, nasal spray and lozenges for smoking cessation. However, the health consequences of nicotine

consumption as a component of tobacco smoke has not been extensively evaluated.

By chemical structure, nicotine is an alkaloid tertiary amine that is composed of a pyridine and pyrrolidine ring (Huber, 1989), as indicated by figure 1.4. Nicotine may exist as one of two stereoisomers (Wonnacott *et al*, 1990). Tobacco contains only (S)-

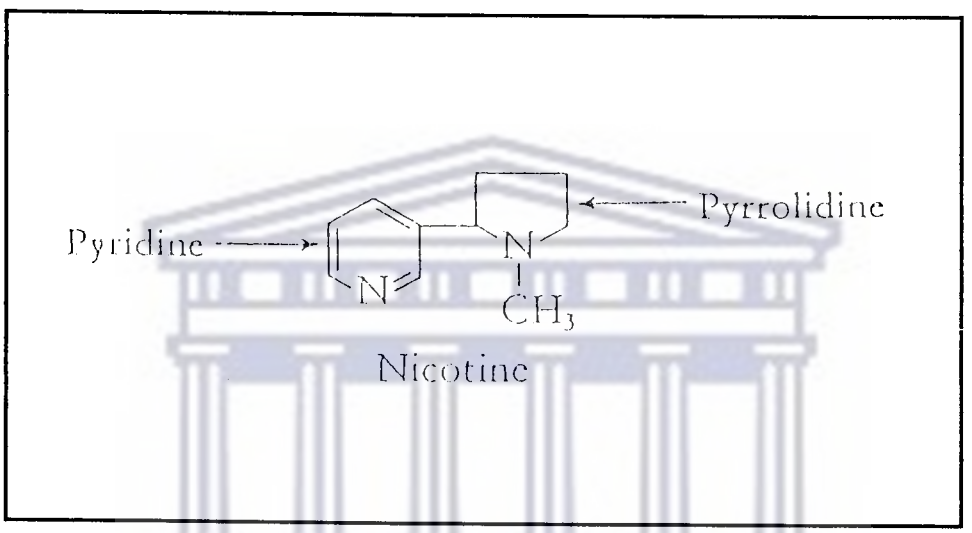

Figure 1.4: Structure of the nicotine molecule (Adapted from Robinson, 1968).

nicotine (also called (-) or levo (*l*)-nicotine), which is the most pharmacologically active form (Huber, 1989; Wonnacott *et al*, 1990). During the smoking process, some of the (S)-nicotine is converted to (R)-nicotine (also called (+) or dextro (*d*)-nicotine), which is much less pharmacologically active than the (S)-isomer (Huber, 1989; Wonnacott *et al*, 1990). About 90% of the nicotine in smoke is (S)-nicotine (Wonnacott *et al*, 1990). On average, modern cigarettes contain 8 - 9mg of nicotine. Low-yield cigarettes do not contain less nicotine, however, they are low yield because they remove tar and nicotine by filtration and or dilute the smoke with air (Benowitz, 1986). In most

commercial cigarettes, only about 15% of the total nicotine appears in the mainstream smoke, where 25% to 40% is released to side stream smoke and 15 to 25% is deposited within the butt (and filter tip). The remainder is pyrolysed to nicotine decomposition products (Huber, 1989).

1.5.2 The Absorption of Nicotine

Nicotine is highly lipid soluble and therefore have a significant influence on biological systems (Meyer *et al*, 1971). Nicotine is a weak base with a pK_a of 8.0 (Wonnacott *et al*, 1990). However, the absorption of nicotine across biological membranes depends on its pH, or state of acid-base dissociation (Huber, 1989). In its ionized state nicotine does not rapidly cross membranes. The smoke from tobaccos found in most cigarettes is acidic (pH 5.5). At this pH the nicotine is primarily ionized. As a consequence, there is little buccal absorption of nicotine from cigarette smoke, even when it is held in the mouth (Benowitz, 1986).

When a cigarette is smoked, micro droplets reach the alveolar spaces and are deposited on the vast internal surface of the lung where they are immediately buffered to a physiological pH of near 7.4. This is close enough to the index of ionic dissociation so that a significant portion (more than 30% of the total amount) of the nicotine is in a nonprotonated state and as such, is rapidly and efficiently absorbed across the air-blood barriers at the alveolar surface (Huber, 1989). This rapid absorption of nicotine from cigarette smoke through the lung is presumably due to the large surface area of the alveoli and small diffusion distance and dissolution of nicotine into a fluid of a normal physiologic range which facilitates transfer across cell membranes (Wonnacott *et al*,

1990). On average, smokers lungs absorb 1mg of nicotine per modern cigarette (Benowitz, 1986). A study by Hoffmann and colleague (1997) indicated however, that nicotine yields have declined per cigarette. In the United States the concentration declined from 2.7mg in 1954 to 0.95mg in 1992 and in the United Kingdom from 2.2mg in 1954 to 1.0mg in 1992.

1.5.3 The Distribution of Nicotine in the Tissues

Nicotine found in the body only after tobacco smoke exposure, has a half-life of two hours (Shaham *et al*, 1993), whilst that of the products of nicotine metabolism, such as cotinine (major metabolite of nicotine) varies from one to two days (Shen *et al*, 1977). Due to its long half-life, cotinine is commonly used as a marker of nicotine intake (Benowitz, 1986). After absorption, nicotine enters the bloodstream where at a pH of 7.4 it is about 69% ionized and 31% un-ionized (Wonnacott *et al*, 1990). In fact, when a given dose of nicotine is ingested, for instance by smoking, about one half is removed from the bloodstream within 15 to 30 minutes (Henningfield, 1985). The liver, lungs and the brain have a high affinity for nicotine whilst the adipose tissue has a relative low affinity for nicotine. Immediately after intra pulmonary injection with nicotine, concentrations in arterial blood, lungs, and the brain are high, while concentrations in tissues such as muscle and adipose tissues (major storage tissues at steady state) are low. The consequence of this distribution pattern is that uptake of nicotine into the brain is rapid, occurring within 1 or 2 minutes of peak arterial concentrations, and blood levels of nicotine fall due to peripheral tissue uptake for 20 or 30 minutes after administration (Duelli *et al*, 1998). Thereafter blood nicotine concentrations decline more slowly, the rate determined by rates of elimination and rates of distribution out of

storage tissues (Wonnacott *et al*, 1990).

Nicotine freely crosses the placenta of pregnant women and has been found in amniotic fluid and in umbilical cord blood of neonates. The amniotic fluid provides a reservoir for continued delivery of nicotine to the fetus, even when maternal levels are low (Wonnacott *et al*, 1990). Ahlsten and colleagues (1990), measured nicotine and cotinine in cord blood sera, and found that high concentrations of nicotine is transferred to the fetuses of smoking mothers. Babies of nursing, smoking mothers, had been found to have a high cotinine concentration in their blood and urine (Charlton, 1994). In children, the level of urine cotinine has been shown to correlate with the number of cigarettes smoked by parents, especially by mothers. Cotinine and nicotine can also be measured in breast milk (Svensson, 1987) and concentrations of nicotine increased 10-fold when the mother smoked just before nursing (Halken *et al*, 1995). It has also been found by Schulte-Hobein and colleagues (1992) that cotinine excretion in urine from non-breast-fed infants of smoking mothers (nicotine exposure via passive smoking only) was even higher than that of adult passive smokers. However, the urinary cotinine excretion in breast-fed infants of smoking mothers was at the same level as that of the mothers themselves.

1.5.4 Metabolism and Elimination of Nicotine

The metabolism of nicotine by mammals has probably been as extensively studied as that of any alkaloid. In general only a small percentage of administered nicotine is excreted unchanged, but the actual figures vary from animal to animal in the range of 4 to 12% (Robinson, 1968). Nicotine is extensively metabolized, primarily in the liver,

but also to a small extend in the lung and kidney (Benowitz, 1986; Wonnacott *et al*, 1990). Figure 1.5 indicates the primary metabolites of nicotine. Once in the blood, nicotine peak levels are reduced fairly rapidly (Huber, 1989). Nicotine is excreted

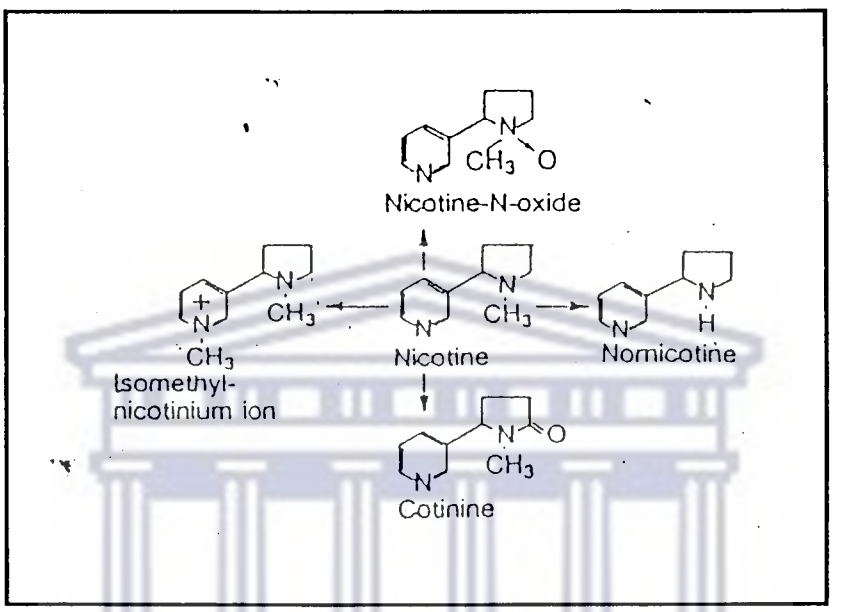

Figure 1.5: The primary metabolites of nicotine (Svensson, 1989).

unchanged in urine in a pH dependent fashion (Svensson, 1987). 30% or more nicotine is lost via the kidney, but more commonly in the range of 5 to 10% of the total absorbed dosage (Huber, 1989). Assuming most nicotine is metabolized by the liver (data in animals indicate only a small contribution by the lung), this means about 70% of the drug is extracted from the blood each time it passes through the liver (Wonnacott *et al*, 1990). The primary metabolites of nicotine are cotinine and nicotine-*N*-oxide. Nicotine-*N*-oxide is however, a minor metabolite. The metabolism of cotinine is much slower than that of nicotine. Therefore, the rate of elimination of cotinine is predicted not to

be substantially influenced by changes in liver blood flow (Wonnacott *et al*, 1990). Nicotine is also excreted in the saliva (Jones *et al*, 1991) and nicotine has also been found to concentrate in breast milk (Svensson, 1987). Cigarette smoking itself may influence the rate of metabolism of nicotine. Clearance of nicotine is slower in non-smokers compared to habitual cigarette smokers which is consistent with the expected effects of cigarette smoking to accelerate drug metabolism (Wonnacott *et al*, 1990).

1.6 Effect of Nicotine and Smoking on Metabolism

Cigarette smoking has the potential to alter the metabolism of individuals repeatedly exposed to nicotine, carbon monoxide and other constituents of cigarette smoke (Hofstetter *et al*, 1986). The smoking mother is at a two-fold increased risk for delivering a low birth weight infant compared to her nonsmoking counterpart (Lambers and Clark, 1996). Maternal smoking is therefore associated with low birth weight of the fetus (Charlton, 1994). Smoking however, is believe to reduce adult body weight, and smoking cessation produces a marked weight gain (Bernstein *et al*, 1996). The effect of smoking and nicotine on reducing body weight results from an increased whole body metabolism (Perkins, 1992).

Nicotine consumption by pregnant mothers via gum or cigarette results in an increase in fetal heart rate (Lambers and Clark, 1996) and increases fetal arterial blood pressure (Clark and Irion, 1992). Arcavi and colleagues (1994), reported that cigarette smoking and nicotine increases adult heart rate and energy expenditure in most smokers. Increased energy expenditure is believed to be an important determinant of lower body weight in smokers compared with nonsmokers (Perkins, 1992). Nicotine absorbed

during smoking increases the discharge of catecholamines from the adrenal medulla and from extra adrenal chromaffin tissue, which causes heart rate and blood pressure to rise (Cryer *et al*, 1976). Even rats, treated with nicotine, lost weight without significant reduction in food intake (Schecter and Cook, 1976). A study done by the Department of Health and Human Services in the United States (1990) proved that chronic administration of nicotine or exposure to cigarette smoke, increases oxygen consumption and results in weight loss of experimental animals.

In nonsmokers, the hormonal responses elicited by exercise or hypoglycemia are sufficient to provide a balance between hepatic glucose output and peripheral glucose utilization during steady state conditions (Wolfe *et al*, 1986). Hofstetter and colleagues (1986) found that acute smoking during rest increases the resting metabolic rate and the respiratory quotient. Nicotine, induces lipolysis (Hellerstein *et al*, 1994), this effect therefore helps explaining why the fat stores of nicotine treated animals decreases (Winders and Grunberg, 1990). In an experiment done by Hellerstein and colleagues (1994), it was observed that acute cigarette smoke had no effect on increasing hepatic glucose production. This lack of hepatic gluconeogenesis production was interesting, in view of the increased glycerol flux (a precursor for hepatic gluconeogenesis), increased free fatty acid (FFA) flux and increased catechol release induced by cigarette smoking (Hellerstein *et al*, 1994). Colberg and colleagues (1994) found in their study, that irrespective of chronic or acute smoking, an increase on the dependence of blood glucose as a fuel during rest and sustained submaximal exercise, occurred. Nicotine increases circulating catecholamines (Cryer *et al*, 1976) and therefore epinephrine may directly raise metabolism by increasing glucose utilization, independent of insulin or

glycogen changes (Staten *et al*, 1989). Measurements with isotopic tracers showed that smokers had augmented rates of glucose appearance, disappearance, clearance, recycling, and oxidation compared with nonsmokers during rest and moderate intensity exercise (Colberg *et al*, 1994). The elevation in catecholamine after smoking (Cryer *et al*, 1976) may help explain the metabolic increase after smoking (Perkins, 1992).

These above mentioned findings may therefore be relating factors that could be responsible for the low birth weight of the fetus. Considering that nicotine readily gains access to the fetal compartment via the placenta, with fetal concentrations generally 15% higher than maternal levels (Koren, 1995). Nicotine also passes freely into breast milk (Luck and Nau, 1984) and is absorbed orally. Therefore *in utero* swallowing and breast feeding both expose the baby to nicotine (Lambers and Clark, 1996). This exposure of the fetus and a neonate of a smoking mother to nicotine during intra-uterine life and neonatal life, if breastfed (Lambers and Clark, 1996), may result in reduced body weight (Charlton, 1994).

Substrate deprivation during fetal life, sufficient to cause low birth weight, can affect the development of the lungs and their postnatal function (Harding, 1995). Maternal smoking reduces blood supply to the placenta, which could result in a reduction in nutrient and oxygen delivery to the developing lungs and thereby affect growth and consequently lung growth as part of its general growth-retarding effect on the fetus (Johnston, 1981). Since carbohydrates play an important role in the structural (Tierney and Levy, 1976) and functional development and maturation of fetal and neonatal lung tissue (Bourbon and Jost, 1982; Gilden *et al*, 1977; Maniscalco *et al*, 1978), interference

with carbohydrate metabolism during late gestation therefore results in retardation of fetal lung growth (Rhoades and Ryder, 1981). *In utero* nicotine exposure is an important component of growth suppression (Eckstein *et al*, 1997) and thus maternal smoking during pregnancy causes fetal lung growth impairment (Charlton, 1994). Research conducted by Maritz (1988), demonstrated that maternal nicotine exposure interferes with neonatal rat lung metabolism and development.

Maritz and Burger (1992) also showed that maternal nicotine exposure during pregnancy and lactation results in an increase in glucose turnover by 86.4% compared to the controls. However, the *in vitro* lactate production by the lung tissue of nicotine exposed rat pups was remarkably lower than that of the control rat pups (Maritz, 1986). From these findings it was concluded that maternal nicotine exposure during pregnancy and lactation results in the inhibition of glycolysis (Maritz, 1986). Maritz and Burger (1992) furthermore showed that the adenine nucleotide pool (ATP+ADP+AMP) was 32.8% higher for the lungs of the three week old neonates exposed to nicotine than that of the control rat lungs. After four weeks of nicotine withdrawal, glycolysis of those animals exposed to nicotine was still inhibited to the same extent than during exposure and the adenine nucleotide pool was 69.95% higher than that of the controls. Maritz and Burger (1992) proposed that the inhibition of glycolysis was due to the high ATP/ADP ratio of the lungs of the nicotine exposed rats.

Since it was demonstrated that maternal nicotine exposure interferes with neonatal rat lung metabolism, the effects being suppression of glycogenolysis and glycolysis (Maritz, 1988), the possibility may therefore exist that interference with the energy

metabolism of a developing lung could lead to serious developmental problems. Maritz and Woolward (1992) found that maternal nicotine exposure impaired elastic tissue synthesis. They (1990) also illustrated an abnormal high lamellar body content in type II cells of lungs of neonatal rats exposed to nicotine via mothers milk and placenta and they furthermore found degenerative changes of type I pneumocytes in these lungs. The high lamellar body content is indicative of a decrease in glycogen, since glycogen and the lamellar bodies have an inverse relationship. It could however, be assumed that there would have been an abnormal increase of surfactant production, since glycogenolysis might be associated with surfactant synthesis (Schellhase *et al*, 1989). A study conducted by Maritz and Thomas (1995), showed that there was an increase in type II cells and a decrease in type I cells in the lungs of nicotine exposed rat pups. It was however, clear that most of the proliferation of the type II cells took place before birth, but after birth the rate of proliferation was very slow. The reduced rate of type II cell proliferation could be related to the inhibition of energy metabolism (Maritz and Thomas, 1995). They (1995) also found that maternal nicotine exposure induced swelling of the mitochondria of the type II cell and absence of microvilli occurred on the alveolar surface of the type II cells of the nicotine exposed lungs, suggesting a change in the functional characteristics of this membrane. These findings clearly illustrate that maternal smoking or nicotine exposure during pregnancy and lactation will interfere with the energy metabolism of a developing lung and could therefore lead to serious developmental and functional problems.

1.7 Motivation for this Study

The number of cigarette smoking women has steadily increased during the last decades.

The number of heavy smokers has also become greater among pregnant women and mothers with infants and young children (Halken *et al*, 1995). It is evident today that children of smokers are likely to suffer from serious respiratory infections and disease (Stein *et al,* 1999; Young, 1992) and that their lung growth is decreased during childhood and adolescence (Moessinger, 1989).

Lung development starts *in utero* (Charlton, 1994) and abnormal development may be initiated, *in utero*, by maternal smoking or specifically nicotine (Merkus *et al*, 1996). Since maternal nicotine exposure results in the inhibition of glycolysis (Maritz, 1986) in the lungs of the offspring and interfere with lung growth and maturation (Maritz and Thomas, 1995), it is imperative to analyse the mechanisms whereby nicotine induces these changes in the cells of the rat lungs.

The influence of maternal nicotine exposure during pregnancy and lactation on the energy metabolism, is still enigmatic. Therefore the aim of this study was to determine the influence of maternal nicotine exposure during pregnancy and lactation on the energy metabolism of developing neonatal lungs of rats at postnatal days 1, 7, 14, 21, and 49.

The objectives were as follows:

With reference to chapter II; to determine the effect of maternal nicotine exposure during gestation and lactation by means of spectrophotometry on the following:

- The activity of selected allosteric enzymes, hexokinase (HK) and phosphofructokinase(PFK).

- The activity of these enzymes in the presence of various ATP concentrations and ATP/ADP ratios within the reaction medium.
- To express the activity of these enzymes as a function of age, and
- To correlate the findings with the stages of neonatal lung structural development as explained in the literature.

With reference to chapter III; to determine the effect of maternal nicotine exposure during gestation and lactation by means of high performance liquid chromatography (HPLC) on the following:

- The adenine nucleotides (ATP, ADP and AMP) and adenosine, and
- To determine the effect of age on the adenine nucleotide pool and adenosine.
- To propose a mechanism whereby nicotine induce an increase in lung ATP content and
- To determine whether the increased ATP/ADP ratio indeed result in inhibition of glycolysis.

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CHAPTER II

Influence of maternal nicotine exposure on the enzymes, hexokinase and phosphofructokinase

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2.1 Introduction

Like most other organs, the lung produces energy in the form of adenosine triphosphate (ATP) via glycolysis and further oxidation of substrates through the tricarboxylic acid cycle and the electron transport chain (Sayeed and Murthy, 1981). Glycolysis is however, one of the major metabolic pathways that supplies the cell with energy and at the same time with material for synthesis (Boiteux and Hess, 1981). The transformation of energy is carried out by enzyme molecules that are integral parts of highly organized assemblies (Stryer, 1975).

Enzymes, are proteins known as catalysts (Stryer, 1975) and although they are subject to the same laws of nature that govern the behaviour of other substances, they differ from ordinary chemical catalysts in several important respects such as, higher reaction rates, milder reaction conditions, greater reaction specificity and the capacity for regulation (Voet and Voet, 1995). This, however, does not indicate that enzymes alter chemical reaction equilibria, it means that an enzyme accelerates the forward and reverse reaction by precisely the same factor (Stryer, 1975). The enzyme is conceptually the trump which allows the cell to exist, and can be seen as fundamental to life, providing a way of keeping ahead of the universal tendency towards disorder (Ferdinand, 1976).

An enzyme structure consists of a protein portion, called the apoenzyme and a non protein portion called a cofactor. Cofactors may be a metal ion, for example magnesium, zinc, calcium or an organic molecule called a coenzyme, which are often vitamin derivatives. The apoenzyme and the cofactor form a holoenzyme or whole enzyme (Tortora and Grabowski, 1993). Enzymes are highly specific catalysts and each particular enzyme affects only specific substrates (Stryer, 1975). A portion of the enzyme, called the active site, is thought to fit the substrate like a key fits in a lock (Voet and Voet, 1995). However, not all active sites are rigid receptors for enzymes therefore, in some cases the active site changes its shape to fit snugly around the substrate once the substrate enters the active site. This is known as an induced fit (Tortora and Grabowski, 1993). An allosteric enzyme is known to change its active site. These enzymes are assumed to be reversibly altered in the molecular structure of the protein, by means of an allosteric effector, which inhibit or activate a particular enzyme (Cohen, 1976). The allosteric effector binds specifically and reversibly to an allosteric site, a binding site on the enzyme (Cohen, 1976; Ferdinand, 1976). The allosteric site is distinct and separate from the active site of an enzyme, however, the allosteric effector binding at the allosteric site causes an alteration in the structure of the active site which may either enhance or reduce the enzyme's ability to act as a catalyst (Ferdinand, 1976). The allosteric effector however, does not activate a reaction that involves the effector itself, but is assumed to bring about a discrete reversible alteration in the molecular structure of the protein, and since it binds at a site altogether distinct from the active site, and it does not participate at any stage of the reaction activated by the protein, it need not bear any particular chemical or metabolic relation of any sort with the substrate itself (Cohen, 1976).

Figure 2.1 indicates the ten enzyme catalysed reactions of glycolysis. Glycolysis may be considered to occur in two stages, stage I (reaction 1-5) and stage II (reaction 6-10). Stage I utilizes two ATPs and stage II produces four ATPs (Voet and Voet, 1995). Several of the glycolytic enzymes are regulated via feedback mechanisms in which positive modifiers increase the apparent affinity of the enzyme for the substrate and negative modifiers decrease the affinity for the substrate (White *et al*, 1973). The observed major controls of the glycolytic pathway are in general, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). These enzymes participate in three irreversible steps as indicated in figure 2.1. This chapter, will only be concentrating on the following two enzymes, hexokinase and phosphofructokinase in the glycolytic pathway, stage I (reactions 1-5). These enzymes are kinases and transfers phosphoryl groups between ATP and a metabolite, however, these kinases are also allosteric enzymes (Tortora and Grabowski, 1993).

Hexokinase (HK) mediates one of the principal reactions in glucose metabolism and catalyses the first step in glucose metabolism (Voet and Voet, 1995), utilizing the first ATP for the phosphorylation of glucose (substrate 1) to glucose-6-phosphate (reaction 1) (figure 2.1), which is a necessary substrate for several pathways, including glycolysis, glycogen synthesis and the hexose monophosphate pathway (Griffin *et al*, 1992). The second substrate for HK, as with other kinases, is a Mg^{2+}-ATP complex. This enzyme catalyses the nucleophilic attack of the C6-OH group of glucose on the γ-phosphate. Uncomplexed ATP is a potent competitive inhibitor of HK (Voet and Voet, 1995). Thus, a high concentration of ATP as well as the presence of the product can inhibit this enzyme's activity (White *et al*, 1973).

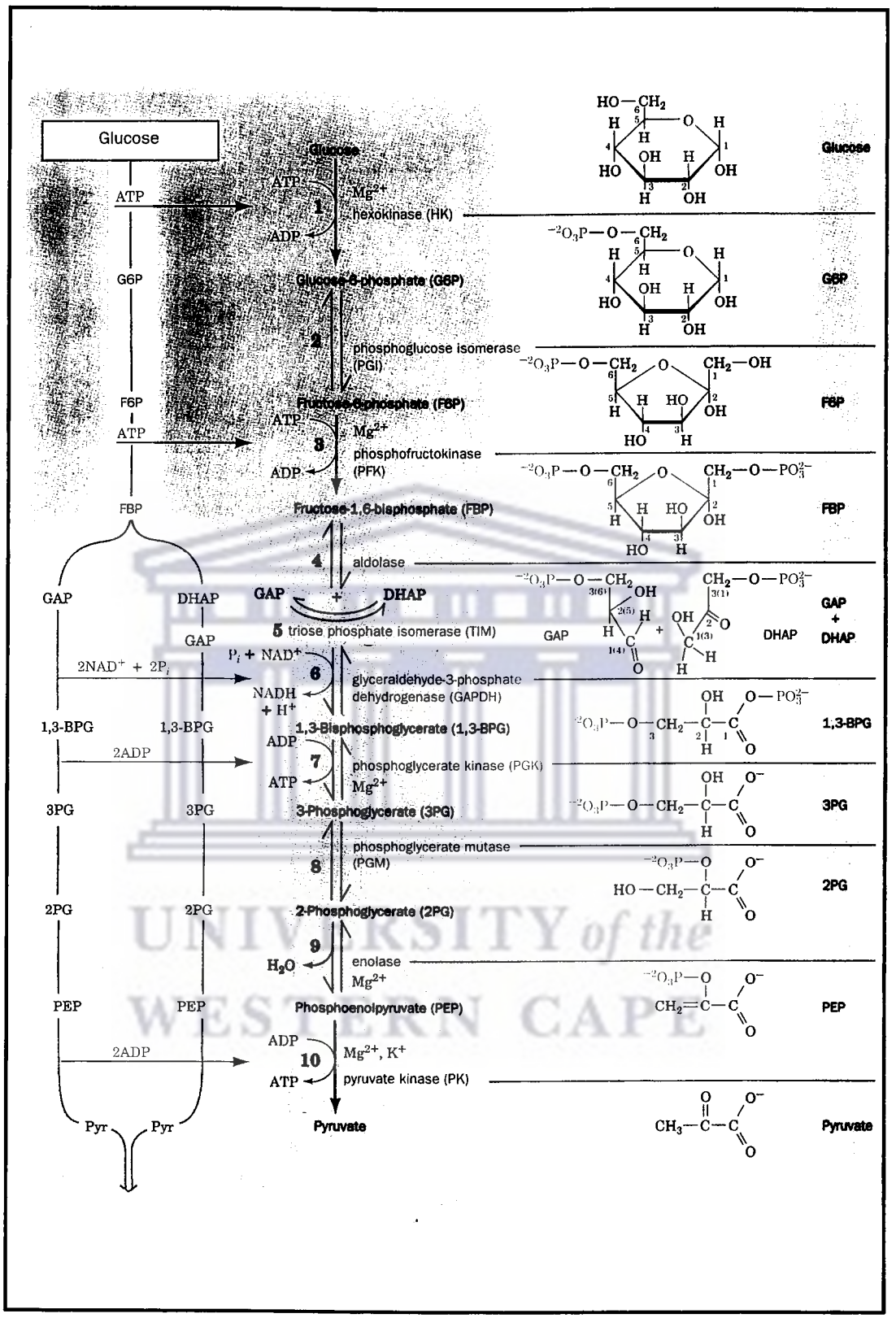

Figure 2.1: Glycolysis may be considered to occur in the following two stages: Stage I (reactions 1-5) and stage II (reactions 6-10) (Voet and Voet, 1995).

The first potentially limiting step in glucose use, is its transport across the cell membrane (Allen *et al*, 1998). This transport can be considered to involve two distinct steps. The first step is movement of glucose across the plasma membrane through transmembrane glucose transporters (GLUT). GLUT-1, GLUT-4, and sodium-glucose cotransporter (SGLT)-1 are the primary transporters found in lung tissue (Basset *et al*, 1987). The GLUT family of transporters is described as facilitative glucose transporters because the glucose diffuses "through" these transporters, driven by the transmembrane glucose concentration gradient. On the other hand, SGLT-1 functions by cotransport of glucose and sodium, with the cotransport driven by the sodium gradient generated primarily by Na-K-adenosinetriphosphatase. The GLUT family of glucose transporters functionally relies on the phosphorylation of intracellular glucose by HK to maintain the glucose gradient across the plasma membrane (Allen *et al*, 1998).

Four isozymes of HK have been described in mammalian tissues, HK types I, II, III and HK IV or glucokinase (Katzen and Schimke, 1965). Types I-III can be distinguished from glucokinase by their low Km and thus higher relative affinities for glucose, and can be further separated from each other on the basis of slightly different properties, including kinetic properties, substrate affinities and responses to hormonal, dietary and metabolic stimuli (Wilson, 1985). In addition, the relative proportions of the isoenzymes vary in different tissues and with the developmental and metabolic state of the organism (Rijksen and Staal, 1985). HK I, II and III will accept several other hexoses as substrates, but HK IV, which predominates in liver, is highly specific for glucose. The HK isoenzymes (HK I, II and III) can catalyse the phosphorylation of both α- and β-D-glucose, although with different kinetic constants. The following three

isoenzymes, HK I, II and III are likely to be found in the lung. HK I has a higher affinity for glucose than the other two isotypes and is the primary isoenzyme involved in phosphorylation of glucose in adult lung. In adult lung tissue HK I is, however, considered to be the predominant isoform. However, the Km for glucose turnover for suckling rat lung is higher than that of adult rats and suckling rat lung has a lower affinity for glucose and therefore possibly a different isoenzyme pattern than in adult lung (Salotra and Singh, 1982).

Phosphofructokinase (PFK) catalysis the phosphorylation of fructose-6-phosphate (F6P) to yield fructose -1,6-bisphosphate (FBP) (Dunaway, 1983). This reaction is similar to the HK reaction (figure 2.1) and utilizes the second ATP in the third step of glycolysis. This enzyme, PFK catalyses the nucleophilic attack by the C1-OH group of F6P on the electrophilic γ-phosphorous atom of the Mg^{2+}-ATP complex (Voet and Voet, 1995).

Regulation of the use of the glucose flux through the glycolytic pathway is largely determined by PFK activity (Dunaway and Kasten, 1985). Since PFK is a major regulatory enzyme of glycolysis (Tortora and Grabowski, 1993). This is mediated by the susceptibility of PFK to be inhibited allosterically by increased levels of citrate and ATP which occur when oxidative metabolism supplies the majority of the energy requirements of the cell. The inhibition of PFK significantly retards glycolysis and enhances the storage of glucose as glycogen (Dunaway and Kasten, 1985). Synergistically PFK is activated by its second substrate F6P (Boitex and Hess, 1981), its product FBP which is the most powerful activator of PFK (Hirata *et al*, 1998) and the effectors ADP and AMP (Boitex and Hess, 1981).

At least four forms of isozymes of PFK have been reported in tumours and normal tissues (Tanaka *et al*, 1971) and at least three of the four isoenzymes are present in all cell types but, in different proportions (Zeitschel *et al*, 1996). The two fundamental isozymes are phosphofructokinase A (muscle-type/M-type) and B (liver-type/L-type). A third form, present in the brain, was designated isozyme C. The fourth isozyme phosphofructokinase III is mainly detected in the spleen and kidney (Kahn *et al*, 1979; Masters and Reid, 1987). The M-type isozymes predominate in cellular situations requiring periods of rapid energy production via glycolysis. In contrast, the L-type isoenzyme predominate in cells that carry out extensive gluconeogenesis and the C-type isoenzyme activity, may be suited to those cellular regions requiring moderate, constant glycolytic rates (Masters and Reid, 1987). In the adult lung nearly equal amounts of the following three subunits M-type, L-type and C-type are found (Dunaway and Kasten, 1987). The activity of PFK as well as the isoenzyme types are affected by alterations in nutritional, hormonal, developmental and pathological states. Tissue specific changes in PFK activity and the nature of the PFK isoenzyme pool contribute significantly to the diversities of glycolytic and gluconeogenic rates (Danaway, 1983).

Enzymatic development can be grouped into three clusters: the late fetal, the neonatal and the late suckling group (Henning, 1981). This metabolic development pattern needs a source of energy. During fetal life, at birth and during postnatal life, the source of energy is glucose (Griffin *et al*, 1992; Henning, 1981). The fetal metabolic pathways however, differ from those in the adult (Henning, 1981) and between species (Faulkner and Jones, 1976).

In fetal lung of the mice, both the HK and PFK activity is high prior to birth. Immediately after birth, the HK activity decreases but again increases in the adult lung. However, the activity of PFK decreases consistently in the course of life (Ràdy *et al*, 1979). The HK activity of human erythrocytes is related to age and the activity varies with age and is much higher in children than in adults. The activity of this enzyme is particularly high during the first year of life, that is 2.5-3 times greater than in adults (Kil'Dema, 1964). Erythrocytes have been shown to have a lower PFK activity in newborns than in adults (Oski, 1969).

Both HK and glucokinase activity are normally present in the liver (Faulkner and Jones, 1976). Glucokinase is considered to be the major glucose phosphorylating enzyme in the liver. The existence of HK is also of physiological significance in the utilization of other hexoses by the hepatic tissue (Dileepan *et al*, 1979). No significant glucokinase activity has been detected in the fetal liver of the rat, guinea pigs or sheep, although low activity has been reported in the liver of the pig (Faulkner and Jones, 1976). Data obtained from rats (Dileepan *et al*, 1979) indicated that traces of hepatic glucokinase activity were noticed in the fetus and the newborn. It was found that activity of glucokinase did not show any significant increase until the 15th day after birth but increased 3 fold between days 9 and 15. A further 15 fold increase was observed between days 15 and 21 after birth which thereafter remain steady throughout adulthood. HK activity in liver on the other hand was high in the fetus and newborn and decreased gradually during suckling (Dileepan *et al*, 1979). PFK activity in the fetal rat liver is 2.5 times greater than adult values before birth. Further, the PFK activity decreased faster before birth than after birth. A gradual decrease in the PFK activity occur after birth until attaining adult values within two weeks (Dunaway, 1983).

In the rat brain, HK I is predominant and according to Griffin and colleagues (1992), HK I is most highly expressed in the brain across all developmental stages in comparison to other tissues such as the heart, kidney, liver, lung and skeletal muscle. HK I expression increases at birth and peaks at postnatal day 7 before decreasing to a constant level at adulthood (Griffin *et al*, 1992). Hommes and Wilmink (1968) reported that total PFK activity in the brain of the rat decreases approximately 50% from seven days prepartum to about 12 days postpartum. PFK activity does not appear to change from 12 days postpartum to 24 days postpartum, although by 25 days postpartum, total PFK activity has usually increased two-fold. The adult brain PFK activity is six-times greater than in fetal brain. The PFK activity within the brain rises to higher adult levels after birth and during the first month of maturation (Danaway, 1983).

Total HK activity is high in the fetal rat heart and skeletal muscle but declines after birth (Griffin *et al*, 1992). Heart muscle contain equal amounts of both types I and II HK. The rat heart has very high levels of both HK I and III isoforms during early stages of development. Postnatally, there is a decrease in HK I and HK III which correspond with the decrease in glucose utilization and the increase in reliance on fatty acids as the primary energy substrate in the adult heart (Coerver *et al*, 1998). The decline in HK I and HK III which was observed in the postnatal heart, may reflect the change in energy metabolism in which glucose is utilized as a secondary substrate in postnatal cardiac muscle, acting as a supplement to the metabolic energy obtained by fatty acid utilization (Wilson, 1995). Skeletal muscle contains predominately HK II. This HK isoenzyme is present in tissues such as the skeletal muscle in which insulin stimulates the uptake of glucose. Skeletal muscle contains the isoenzymes HK I and III. Development of HK I and III in skeletal muscle are diffusely intense during gestation and after birth. Postnatal

animals show a decrease in HK I protein. The HK activity in postnatal muscle has also proven that, following birth, there is an increase in glycogen synthesis and a subsequent decrease in glycolytic metabolism. This change is reflected in a shift from HK I to HK II as the predominant isoform in skeletal muscle (Coerver *et al*, 1998). Fetal rat heart-PFK-activity is approximately 56% of the adult PFK activity. In the fetal heart, PFK-L_2 was the predominant species. PFK-M and PFK-L_2 in adult heart were present in a 70:30 ratio. The fetal heart is primarily dependent on glycolysis for energy production and highly tolerant of anoxia. During this period PFK-L_2 appears to be the major PFK isoenzyme. From 10-12 days postpartum, nearly normal contraction rates can be maintained by glucose. During this interval, PFK-L_2 levels appeared to remain constant, but PFK-M levels appear to increase. This elevation in total PFK activity was therefore due to an increase in PFK-M levels. The increase in PFK-M very likely contributes to the ability of the heart to regulate glycolysis in response to the availability of fatty acids which are used preferentially for energy production via oxidative phosphorylation (Dunaway, 1983). In rat skeletal muscle, total PFK activity increased from fetal levels of 5.8 U/g to adult levels of 31.6 U/g. Both PFK-L_2 and PFK-M were present in fetal and neonatal muscle. PFK-L_2 which was 40% of the PFK muscle activity, decreased during maturation and was not detectable in adult lung. The increase in muscle PFK activity during maturation was due to an increase in the level of PFK-M and correlated with the increased contractile ability of maturating skeletal muscle (Dunaway, 1983).

The changes in the enzyme activity are different for different tissues (Griffin *et al*, 1992). The increase or decrease of an enzyme's activity may be correlated with the increase or decrease in physiological demand for glycolysis and energy in the tissues. The enzyme activity of the tissues at all developmental stages is, associated with the

cell structure (Masters and Reid, 1987). However, the changes in the enzyme activity is influenced by the alterations in nutritional, hormonal, developmental and pathological states (Danaway, 1983). In addition, the relative proportions of the isoenzymes vary in different tissues and with the developmental and metabolic state of the organism (Griffin *et al*, 1992).

Research conducted by Maritz (1988), shows that maternal nicotine exposure during pregnancy and lactation results in the suppression of glycogenolysis and an irreversible inhibition of glycolysis (Maritz, 1987). Furthermore, it is suggested that the observed increase in ATP/ADP ratio of lung tissue of nicotine exposed rat pups (Maritz and Burger, 1992) may be responsible for the suppression of the glycolytic pathway.

In the cells, the rate of glycolysis is limited by the rate at which ATP is used. Thus the glycolytic flux depends on the cellular levels of ATP and ADP (Fisher, 1984). The overall rate of glycolysis is therefore determined by the availability of substrate, utilization of ATP and the concentrations of the various enzymes (White *et al*, 1973). In the lung, the ATP produced can be utilized for the synthesis of surfactant phospholipids and normal development of pulmonary function. The ability of the tissues to utilize glucose and generate the stored energy (ATP), depends on the availability of the enzymes involved in the biochemical pathways (Bhavnani and Wallace, 1990). It is also dependent on the fact that the enzyme keeps ahead of the universal tendency towards disorder (Ferdinand, 1976) because, any alteration or absence of a specific enzyme, could result either in death or a serious disease (Dressler and Potter, 1991).

Glycolysis plays an important role in lung growth and development (Maritz and Najaar, 1995). Inhibition thereof is accompanied by inhibition of lung growth and development. In a previous study it was found that maternal nicotine exposure during gestation resulted in irreversible inhibition of glycolysis (Maritz, 1987). Although it was suggested that this inhibition is due to a high ATP/ADP ratio in the lungs of the offspring, no direct evidence exists to support this suggestion.

Therefore the aim of this study was to determine the influence of maternal nicotine exposure during pregnancy and lactation on the energy metabolism of developing neonatal lungs of rats at postnatal days 1, 7, 14, 21, and 49.

The objectives were to determine the effect of maternal nicotine exposure during gestation and lactation by means of spectrophotometry on the following:

- The activity of selected allosteric enzymes, hexokinase (HK) and phosphofructokinase (PFK).
- The activity of these enzymes in the presence of various ATP concentrations and ATP/ADP ratios within the reaction medium.
- To express the activity of these enzymes as a function of age, and
- To correlate the findings with the stages of neonatal lung structural development as explained in the literature.

2.2 Materials and Methods

2.2.1 Sample selection and management

White virgin female rats (Wistar descendants) of 200-250 grams were used in this investigation. They were fed on a stock diet (Epol rat cubes) throughout the experiment and received food and tap water as required. A day-night cycle of 12 hours was maintained and the room temperature was kept at 22 ± 1^0C.

We maintained our own breeding program for both control and experimental animals. The animals were mated overnight (12 hours) after which the sires were removed. The females were then randomly assigned to control and experimental groups. The body weight of each female was recorded daily for the next 7 days. A significant mass increase over this time indicated that mating was successful. The pregnant rats were placed in individual straw lined cages for the duration of the study.

The length of gestation averaged 22 days for both control and experimental groups. The treatment procedure and dosage was as follows; the experimental dams received a single dose of 1mg nicotine/kg body weight/day, subcutaneously during pregnancy and lactation. The control dams received the same volume of saline instead of nicotine. This dosage was recommended since research shows that the daily nicotine intake of men and women ranged from 10.5 to 78.6mg (Benowitz and Jacobs, 1984). Thus the nicotine intake of a 60kg female will range from 0.16 to 1.18mg/kg body weight per day (assuming that approximately 90% of the drug is absorbed on inhalation). The exposure to the treatment of nicotine, however, commenced on day 7 of gestation to avoid interference of nicotine with the blastocyte implantation and initial embryonic growth (Maritz and Thomas, 1995) and was continued during lactation up to weaning on day

21 after birth.

2.2.2 Sampling of lung tissue

Lung tissue from 1, 7, 14, 21 and 49 day old rat pups were used in this investigation. The day of birth was designated as day 0. Only lung tissue totally free of overt signs of disease were used for experimentation. Rat pups from each of the five age groups were randomly selected from each of the 10 control litters and 10 nicotine exposed litters. The pups were sacrificed by peritoneally injecting saggital (Kyron Laboratories) in an overdose (5ml/kg body weight).

The lung was rapidly extracted from the thoracic-cavity and immediately placed in a cryo vial (Greiner). Cryo freezing was done by submerging the vial in liquid nitrogen for ± 30 seconds. The procedure was done in less than a minute to prevent enzymatic degradation. After collecting all samples the vials were removed from the liquid nitrogen and stored in a -80 ^{0}C freezer, until used for analysis.

2.2.3 Chemicals

All chemicals used in these assays were purchased from Boehringer Mannhein (Germany).

2.2.4 Preparation of lung tissue

The stored lung samples were removed from the -80^{0}C freezer. The tissue was then homogenized (10mg lung tissue/ml water) for 30 seconds using a Polytron Kinematica PT10/35 and then placed on ice. Tissue debri was removed by centrifuging the homogenate for 20 minutes at 3000g (gravity units) in a Beckman TJ-6 benchtop

centrifuge. The supernatant was stored on ice and was then used to determine the activity of the allosteric enzymes spectrophotometrically using a Beckman DU-640 spectrophotometer with circulating bath to keep the temperature constant. Note, it is essential that the reaction medium within the cuvette and the cuvette be maintained at a constant temperature because, enzyme activity changes with a change in temperature.

2.2.5 Determination of activity of selected allosteric enzymes of lung homogenate of neonatal rat pups

2.2.5.1 Hexokinase

2.2.5.1.1 Principle of method

This assay was based on the protocol described by Joshi and Jagganathan as cited by Wood (1966). The principle of the method was based on the following:

$$\text{Hexose} + \text{ATP} \xrightarrow{Hexokinase} \text{Hexose-6-phosphate} + \text{ADP} \quad \text{(reaction 1)}$$

$$\text{Hexose-6-phosphate} + \text{NADP} \xrightarrow{G6PDH} \text{6-phosphogluconate} + \text{NADPH} \quad \text{(reaction 2)}$$

The assay was performed at 30^0C, the absorption was read at 340nm using a quartz cuvette with a path length of 1cm.

2.2.5.1.2 Procedure

Reaction medium (1.514ml) was made up by adding 0.3ml of each of the following into the cuvette:

- 0.15M Glucose

- 0.20M $MgCl_2.H_2O$
- 0.20M Tris-HCL (pH 7.6)
- 0.10mM Disodium EDTA
- 1.30mM NADP and
- 14*ul* of 2U/ml G6PDH (140U/mg: 1mg/ml)

To the reaction medium the following was added

- 0.6ml distilled water and
- 0.5ml supernatant

The reference blank contained the reaction medium and 1.1ml distilled water of which 0.5ml of the 1.1ml distilled water replaced the supernatant.

To all cuvettes including the reference blank, 0.1ml adenine nucleotide solution (table 2.1 indicate the concentrations of adenine nucleotide solutions used to determine the enzyme activity) was added. All these solutions in the table, had a pH of 7.6.

- Mixed by inversion only
- Total cuvette volume 2.714ml
- Absorbancy was read at 1 minute intervals from the second to the tenth minute after adding ATP

The changes in optical density (OD) was linear. The average changes in OD, due to the conversion of NADP to NADPH (reaction 2; p59), was determined by calculating the difference in value between the final and the initial times for this specific assay.

The activity of the enzyme was calculated as follows: (Method of calculation was supplied by Boehringer)

$\Delta A \times 2.2 = \text{activity (U/L)}$

Table 2.1: Adenine nucleotide concentrations.

Adenine nucleotide	Concentrations
ATP	20mM
ATP	20*u*M
ATP/ADP = 12.5	20*u*M + 1.6*u*M
ATP/ADP = 6.2	20*u*M + 3.2*u*M

2.2.5.2 Phosphofructokinase

2.2.5.2.1 Principle of method

This assay was based on the protocol described by Ling and colleagues as cited by Wood (1966). The principle of the method was based on the following:

Fructose-6-phosphate + ATP $\xrightarrow{PFK}$ fructose-1,6-bisphosphate + ADP (reaction 1)

Fructose-1,6-bisphosphate $\xrightarrow{Aldolase/TIM}$ 2 glyceraldehyde-3-P (reaction 2)

Glyceraldehyde-3-P + $2NAD^+$ $\xrightarrow{GAPDH}$ 2 NADH + $2H^+$ + 1,3-bisphosphoglycerate (reaction 3)

The assay was performed at 25^0C, the absorption was read at 340nm using a quartz cuvette with a path length of 1cm.

2.2.5.2.2 Procedure

Reaction medium (2.355ml) in the cuvette was made up as follows:

- 0.5ml 0.20M Tris-HCL (pH 8.0)
- 0.3ml Adenine nucleotide solution (table 2.1 indicates the concentrations of adenine nucleotide solutions used to determine the enzyme activity) all of these solutions had a pH of 7.0.
- 0.075ml 0.20M $MgSO_4$
- 0.3ml 0.02M Fructose-6-phosphate
- 0.2ml 2.40mM NADH
- 0.75ml 0.20M KCl
- 0.03ml 0.1M Dithiothreitol
- 0.2ml of the following enzyme mixture:

  0.25ml Aldolase (9U/mg: 2mg/2ml) and a 0.5ml solution of TIM (5000U/mg: 2mg/ml) and GAPDH (80U/mg: 100mg/10ml) where the TIM and GAPDH was mixed in a 1:10 dilution. This solution of aldolase, TIM and GAPDH was added to a 4.7ml 0.01M Tris-HCL, pH 8.0 solution.

Added to the reaction medium:

- 0.06ml of supernatant

The reference blank containing the reaction medium and 0.06ml distilled water, the 0.06ml water replaced the supernatant

- Mixed by inversion only
- Total cuvette volume 2.415ml
- Absorbancy was read at 1minute intervals for 3minutes

The changes in OD was linear. The average changes in OD, due to the conversion of NAD^+ to NADH + H^+ (reaction 3; p61), was determined by calculating the difference in value between the initial and the final times for this specific assay.

The activity of the enzyme was calculated as follows: (Method of calculation was supplied by Boehringer Mannheim)

$$\triangle A \times 0.24 = \text{activity (U/L)}$$

2.2.6 Calculations

The final calculation to express the activity of the specific enzymes in U/g lung tissue instead of U/L homogenate was determined as described by the following hypothetical example:

If the lung homogenate was composed of 10mg tissue/ml water, and 60*ul* of supernatant from the homogenate was used in the assay. Thus 0.6mg lung tissue occurred in 60*ul* of supernatant. The total volume (supernatant + reaction medium) in the cuvette was 3ml. Therefore, the weight of the tissue/ml reaction medium in cuvette would thus be:

$$\frac{\text{0.6mg lung tissue}}{\text{3ml cuvette volume}}$$

= 0.2mg lung tissue in 1ml reaction medium (0.2g tissue/L)

The activity of the enzyme was calculated to be 0.003U/L. The final absorbance ($\triangle A$) was multiplied by the specified factor (see method for calculation of activity for each enzyme). If the dilution factor was for example, 50 this was calculated as follow:

$$\frac{\text{total volume of cuvette}}{\text{volume of supernatant used in assay}}$$

(note units should be the same). The activity (0.003U/L) was then multiplied by 50 (the dilution factor) to obtain 0.15U/L. Since there was 0.2g tissue/L and 0.15U/L, there was 0.15U/0.2g lung tissue. Therefore the final activity for the enzyme was calculated to be:

$$\frac{0.15U}{0.2g}$$

= 0.75U/g lung tissue

2.2.7 Statistical analysis

Results were recorded throughout as the mean ± standard error of the mean. For statistical evaluation the unpaired Wilcoxon Rank t-test was employed. The medical statistical package Medcalc (Schoonjans *et al*, 1995) was used. The probability level of $P < 0.05$ was designated as being statistically significant between the mean values of control and experimental (nicotine) groups.

2.3 Results

The objectives of this study was to investigate:

A) the changes in the activity of HK and PFK at different ATP concentrations and ATP/ADP ratios as the lung matures between postnatal days 1 and 49

B) the effect of maternal nicotine exposure during gestation and lactation on the changes in HK and PFK activity

The large number of variables in the study, namely, 5 different age groups (postnatal days 1, 7, 14, 21 and 49), 2 ATP concentrations, 2 ATP/ADP ratios, and the effect of maternal nicotine exposure, makes reporting of the data difficult and could result in confusion. To overcome this, and to prevent loss of data, it was decided to present the data obtained for HK and PFK, in this chapter, as follows:

a) the effect of age on HK and PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups (2.3.1.1 HK and 2.3.2.1 PFK)

b) a comparison of HK and PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group (2.3.1.2 HK and 2.3.2.2 PFK)

c) the effect of age on HK and PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups (2.3.1.3 HK and 2.3.2.3 PFK)

d) a comparison of HK and PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group (2.3.1.4 HK and 2.3.2.4 PFK)

e) the effect of maternal nicotine exposure on HK and PFK activity: a comparison

with the HK and PFK of lungs of control rats (2.3.1.5 HK and 2.3.2.5 PFK)

2.3.1 Hexokinase

2.3.1.1 The effect of age on HK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups

The data in table 2.2 and figure 2.2 show the activity of HK of lung tissue of control rat pups at postnatal days 1, 7, 14, 21 and 49 at 20mM and 20*u*M ATP as well as at ATP/ADP ratios of 12.5 and 6.2. The data clearly show that the HK activity at both ATP concentrations and both ATP/ADP ratios increased ($P<0.001$) between postnatal days 1 and 14. After postnatal day 14 the HK activity gradually decreased ($P<0.001$) to levels on postnatal day 49 that were either lower ($P<0.001$) or equal ($P<0.01$) to that on postnatal day 1. This trend is illustrated by figure 2.2. This figure also clearly illustrates the marked difference in HK activity at 20mM ATP compared to the lower ATP concentration and ATP/ADP ratios. It furthermore shows that the difference in response at 20mM ATP and the lower ATP concentration and ATP/ADP ratios increased as the lung tissue mature up to postnatal day 14.

A more detailed analysis of the data show that the activity of HK at a concentration of 20mM ATP on postnatal day 7 was at 1.03 ± 0.03 U/g tissue, 3 times higher ($P<0.001$) than the 0.34 ± 0.02 U/g tissue of postnatal day 1 old rat pups. On postnatal day 14 the HK activity was at 1.65 ± 0.05 U/g lung tissue, a 4.8 fold increase ($P<0.001$) compared to that of the enzyme activity on postnatal day 1. The enzyme activity only increased 1.6 fold ($P<0.001$) between postnatal days 7 and 14. This implies that most of the activity increase occurred between postnatal days 1 and 7. On postnatal day 21 the

Table 2.2: The effect of age on HK activity (U/g lung tissue ± SEM) of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.34 ± 0.02 (n = 10)	1.03 ± 0.03 (n = 8) P<0.001	1.65 ± 0.05 (n = 8) P<0.001	0.90 ± 0.07 (n = 7) P<0.001	0.20 ± 0.01 (n = 7) P<0.001
20*u*M	0.09 ± 0.006 (n = 9)	0.28 ± 0.02 (n = 7) P<0.001	0.39 ± 0.009 (n = 7) P<0.001	0.23 ± 0.02 (n = 8) P<0.001	0.12 ± 0.004 (n = 8) P<0.01
ATP/ADP					
12.5	0.12 ± 0.005 (n = 7)	0.31 ± 0.02 (n = 7) P<0.001	0.55 ± 0.01 (n = 6) P<0.001	0.17 ± 0.02 (n = 7) P<0.10	0.05 ± 0.003 (n = 10) P<0.001
6.2	0.17 ± 0.003 (n = 7)	0.29 ± 0.02 (n = 6) P<0.01	0.34 ± 0.06 (n =10) P<0.10	0.25 ± 0.01 (n = 9) P<0.001	0.05 ± 0.003 (n = 7) P<0.001

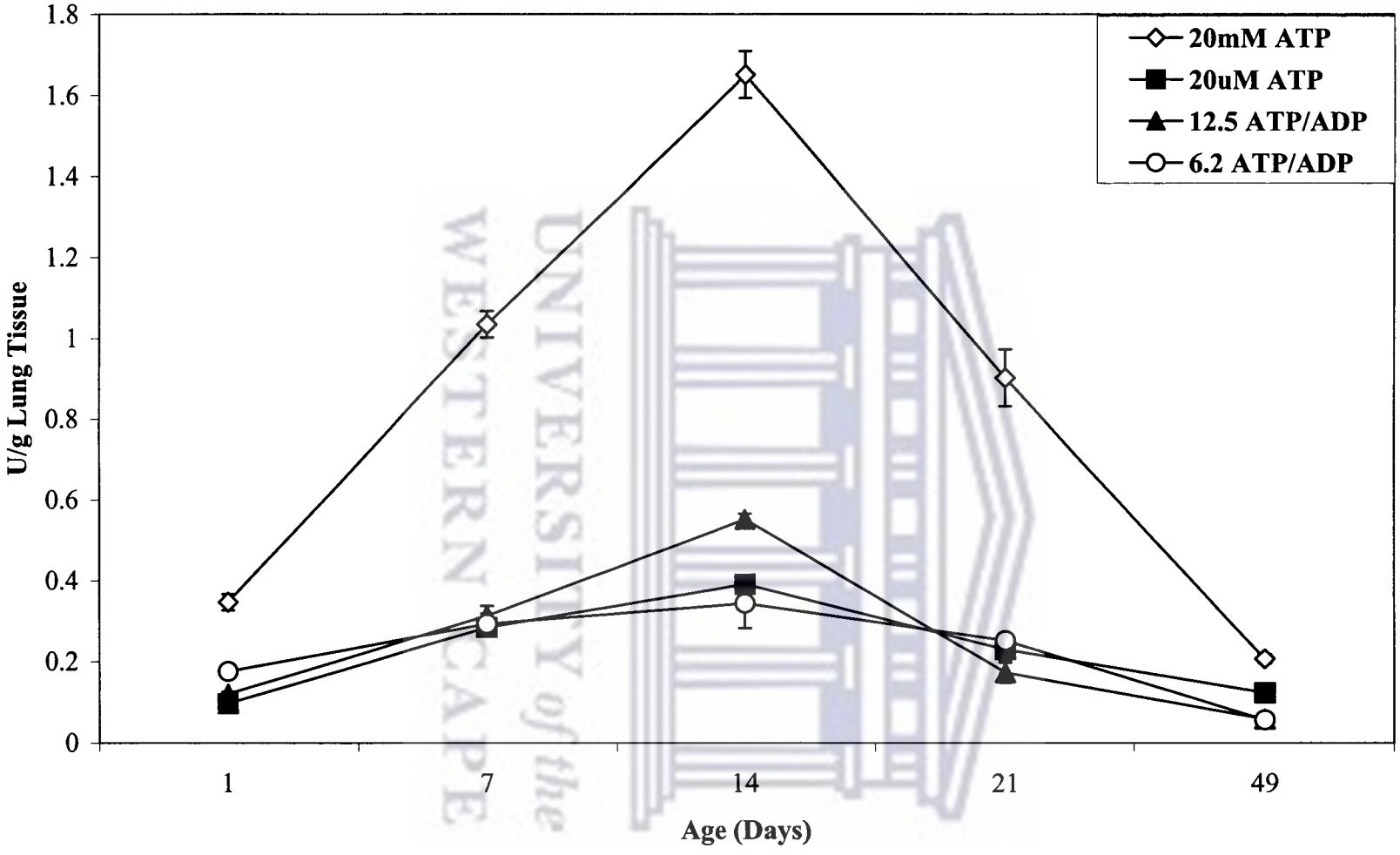

Figure 2.2: The effect of age on HK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

enzyme activity was at 0.90 ± 0.07 U/g tissue, 2.6 times higher (P<0.001) than that of the enzyme activity on postnatal day 1 but 1.8 times lower (P<0.001) than on postnatal day 14. On postnatal day 49 the HK activity was at 0.20 ± 0.01 U/g tissue, 1.7 fold lower (P<0.001), compared to that of the enzyme activity on postnatal day 1. Between postnatal days 21 and 49 the enzyme activity showed a 4.4 fold decrease (P<0.001).

The activity of HK, at 20*u*M ATP was 0.28 ± 0.02 U/g lung tissue on postnatal day 7. This was 2.9 times higher (P<0.001) than the 0.09 ± 0.006 U/g lung tissue of postnatal day 1 pups and on postnatal day 14, the HK activity was at 0.39 ± 0.009 U/g tissue, 4 fold higher (P<0.001) than the activity of the enzyme on postnatal day 1. This means that the enzyme activity showed a 1.4 fold increase (P<0.01) between postnatal days 7 and 14. These results again imply that the activity increase between postnatal days 1 and 7 exceeded the increase between postnatal days 7 and 14. On postnatal day 21 the enzyme activity was at 0.23 ± 0.02 U/g lung tissue, 2.4 times higher (P<0.001) than the enzyme activity on postnatal day 1 so that the activity of HK on postnatal day 7 resembled that activity on postnatal day 21. However, the activity of HK also decreased (P<0.01) by 1.7 fold between postnatal days 14 and 21. On postnatal day 49 the HK activity was at 0.12 ± 0.004 U/g lung tissue, 1.3 fold higher (P<0.01) than that of the enzyme activity on postnatal day 1 despite a decrease (P<0.001) in the enzyme activity of 1.9 fold between postnatal days 21 and 49.

The activity of HK at an ATP/ADP ratio of 12.5 on postnatal day 7 was 0.31 ± 0.02 U/g lung tissue. This was 2.6 times higher (P<0.001) than the 0.12 ± 0.005 U/g lung tissue on postnatal day 1. On day 14 after birth, the HK activity was at 0.55 ± 0.01 U/g lung

tissue, 4.6 times higher ($P<0.001$) when compared to the enzyme activity on day 1 after birth. This means that at this ATP/ADP ratio, the increase in HK activity between postnatal days 1 and 14 resembled the increase at the ATP concentration of 20*u*M. The only difference was that the activity of HK on postnatal day 14 in the presence of an ATP/ADP ratio of 12.5 was higher than at 20*u*M ATP. The enzyme activity increased ($P<0.01$) 1.8 fold between postnatal days 7 and 14. On postnatal day 21 the enzyme activity was at 0.17 ± 0.02 U/g lung tissue, 1.4 times higher ($P<0.10$) than that of day 1 old rat pups after birth, despite the 3.2 fold decrease ($P<0.01$) between postnatal days 14 and 21. The HK activity on postnatal day 49 was at 0.05 ± 0.003 U/g lung tissue, 2.1 times lower ($P<0.001$) than the activity on day 1 after birth. This could be attributed to the fact that the HK activity increased 4.58 fold between postnatal days 1 and 14, but decreased 11 fold between postnatal days 14 and 49.

At a reduced ATP/ADP ratio of 6.2, the activity of HK of lung tissue on postnatal day 7 was 0.29 ± 0.02 U/g lung tissue. This was 1.7 times higher ($P<0.01$) than that of 1 day old pups at 0.17 ± 0.003 U/g lung tissue. On postnatal day 14 the HK activity was at 0.34 ± 0.06 U/g lung tissue, 2 times higher ($P<0.10$) than the enzyme activity of the lung on day 1 after birth. However, the HK activity on postnatal days 7 and 14 was not statistically significant ($P>0.05$). This implies that with the ATP/ADP ratio of 6.2, optimal activity was achieved on postnatal day 7 while optimal activity was achieved on postnatal day 14 at 20*u*M and 20mM ATP and an ATP/ADP ratio of 12.5. On postnatal day 21 the enzyme activity was at 0.25 ± 0.01 U/g lung tissue, 1.4 times higher ($P<0.001$) than the enzyme activity on day 1 after birth despite the decrease ($P<0.001$) of 1.4 fold between postnatal days 14 and 21. On postnatal day 49 the HK activity was

at 0.05 ± 0.003 U/g lung tissue, 3.1 times lower (P<0.001), when compared to that of the enzyme activity on postnatal day 1. The enzyme activity also decreased 4.5 fold (P<0.001) between postnatal days 21 and 49. The activity of HK at this ratio on postnatal days 7, 14 and 21 did not differ significantly from the HK activity at 20*u*M ATP.

2.3.1.2 A comparison of HK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group

From the data summarized in table 2.3 and figure 2.3 it was clear that the highest activity of HK was achieved at 20mM ATP (P<0.001) for all age groups involved. The HK activity at both ATP concentrations and ATP/ADP ratios gradually increased to maximum on postnatal day 14 whereafter it again decreased to relatively low values on postnatal day 49. A more detailed analysis of the data showed that the activity of HK on day 1 after birth was at 0.34 ± 0.02 U/g lung tissue at an ATP concentration of 20mM, 3.6 times higher (P<0.001) than the 0.09 ± 0.006 U/g lung tissue at 20*u*M ATP. At the ATP/ADP ratio of 12.5 the HK activity was 1.2 times higher (P<0.05) at 0.12 ± 0.005 U/g lung tissue than at 20*u*M ATP. At the ATP/ADP ratio of 6.2, the activity of HK was by 1.5 times higher (P<0.001) at 0.17 ± 0.003 U/g lung tissue than at an ATP/ADP ratio of 12.5.

On postnatal day 7 (figure 2.3) at an ATP concentration of 20mM, the HK activity was at 1.03 ± 0.03 U/g lung tissue. This was 3.6 times higher (P<0.001) than the 0.28 ± 0.02 U/g lung tissue at an ATP concentration of 20*u*M. The HK activity at an ATP concentration of 20*u*M and an ATP/ADP ratio of 12.5 and 6.2 were not statistically

Table 2.3: A comparison of HK activity (U/g lung tissue ± SEM) of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.34 ± 0.02 (n = 10)	1.03 ± 0.03 (n = 8)	1.65 ± 0.05 (n = 8)	0.90 ± 0.07 (n = 7)	0.20 ± 0.01 (n = 7)
20*u*M	0.09 ± 0.006 (n = 9) P<0.001	0.28 ± 0.02 (n = 7) P<0.001	0.39 ± 0.009 (n = 7) P<0.001	0.23 ± 0.02 (n = 8) P<0.001	0.12 ± 0.004 (n = 8) P<0.001
ATP/ADP					
12.5	0.12 ± 0.005 (n = 7) P<0.001	0.31 ± 0.02 (n = 7) P<0.001	0.55 ± 0.01 (n = 6) P<0.001	0.17 ± 0.02 (n = 7) P<0.001	0.05 ± 0.003 (n = 10) P<0.001
6.2	0.17 ± 0.003 (n = 7) P<0.001	0.29 ± 0.02 (n = 6) P<0.001	0.34 ± 0.06 (n =10) P<0.001	0.25 ± 0.01 (n = 9) P<0.001	0.05 ± 0.003 (n = 7) P<0.001

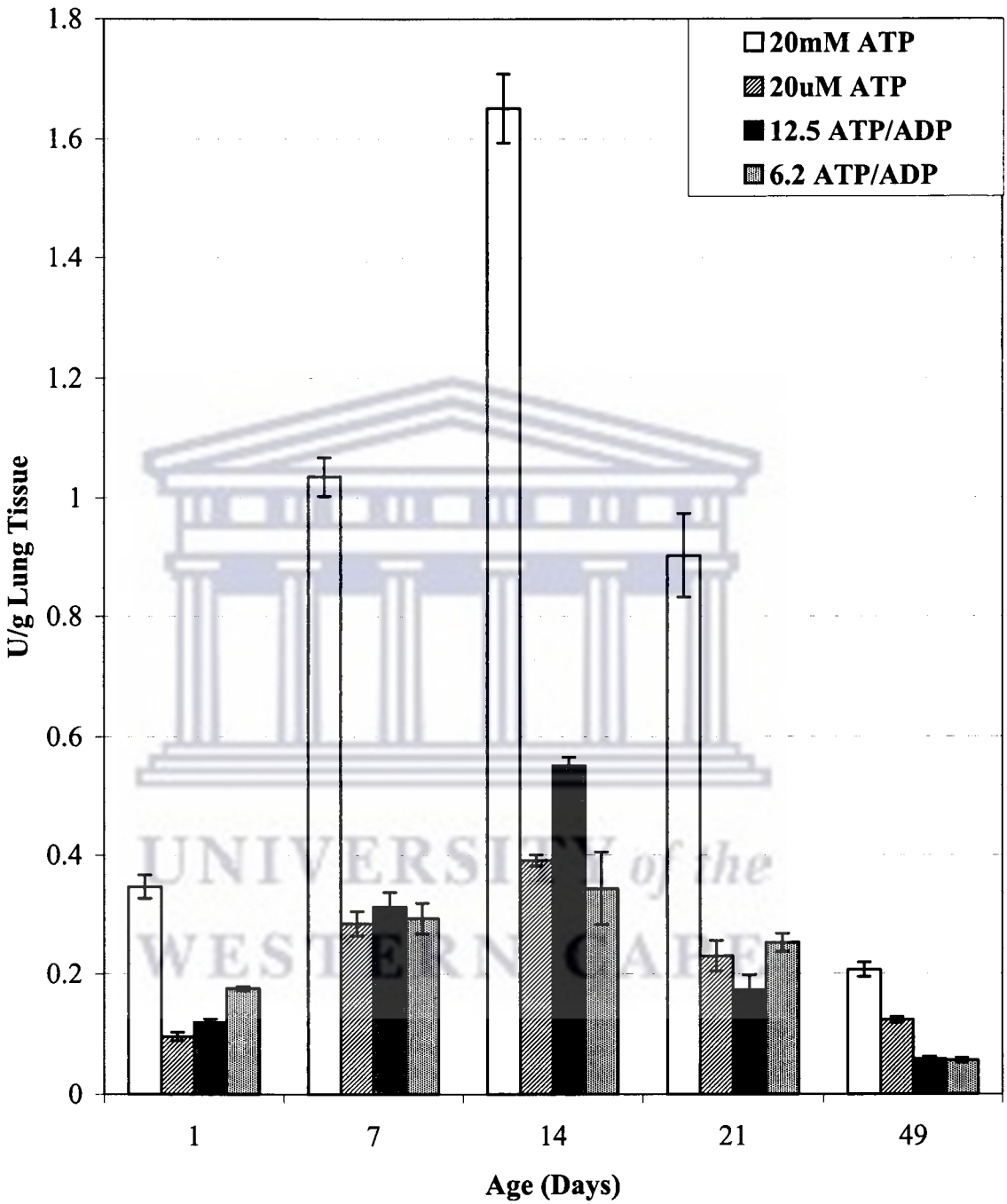

Figure 2.3: A comparison of HK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group.

significant (P>0.10). The activity of HK on postnatal day 14 was 4.2 times higher (P<0.001) at an ATP concentration of 20mM (1.65 ± 0.05 U/g lung tissue), than at a concentration of 20*u*M (0.39 ± 0.009 U/g lung tissue). At the ATP/ADP ratio of 12.5 the HK activity was at 0.55 ± 0.01 U/g lung tissue, 1.4 times higher (P<0.01) than at 20*u*M ATP. However, the enzyme activity was at an ATP/ADP ratio of 12.5, 3 times lower (P<0.001) than the enzyme activity at a concentration of 20mM ATP. At an ATP/ADP ratio of 6.2, the activity of HK was 1.6 times lower (P<0.01) than at an ATP/ADP ratio of 12.5 but corresponds with the 0.39 ± 0.009 U/g lung tissue at 20*u*M ATP. The activity of HK was 4.8 times lower (P<0.001) at an ATP/ADP ratio of 6.2 than the activity of the enzyme at 20mM ATP.

On postnatal day 21 the activity of HK at 20mM ATP was 3.9 times higher (P<0.001) at 0.90 ± 0.07 U/g lung tissue than the 0.23 ± 0.02 U/g lung tissue at 20*u*M ATP. At an ATP/ADP ratio of 12.5 and 6.2, the HK activity of lung tissue was 5.2 and 3.6 times respectively lower (P<0.001) than at 20mM ATP. The HK activity at 20*u*M ATP and an ATP/ADP ratio of 6.2 was not significantly different from each other (P>0.01).

On postnatal day 49, at an ATP concentration of 20mM, the HK activity (0.20 ± 0.01 U/g lung tissue) was 1.7 times higher (P<0.001) than at an ATP concentration of 20*u*M (0.12 ± 0.004 U/g lung tissue). At an ATP/ADP ratio of 12.5 the HK activity was 2.1 times lower (P<0.001) than at a concentration of 20*u*M ATP, and 3.5 times lower (P<0.001) than the enzyme activity at 20mM ATP. At an ATP/ADP ratio of 6.2 the activity of HK was not significantly (P>0.10) different, from that of the enzyme activity at an ATP/ADP ratio of 12.5 (P>0.10).

2.3.1.3 The effect of age on HK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups

The data in table 2.4 and figure 2.4 show the HK activity of nicotine exposed rat pups during pregnancy and lactation on postnatal days 1, 7, 14, 21 and 49 at 20mM and 20*u*M ATP as well as, ATP/ADP ratios of 12.5 and 6.2. The data clearly show that the HK activity at an ATP concentration of 20mM and an ATP/ADP ratio of 6.2 gradually increased ($P<0.001$) between postnatal days 1 and 14 to reach maximum activity on postnatal day 14. However, at an ATP concentration of 20*u*M and an ATP/ADP ratio of 12.5, maximum activity of the enzyme was reached on postnatal day 7 and it plateaued up to day 14. After postnatal day 14 the HK activity gradually decreased to levels on postnatal day 49 that were lower ($P<0.001$) than on postnatal day 1.

A more detailed analysis of the data show that the activity of HK at a concentration of 20mM ATP increased rapidly from 0.41 ± 0.02 U/g lung tissue on postnatal day 1 to 1.68 ± 0.05 U/g lung tissue on postnatal day 7 ($P<0.001$) and to 2.54 ± 0.12 U/g lung tissue on postnatal day 14 ($P<0.001$). This represents a 6.2 fold increase between postnatal days 1 and 14. Most of the increase however, occurred between postnatal days 1 and 7 (4.1 fold). On postnatal day 21 the enzyme activity was at 0.78 ± 0.04 U/g lung tissue, 1.9 times higher ($P<0.001$) than the enzyme activity on postnatal day 1 despite the 3.2 fold decrease ($P<0.01$) between postnatal days 14 and 21. The enzyme activity however, decreased ($P<0.001$) further by 3.7 fold between postnatal days 21 and 49 to 0.21 ± 0.01 U/g lung tissue. On postnatal day 49 the HK activity at 20mM ATP was 1.9 times lower ($P<0.001$) than the enzyme activity on postnatal day 1.

Table 2.4: The effect of age on HK activity (U/g lung tissue ± SEM) of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.41 ± 0.02 (n = 10)	1.68 ± 0.05 (n = 7) P<0.001	2.54 ± 0.12 (n = 6) P<0.001	0.78 ± 0.04 (n = 7) P<0.001	0.21 ± 0.01 (n = 7) P<0.001
20*u*M	0.15 ± 0.006 (n = 9)	0.47 ± 0.04 (n = 9) P<0.001	0.42 ± 0.06 (n = 8) P<0.001	0.14 ± 0.008 (n = 7) P<0.10	0.04 ± 0.002 (n = 9) P<0.001
ATP/ADP					
12.5	0.14 ± 0.009 (n = 10)	0.43 ± 0.02 (n = 6) P<0.001	0.42 ± 0.02 (n = 7) P<0.001	0.20 ± 0.01 (n = 8) P<0.01	0.07 ± 0.003 (n = 9) P<0.001
6.2	0.16 ± 0.008 (n = 10)	0.42 ± 0.02 (n = 6) P<0.001	0.54 ± 0.05 (n = 10) P<0.001	0.22 ± 0.008 (n = 8) P<0.001	0.04 ± 0.001 (n = 6) P<0.001

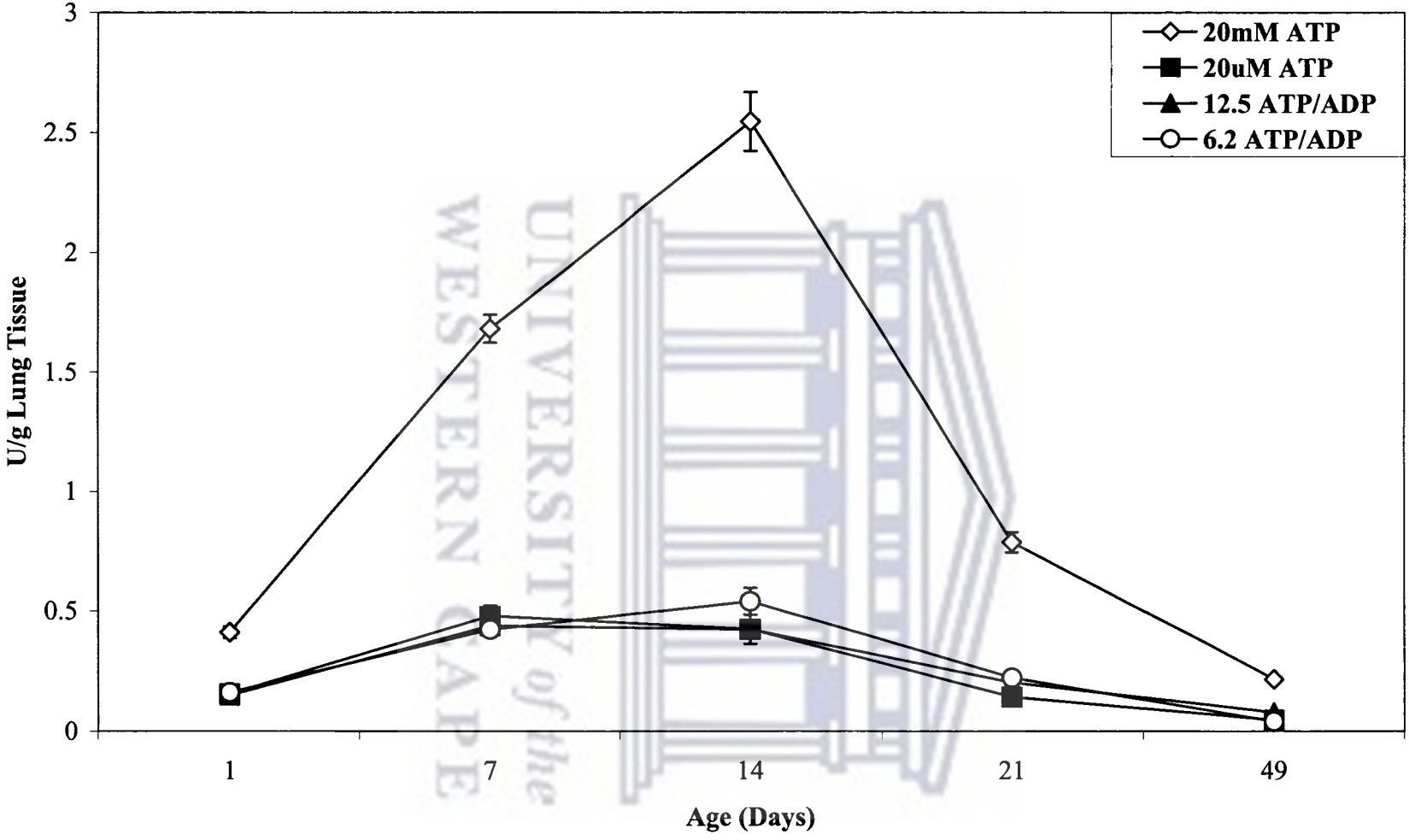

Figure 2.4: The effect of age on HK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

At a concentration of 20*u*M ATP, the activity of HK on postnatal day 7 was 0.47 ± 0.04 U/g lung tissue. This was 3.1 times higher ($P<0.001$) than the activity of the enzyme of 0.15 ± 0.006 U/g lung tissue at day 1 after birth. The enzyme activity on postnatal day 14 at 0.42 ± 0.06 U/g lung tissue did not differ significantly ($P>0.01$) from the activity on postnatal day 7. After postnatal day 14, the HK activity decreased ($P<0.001$) 3 fold to 0.14 ± 0.008 U/g lung tissue on postnatal day 21 which resembled the activity of postnatal day 1 ($P>0.05$). The HK activity was on postnatal day 49 (0.04 ± 0.002 U/g lung tissue) 3.3 times lower ($P<0.001$) than the activity on day 1 after birth. This was due to a 3 fold decrease in HK activity ($P<0.001$) between postnatal days 21 and 49.

At an ATP/ADP ratio of 12.5, the HK activity of lung tissue of one day old nicotine exposed rat pups was 0.14 ± 0.009 U/g lung tissue. On postnatal day 7 it was 0.43 ± 0.02 U/g lung tissue. This means that the activity of HK increased ($P<0.001$) 2.9 fold during the first 7 days after birth. However, between postnatal days 7 and 14 the activity of HK remained unchanged ($P>0.10$). After postnatal day 14 the enzyme activity decreased ($P<0.001$) to 0.20 ± 0.01 U/g lung tissue on postnatal day 21. This was still 1.4 times higher ($P<0.01$) than the activity of the enzyme on day 1 after birth. However, due to a constant decline in HK activity between postnatal days 14 and 49, the HK activity on postnatal day 49 was at 0.07 ± 0.003 U/g lung tissue, 1.9 fold ($P<0.001$) less than on postnatal day 1.

At an ATP/ADP ratio of 6.2 the activity of HK increased ($P<0.001$) from postnatal day 1 (0.16 ± 0.008 U/g lung tissue) to postnatal day 7 (0.42 ± 0.02 U/g lung tissue). This

represents a 2.6 fold increase in HK. Between postnatal days 7 and 14 the HK activity increased to 0.54 ± 0.05 U/g lung tissue to give a total of 3.4 fold increase between postnatal days 1 and 14. Although the 1.3 fold increase between postnatal days 7 and 14 was only half of the 2.6 fold increase between postnatal days 1 and 7, it was still significant ($P<0.05$). After postnatal day 14 the HK activity decreased ($P<0.001$) to 0.22 ± 0.008 U/g lung tissue on postnatal day 21, but was still 1.4 times higher ($P<0.001$) than on postnatal day 1. The HK activity was 4 times lower ($P<0.001$) on postnatal day 49 at 0.04 ± 0.001 U/g lung tissue compared to postnatal day 1. Between postnatal day 21 and postnatal day 49 the HK activity decreased 5.5 fold ($P<0.001$).

2.3.1.4 A comparison of HK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group

The data in table 2.5 and figure 2.5 displays the influence of the change in the ATP concentrations and the ATP/ADP ratios on the HK activity for nicotine exposed rat pups. It was interesting to note that at an ATP concentration of 20mM, the HK activity was markedly higher ($P<0.001$) than at 20*u*M ATP or at the ATP/ADP ratios of 12.5 and 6.2. The HK activity also gradually increased from postnatal day 1 to reach maximum activity on postnatal day 14 at an ATP concentration of 20mM and at an ATP/ADP ratio of 6.2. However, maximum activity was reached on postnatal day 7 and plateaued up to postnatal day 14 at an ATP concentration of 20*u*M and an ATP/ADP ratio of 12.5.

Further analysis of the data show that the activity of HK on postnatal day 1 was 2.6 times higher ($P<0.001$) at a concentration of 20mM ATP (0.41 ± 0.02 U/g lung tissue)

Table 2.5: A comparison of HK activity (U/g lung tissue ± SEM) of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.41 ± 0.02 (n = 10)	1.68 ± 0.05 (n = 7)	2.54 ± 0.12 (n = 6)	0.78 ± 0.04 (n = 7)	0.21 ± 0.01 (n = 7)
20*u*M	0.15 ± 0.006 (n = 9) P<0.001	0.47 ± 0.04 (n = 9) P<0.001	0.42 ± 0.06 (n = 8) P<0.001	0.14 ± 0.008 (n = 7) P<0.001	0.04 ± 0.002 (n = 9) P<0.001
ATP/ADP					
12.5	0.14 ± 0.009 (n = 10) P<0.001	0.43 ± 0.02 (n = 6) P<0.01	0.42 ± 0.02 (n = 7) P<0.01	0.20 ± 0.01 (n = 8) P<0.001	0.07 ± 0.003 (n = 9) P<0.001
6.2	0.16 ± 0.008 (n = 10) P<0.001	0.42 ± 0.02 (n = 6) P<0.01	0.54 ± 0.05 (n = 10) P<0.001	0.22 ± 0.008 (n = 8) P<0.001	0.04 ± 0.001 (n = 6) P<0.01

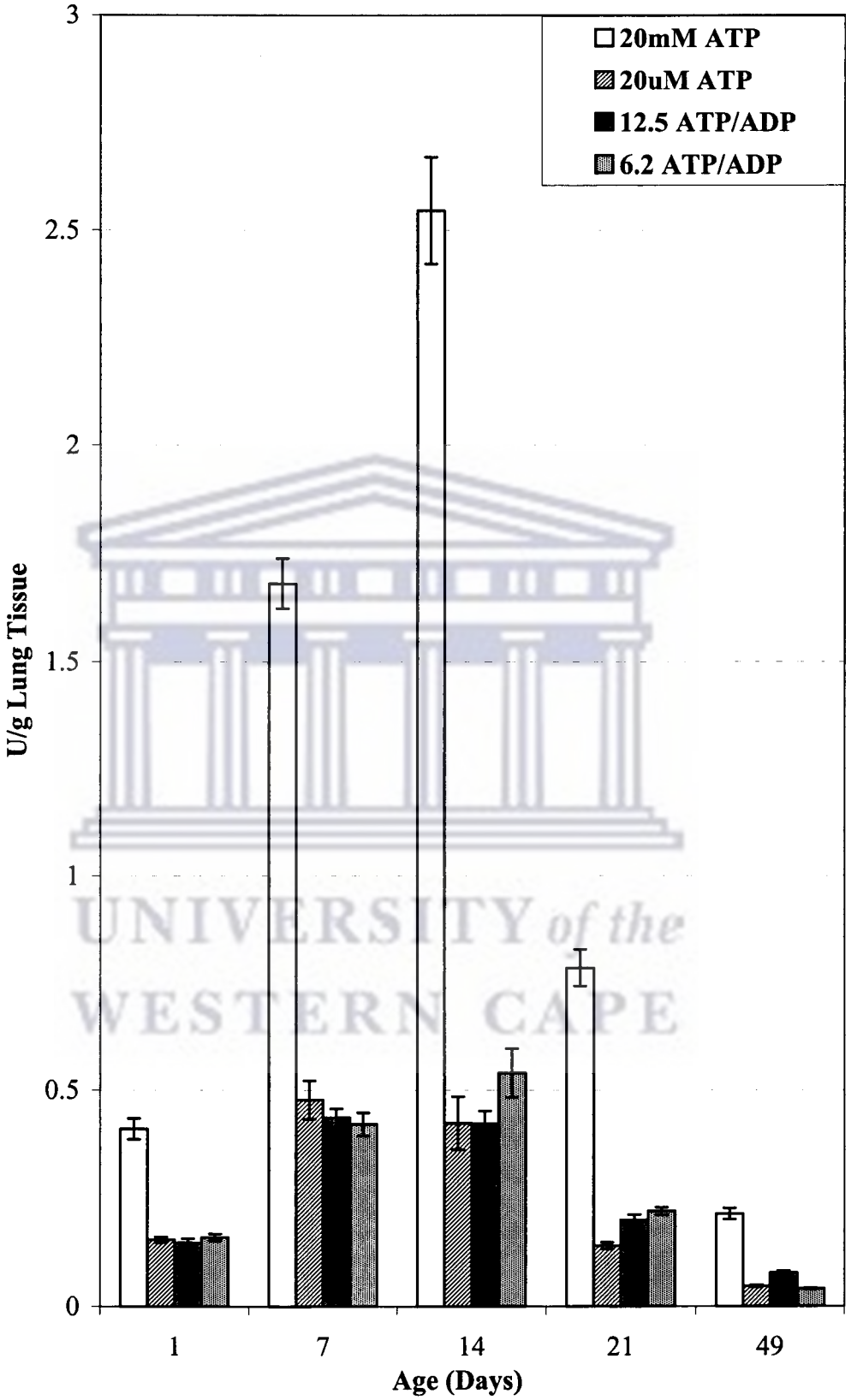

Figure 2.5: A comparison of HK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group.

than at 20*u*M ATP (0.15 ± 0.006 U/g lung tissue). There was no significant difference (P>0.01) in the HK activity between 20*u*M ATP and the ATP/ADP ratios of 12.5 and 6.2.

On postnatal day 7 the HK activity was at an ATP concentration of 20mM (1.68 ± 0.05 U/g lung tissue), 3.5 times higher (P<0.001) compared to the enzyme activity at an ATP concentration of 20*u*M (0.47 ± 0.04 U/g lung tissue). However, no significant difference was observed between the HK activity of 20*u*M ATP, an ATP/ADP ratio of 12.5 and 6.2 (P>0.10).

On postnatal day 14 the HK activity at an ATP concentration of 20mM was at 2.54 ± 0.12 U/g lung tissue, 6 times higher (P<0.001) than the enzyme activity at an ATP concentration of 20*u*M (0.42 ± 0.06 U/g lung tissue). However, no significant difference was observed between the HK activity of 20*u*M ATP and an ATP/ADP ratio of 12.5 (P>0.10). The HK activity was also 4.7 times lower (P<0.001) at an ATP/ADP ratio of 6.2 than at 20mM ATP.

On postnatal day 21 the activity of HK at a 20mM concentration of ATP was at 0.78 ± 0.04 U/g lung tissue, 5.6 times higher (P<0.001) than the enzyme activity of 0.14± 0.008 U/g lung tissue at 20*u*M ATP. At an ATP/ADP ratio of 6.2 the activity of the enzyme was not significantly different than the enzyme activity at an ATP/ADP ratio of 12.5 (P>0.10). It therefore means that at both the ratios, the activity of HK was 3.9 times lower (P<0.001)than in the presence of 20mM ATP. On the other hand, the HK activity at these two ATP/ADP ratios was 1.4 times higher (P<0.01) than at 20*u*M ATP.

On postnatal day 49, at an ATP concentration of 20mM, the HK activity of 0.21 ± 0.01 U/g lung tissue was 4.6 times higher (P<0.001) than the activity of 0.04 ± 0.002 U/g lung tissue at 20*u*M ATP. At an ATP/ADP ratio of 12.5 the HK activity was 1.7 times higher (P<0.001) at 0.07 ± 0.003 U/g lung tissue than the activity of the enzyme at an ATP concentration of 20*u*M and 2.8 times lower (P<0.001) than the enzyme activity at 20mM ATP. At an ATP/ADP ratio of 6.2 the activity of HK had decreased (P<0.001) 1.9 fold from an ATP/ADP ratio of 12.5 and the activity of HK was 5.3 times lower (P<0.01) at an ATP/ADP ratio of 6.2 than the activity of the enzyme at 20mM ATP.

2.3.1.5 The effect of maternal nicotine exposure on HK activity: a comparison with the HK activity of lungs of control rats

The data in figures 2.6 to 2.9 clearly shows that the HK activity of lung tissue of control rat pups increased between postnatal days 1 and 14 . Between postnatal days 14 and 49 the HK activity gradually decreased. This trend in the development of HK activity was the same for both ATP concentrations and both the ATP/ADP ratios. Unlike the HK activity of lung tissue of control rat pups, the trend in the change in HK activity of lung tissue of nicotine exposed rat pups were not the same in the presence of different ATP concentrations and ATP/ADP ratios.

At 20mM ATP (figure 2.6), the activity of HK in nicotine exposed lung followed the same developmental trend than in lung tissue of control rats. However, on postnatal days 7 and 14, the HK activity of lung tissue of nicotine exposed lungs was higher (P<0.001) than that of control animals. No difference occurred on postnatal days 1, 21, and 49. At an ATP concentration of 20*u*M (figure 2.7), the HK in nicotine exposed lung

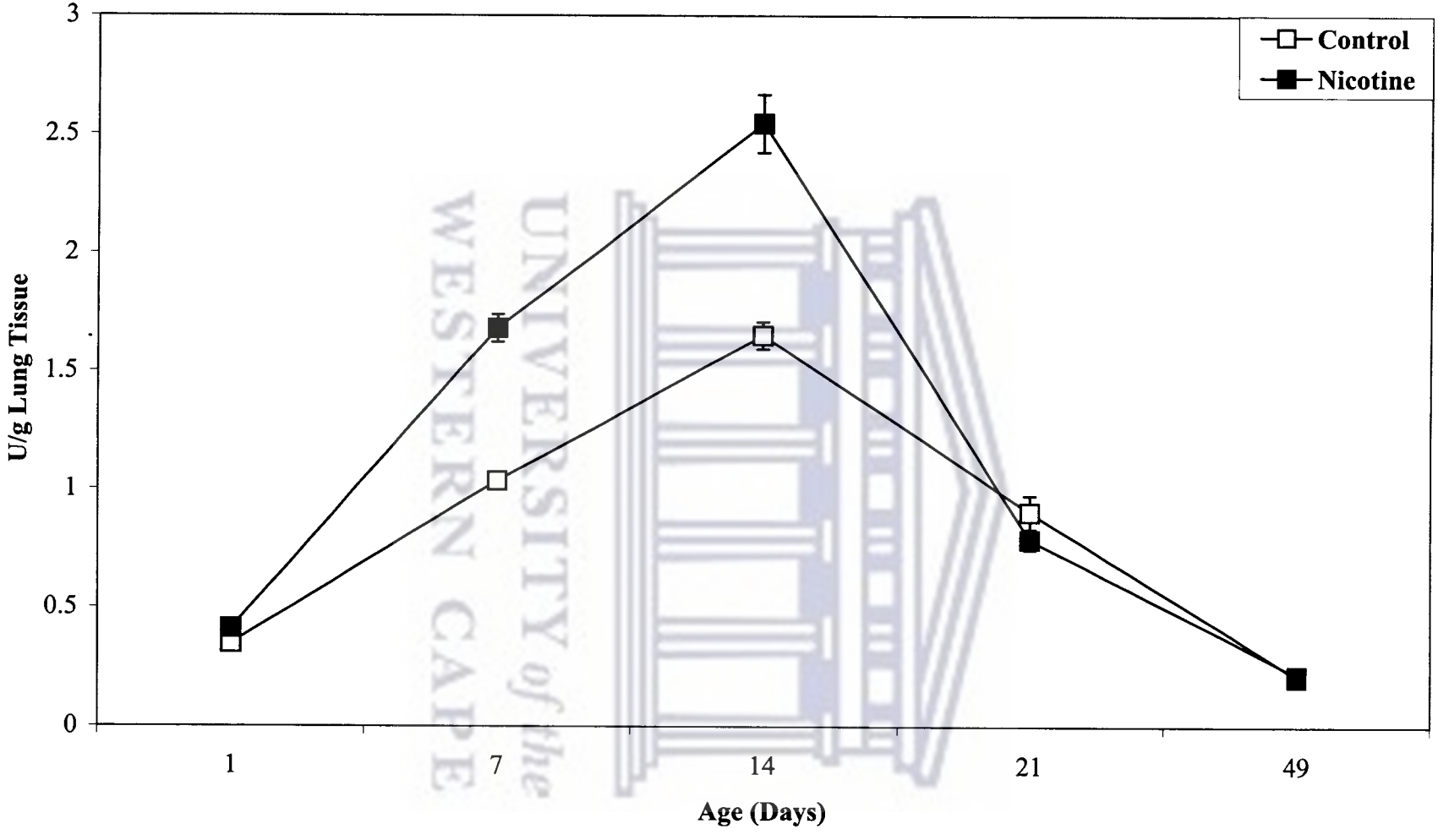

Figure 2.6: The influence of maternal nicotine exposure on the HK activity at an ATP concentration of 20mM.

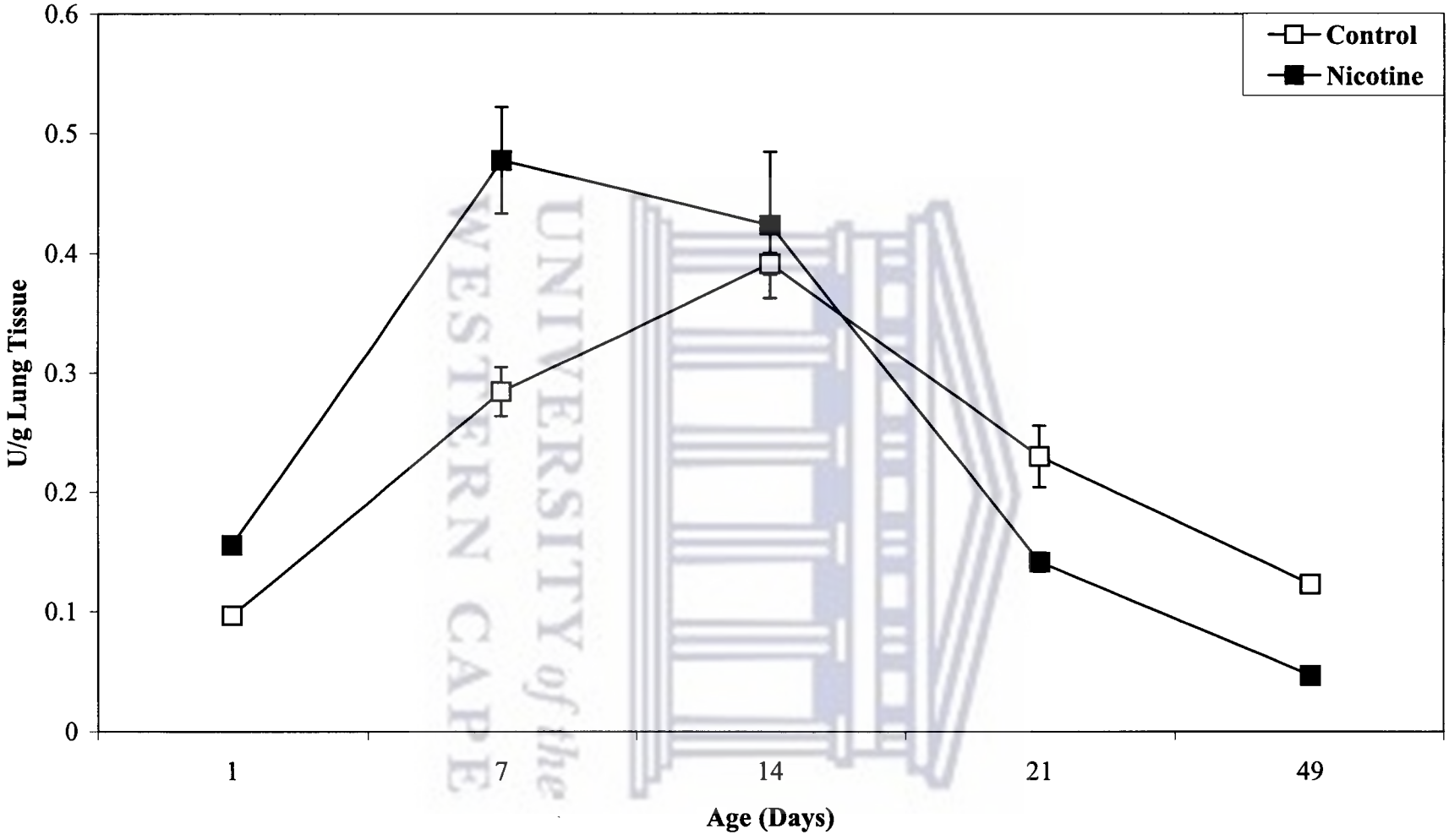

Figure 2.7: The influence of maternal nicotine exposure on the HK activity at an ATP concnetration of 20uM.

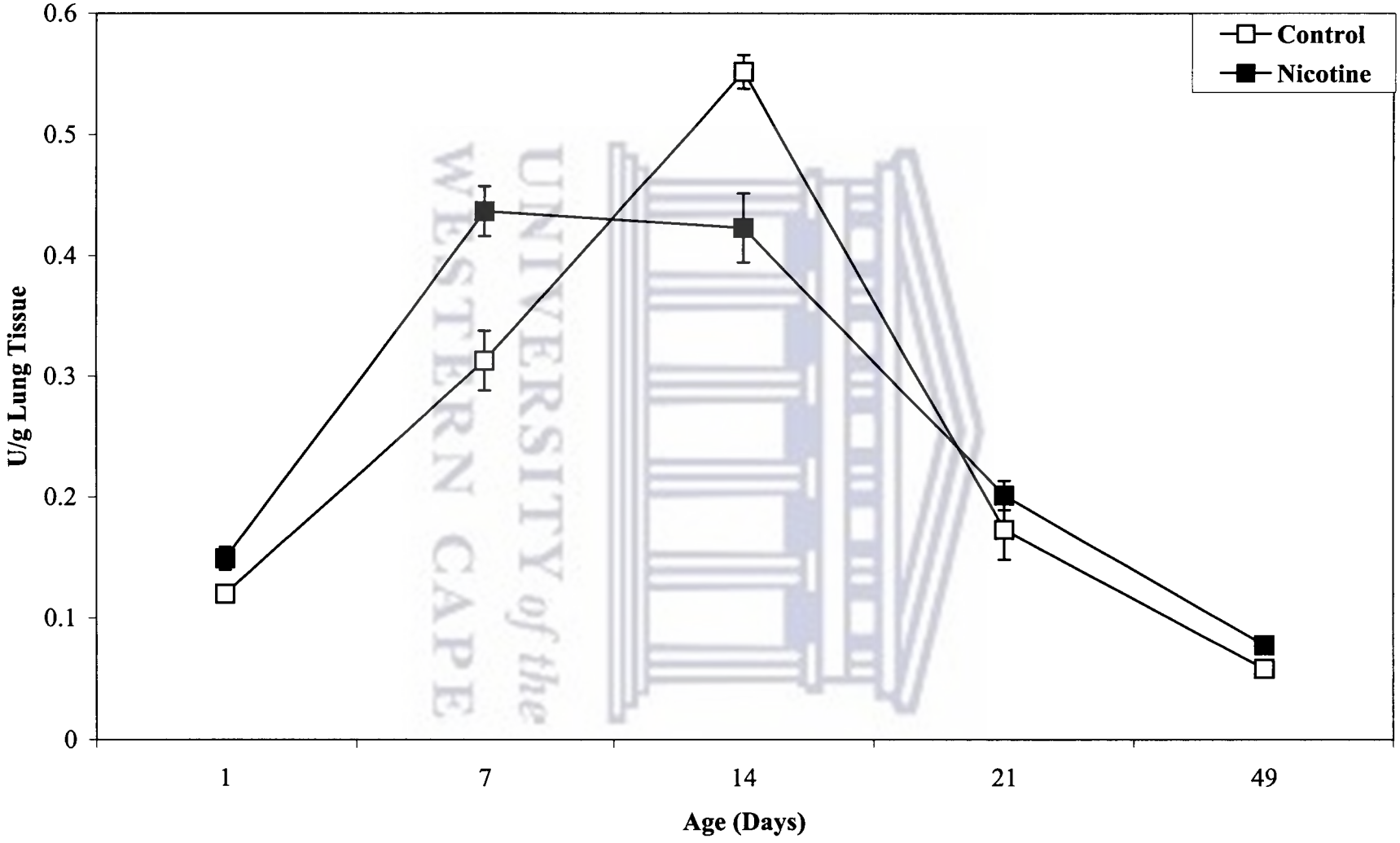

Figure 2.8: The influence of maternal nicotine exposure on the HK activity at an ATP/ADP ratio of 12.5.

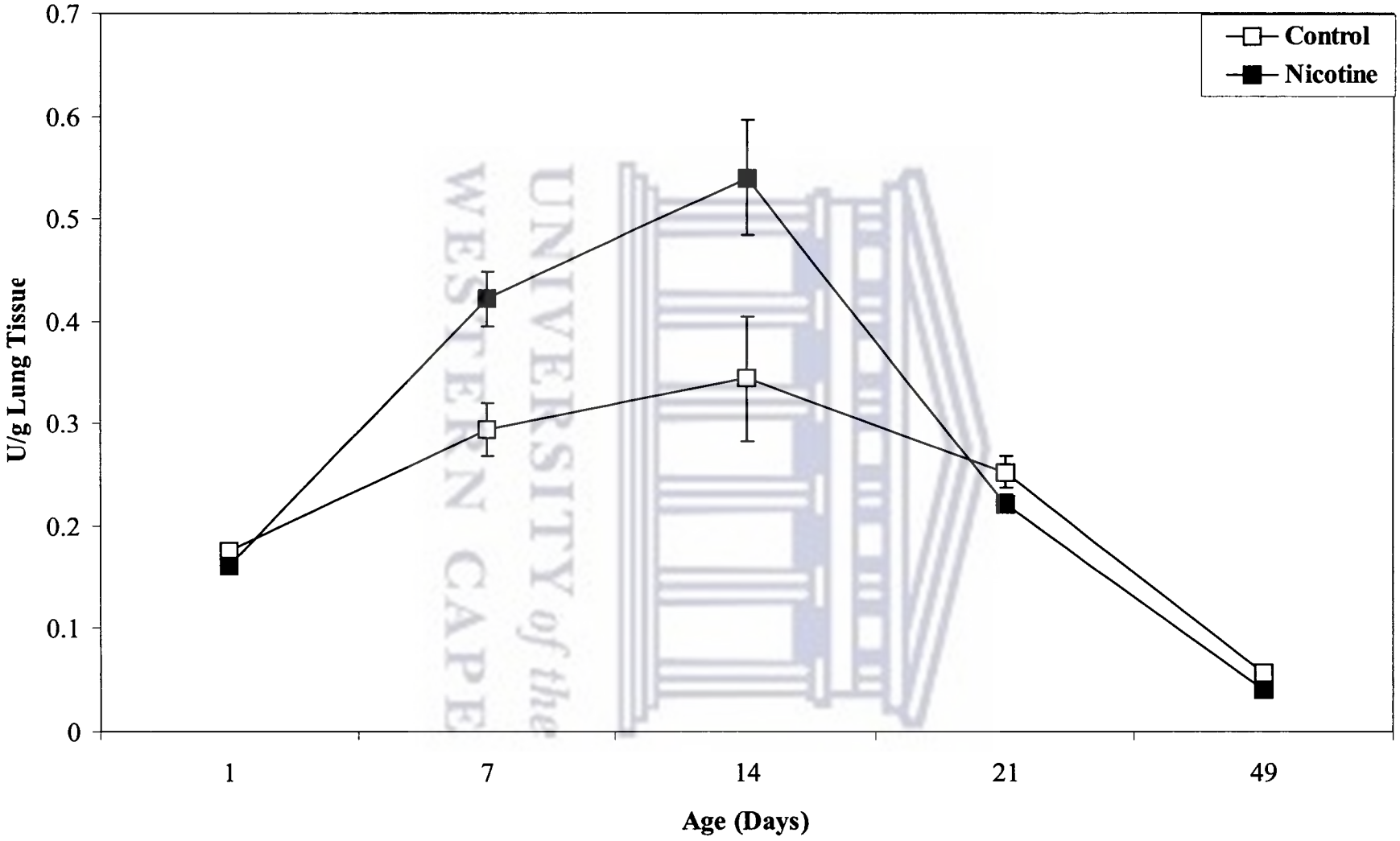

Figure 2.9: The influence of maternal nicotine exposure on the HK activity at an ATP/ADP ratio of 6.2.

reached maximum activity on postnatal day 7 and remained at this level up to postnatal day 14. This is in contrast to the HK activity of control lung which reached maximal activity on postnatal day 14. The activity of HK in lung tissue of 7 and 14 day old nicotine exposed animals was not different from that of the 14 day old control rats. However, between postnatal days 14 and 49 the HK activity of lung tissue of nicotine exposed rats decreased faster than in control lungs over the same period. Consequently the HK activity of lung tissue of control animals, was higher on postnatal days 21 and 49 than that of the nicotine exposed animals.

As for the activity of HK of lung tissue of nicotine exposed rat pups at 20*u*M ATP, the activity of HK at an ATP/ADP ratio of 12.5 also reached maximal activity on postnatal day 7, and also maintained the same level of activity between postnatal days 7 and 14 (figure 2.8). However, while the activity of HK of lung tissue of control rats was lower than that of 1 and 7 day old nicotine exposed rat pups, it was significantly higher ($P<0.001$) on postnatal day 14. This is the only time that the activity of HK of lung tissue of 14 day old control animals was higher than that of the nicotine exposed animals. On postnatal day 21 there was no difference in HK activity of control and nicotine exposed lung. However, on postnatal day 49 the HK activity of control lung was lower ($P<0.01$) than that of the nicotine exposed animals.

Similar trends in the change of HK activity of lung tissue in nicotine exposed animals in the presence of an ATP/ADP ratio of 6.2 (figure 2.9) and the 20mM ATP (figure 2.6) could be seen. Maximal activity was also reached on postnatal day 14 and the activity of lung tissue of postnatal day 1 nicotine exposed rat pups was not different from that

of the control animals (figures 2.6 and 2.9). However, the rate at which the activity of HK increase between postnatal days 1 and 14 was higher in lung tissue of nicotine exposed rats than in the control animals so that the difference on postnatal day 14 was higher than on postnatal day7 (figures 2.6 and 2.9). Between postnatal days 14 and 49, the HK activity again decreased so that on postnatal day 49 no difference was observed between control and nicotine exposed animals.

2.3.2 Phosphofructokinase

2.3.2.1 The effect of age on PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups

The data in table 2.6 and figure 2.10 show the activity of PFK of lung tissue of control rat pups at postnatal days 1, 7, 14, 21 and 49 at 20mM and 20*u*M ATP as well as at ATP/ADP ratios of 12.5 and 6.2. The data summarized in table 2.6 and figure 2.10 show that the PFK activity at a concentration of 20mM ATP, start to increase on day 1 after birth and continue to increase to day 14 after birth ($P<0.001$). While maximum activity at 20mM ATP was achieved on postnatal day 14, the PFK activity at 20*u*M ATP reached maximum activity on postnatal day 7. The PFK activity at both the ATP/ADP ratios decreased between postnatal days 1 and 7, but reached it's maximum activity on postnatal day 14. In all instances the PFK activity decreased between postnatal days 14 and 21 after which it again increased between postnatal days 21 and 49. This trend is illustrated by figure 2.10.

Further analysis of the data show that the activity of PFK at an ATP concentration of 20mM on postnatal days 1 and 7 after birth did not significantly differ ($P>0.10$).

Table 2.6: The effect of age on PFK activity (U/g lung tissue ± SEM) of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.07 ± 0.008 (n = 9)	0.08 ± 0.004 (n = 7) P>0.10	3.51 ± 0.45 (n = 8) P<0.001	0.09 ± 0.007 (n = 9) P>0.10	0.86 ± 0.05 (n = 8) P<0.001
20*u*M	0.09 ± 0.008 (n = 10)	1.36 ± 0.07 (n = 8) P<0.001	1.07 ± 0.15 (n = 10) P<0.001	0.32 ± 0.06 (n = 9) P<0.001	0.75 ± 0.04 (n = 10) P<0.001
ATP/ADP					
12.5	0.43 ± 0.02 (n = 6)	0.34 ± 0.03 (n = 7) P<0.10	2.27 ± 0.38 (n = 7) P<0.01	0.09 ± 0.02 (n = 10) P<0.001	0.34 ± 0.04 (n = 8) P>0.10
6.2	0.79 ± 0.07 (n = 10)	0.24 ± 0.02 (n = 9) P<0.001	2.34 ± 0.22 (n = 7) P<0.001	0.10 ± 0.01 (n = 10) P<0.001	0.33 ± 0.02 (n = 9) P<0.001

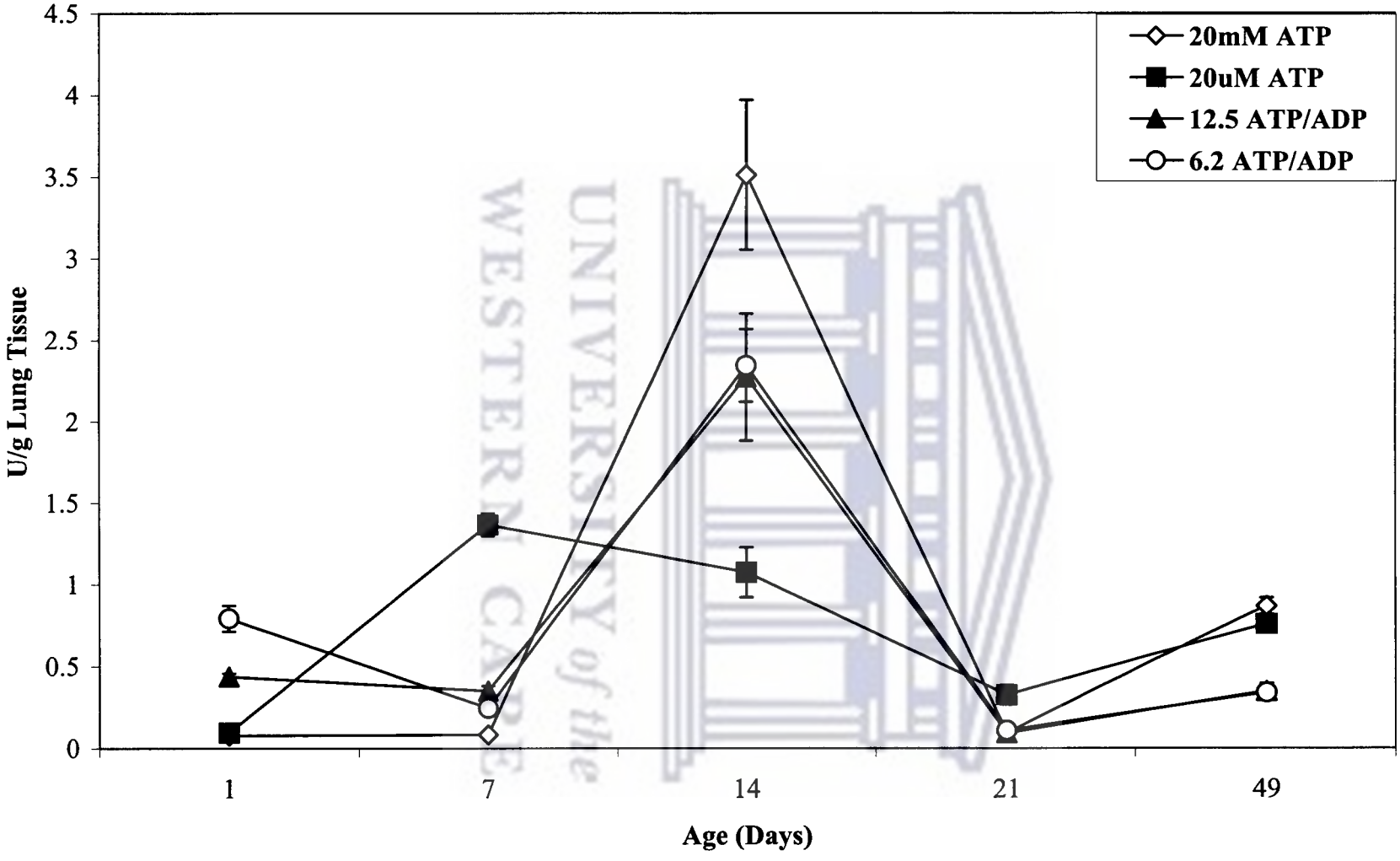

Figure 2.10: The effect of age on PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

However, between postnatal days 7 and 14 activity of PFK rapidly increased to 3.51 ± 0.45 U/g lung tissue resulting in a 45.7 fold higher ($P<0.001$) enzyme activity on postnatal day 14 than on day 1 after birth. Between postnatal days 14 and 21 the PFK activity again drastically decreased 37 fold to 0.09 ± 0.007 U/g lung tissue, to resemble the activity ($P>0.10$) on day 1 after birth. On postnatal day 49 the PFK activity was at 0.86 ± 0.05 U/g lung tissue, 11.3 times higher ($P<0.001$) than the enzyme activity on postnatal day 1 and 9.2 times higher ($P<0.001$) than on day 21 after birth.

At an ATP concentration of 20*u*M, the PFK activity was at 1.36 ± 0.07 U/g lung tissue on postnatal day 7, 14.3 times higher ($P<0.001$) than the lung tissue of postnatal day 1 pups at 0.09 ± 0.008 U/g lung tissue. The activity of PFK on postnatal day 14 was 1.07 ± 0.15 U/g lung tissue. Although the activity of PFK on day 14 appears to be lower than on postnatal day 7, the difference was not significant ($P>0.10$) which implies the activity remained unchanged during this phase of lung development. On postnatal day 21 the enzyme activity was at 0.32 ± 0.06 U/g lung tissue, 3.4 times higher ($P<0.001$) than the enzyme activity on postnatal day 1 but 3.3 times lower ($P<0.001$) than on postnatal day 14. On postnatal day 49 the PFK activity which was at 0.75 ± 0.04 U/g lung tissue was 7.9 times higher ($P<0.001$) than the enzyme activity at day 1 after birth. Thus, between postnatal days 21 and 49 the PFK activity had increased ($P<0.001$) by 2.3 fold.

PFK activity of both ATP/ADP ratios displayed the same trend as for the 20mM ATP concentration. At an ATP/ADP ratio of 12.5 the PFK activity decreased ($P<0.10$) from 0.43 ± 0.02 U/g lung tissue on postnatal day 1 to 0.34 ± 0.03 U/g lung tissue on

postnatal day 7. On postnatal day 14 the PFK activity was at 2.27 ± 0.38 U/g lung tissue, 5.2 times higher ($P<0.01$) than on day 1 after birth. Between postnatal days 7 and 14, the PFK activity increased ($P<0.001$) 6.5 fold. Contrary to the PFK activity between postnatal days 7 and 14, the activity of the enzyme drastically decreased 24.3 fold ($P<0.001$) between days 14 and 21(0.09 ± 0.02 U/g lung tissue). On postnatal day 49, the PFK activity at 0.34 ± 0.04 U/g lung tissue was not different from the enzyme activity at postnatal day 1. However, between day 21 after birth and day 49 after birth, the PFK activity again increased ($P<0.001$), 3.7 fold to 0.34 ± 0.04 U/g lung tissue. The PFK activity on postnatal days 1 and 7 after birth thus corresponds with the activity on day 49 after birth.

At a reduced ATP/ADP ratio of 6.2, the PFK activity of lung tissue of postnatal day 1 rat pups was at 0.79 ± 0.07 U/g lung tissue, 3.2 times higher ($P<0.001$) than that of postnatal day 7 pups at 0.24 ± 0.02 U/g lung tissue. The enzyme activity on postnatal day 14 was at 2.34 ± 0.22 U/g lung tissue, 3 times higher ($P<0.001$) than that of postnatal day 1, but due to the decrease in activity between postnatal days 1 and 7 it was 9.6 times higher ($P<0.001$) than on day 7 after birth. On postnatal day 21 the PFK activity was at 0.10 ± 0.01 U/g lung tissue, 7.2 times lower ($P<0.001$) than on postnatal day 1. The activity of PFK however, again increased ($P<0.001$) 3.1 fold to 0.33 ± 0.02 U/g lung tissue from postnatal day 21, but the PFK activity at postnatal day 49 was at 0.33 ± 0.02 U/g lung tissue, 2.3 times lower ($P<0.001$) than that of day 1 after birth at 0.79 ± 0.07 U/g lung tissue.

2.3.2.2 A comparison of PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group

From the data summarized in the table 2.7 and figure 2.11 it was clear that the highest activity of PFK was achieved at 20mM ATP, on postnatal day 14. However, on postnatal days 1, 7, 21 and 49 the activity of PFK at a concentration of 20mM ATP was either lower ($P<0.001$) or equal ($P>0.10$) to 20*u*M ATP or an ATP/ADP ratio of 12.5 or 6.2.

The data furthermore showed that the activity of PFK on day 1 after birth, at 0.07 ± 0.008 U/g lung tissue at an ATP concentration of 20mM was 1.2 times lower ($P<0.10$) than that of a concentration of 20*u*M ATP at 0.09 ± 0.008 U/g lung tissue. At an ATP/ADP ratio of 12.5 the PFK activity was at 0.43 ± 0.02 U/g lung tissue 4.6 times higher than at a concentration of 20*u*M ATP, and was 5.7 times higher ($P<0.001$) than the enzyme activity at a concentration of 20mM ATP. At an ATP/ADP ratio of 6.2 the activity of PFK was at 0.79 ± 0.07 U/g lung tissue, 1.8 times higher ($P<0.05$) than at an ATP/ADP ratio of 12.5. The activity of PFK was 10.3 times higher ($P<0.001$) at an ATP/ADP ratio of 6.2 than the activity of the enzyme at 20mM ATP.

On postnatal day 7 and at an ATP concentration of 20mM, the PFK activity was 16.5 times lower ($P<0.001$) than at a concentration of 20*u*M ATP. At an ATP/ADP ratio of 12.5 the PFK activity was 3.9 times lower ($P<0.001$) than at a concentration of 20*u*M ATP. However, the enzyme activity at this ratio was 4.2 times higher ($P<0.001$) than the enzyme activity at 20mM ATP. At a ratio of 6.2 the activity was 1.4 times lower ($P<0.05$) than at a ratio of 12.5. The activity of PFK was 3 times higher ($P<0.001$) at an

Table 2.7: A comparison of PFK activity (U/g lung tissue ± SEM) of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.07 ± 0.008 (n = 9)	0.08 ± 0.004 (n = 7)	3.51 ± 0.45 (n = 8)	0.09 ± 0.007 (n = 9)	0.86 ± 0.05 (n = 8)
20*u*M	0.09 ± 0.008 (n = 10) P<0.10	1.36 ± 0.07 (n = 8) P<0.001	1.07 ± 0.15 (n = 10) P<0.001	0.32 ± 0.06 (n = 9) P<0.001	0.75 ± 0.04 (n = 10) P>0.10
ATP/ADP					
12.5	0.43 ± 0.02 (n = 6) P<0.001	0.34 ± 0.03 (n = 7) P<0.001	2.27 ± 0.38 (n = 7) P<0.10	0.09 ± 0.02 (n = 10) P>0.05	0.34 ± 0.04 (n = 8) P<0.001
6.2	0.79 ± 0.07 (n = 10) P<0.001	0.24 ± 0.02 (n = 9) P<0.001	2.34 ± 0.22 (n = 7) P<0.05	0.10 ± 0.01 (n = 10) P>0.05	0.33 ± 0.02 (n = 9) P<0.001

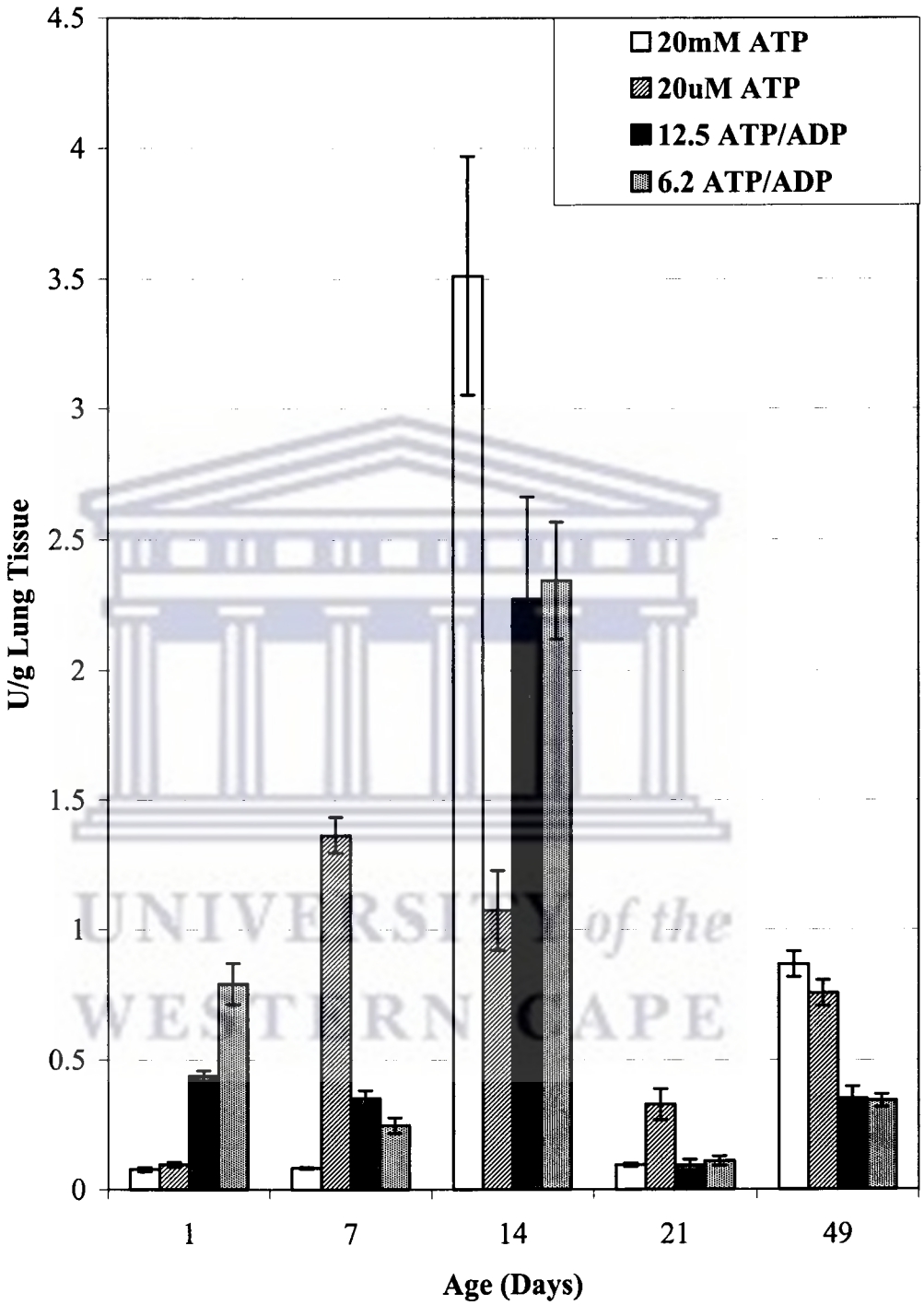

Figure 2.11: A comparison of PFK activity of lung tissue of control rats at different ATP concentrations and ATP/ADP ratios within each age group.

ATP/ADP ratio of 6.2 than the activity of the enzyme at 20mM ATP.

The PFK activity of lung tissue on postnatal day 14, and at an ATP concentration of 20mM was 3.51 ± 0.45 U/g lung tissue. Thus was 3.3 times higher ($P<0.001$) than the enzyme activity at 1.07 ± 0.15 U/g lung tissue at a concentration of 20*u*M ATP. At an ATP/ADP ratio of 12.5 the activity of PFK was the same as at a ratio of 6.2 ($P>0.10$). Therefore at ATP/ADP ratios of 12.5 and 6.2 the PFK activity was 2.1 times higher ($P<0.01$) than at a concentration of 20*u*M ATP, and 1.5 times lower ($P<0.10$) than at a concentration of 20mM ATP.

On postnatal day 21 the PFK activity was 3.4 times lower ($P<0.001$) at an ATP concentration of 20mM than at a concentration of 20*u*M ATP. The PFK activity at 20mM ATP and at the ATP/ADP ratios of 12.5 and 6.2 was not significantly different ($P>0.05$). It was however, 3.4 times lower ($P<0.01$) than at 20*u*M ATP.

The PFK activity of lung tissue on postnatal day 49 at an ATP concentration of 20mM (0.86 ± 0.05 U/g lung tissue) was not significantly different from the enzyme activity at a reduced concentration of 20*u*M ATP (0.75 ± 0.04 U/g lung tissue) ($P>0.10$). At both the ATP/ADP ratios the PFK activity was lower ($P<0.001$) than at either 20mM or 20*u*M ATP. As for the 2 ATP concentrations, the PFK activity was not affected by a change in the ATP/ADP ratio.

2.3.2.3 The effect of age on PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups

A summary of the data in table 2.8 and figure 2.12 show the PFK activity of nicotine exposed rat pups during pregnancy and lactation at postnatal days 1, 7, 14, 21 and 49 and at 20mM, 20*u*M ATP as well as, ATP/ADP ratios of 12.5 and 6.2. The data in the table and as illustrated in the figure clearly show that maximum PFK activity was achieved on day 14 after birth. The PFK activity on day 1 after birth was the same for all the treatment groups. The highest PFK activity was obtained at an ATP/ADP ratio of 12.5 in the lung tissue of 14 day old rat pups. Between postnatal days 21 and 49, the PFK activity increased except for the activity obtained in the presence of 20mM ATP where the activity actually decreased further.

A more detailed description of the data show that the activity of PFK of rat pups on day 1 after birth, at an ATP concentration of 20mM, was at 0.08 ± 0.01 U/g lung tissue not significantly different ($P>0.05$) from the activity on postnatal day 7 (0.10 ± 0.01 U/g lung tissue). However, the PFK activity was at 0.65 ± 0.15 U/g lung tissue, 6.3 times higher ($P<0.01$) on postnatal day 14 than on day 7 after birth. Between postnatal day 14 and 21 the PFK activity again decreased to 0.36 ± 0.02 U/g lung tissue. While this was still 4.1 times higher ($P<0.001$) than the activity of the enzyme on postnatal day 1, the activity was only half the activity on day 14 after birth ($P<0.001$). The enzyme activity decreased even further to 0.21 ± 0.02 U/g lung tissue between postnatal day 21 and postnatal day 49 ($P<0.001$). The PFK activity on day 49 after birth was however, still 2.5 times higher than on postnatal day 1.

Table 2.8: The effect of age on PFK activity (U/g lung tissue ± SEM) of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.08 ± 0.01 (n = 10)	0.10 ± 0.01 (n = 8) P>0.10	0.65 ± 0.15 (n = 8) P<0.01	0.36 ± 0.02 (n = 10) P<0.001	0.21 ± 0.02 (n = 8) P<0.001
20*u*M	0.10 ± 0.008 (n = 10)	0.44 ± 0.03 (n = 7) P<0.001	0.58 ± 0.08 (n = 8) P<0.001	0.19 ± 0.03 (n = 10) P>0.10	0.47 ± 0.03 (n = 10) P<0.001
ATP/ADP					
12.5	0.08 ± 0.01 (n = 8)	0.29 ± 0.02 (n = 6) P<0.001	1.06 ± 0.06 (n = 7) P<0.001	0.10 ±0.01 (n = 10) P>0.10	0.58 ± 0.07 (n = 8) P<0.001
6.2	0.11 ± 0.01 (n = 10)	0.15 ± 0.02 (n = 9) P>0.10	0.35 ± 0.05 (n = 7) P<0.01	0.16 ± 0.01 (n = 9) P<0.05	0.28 ± 0.02 (n = 9) P<0.001

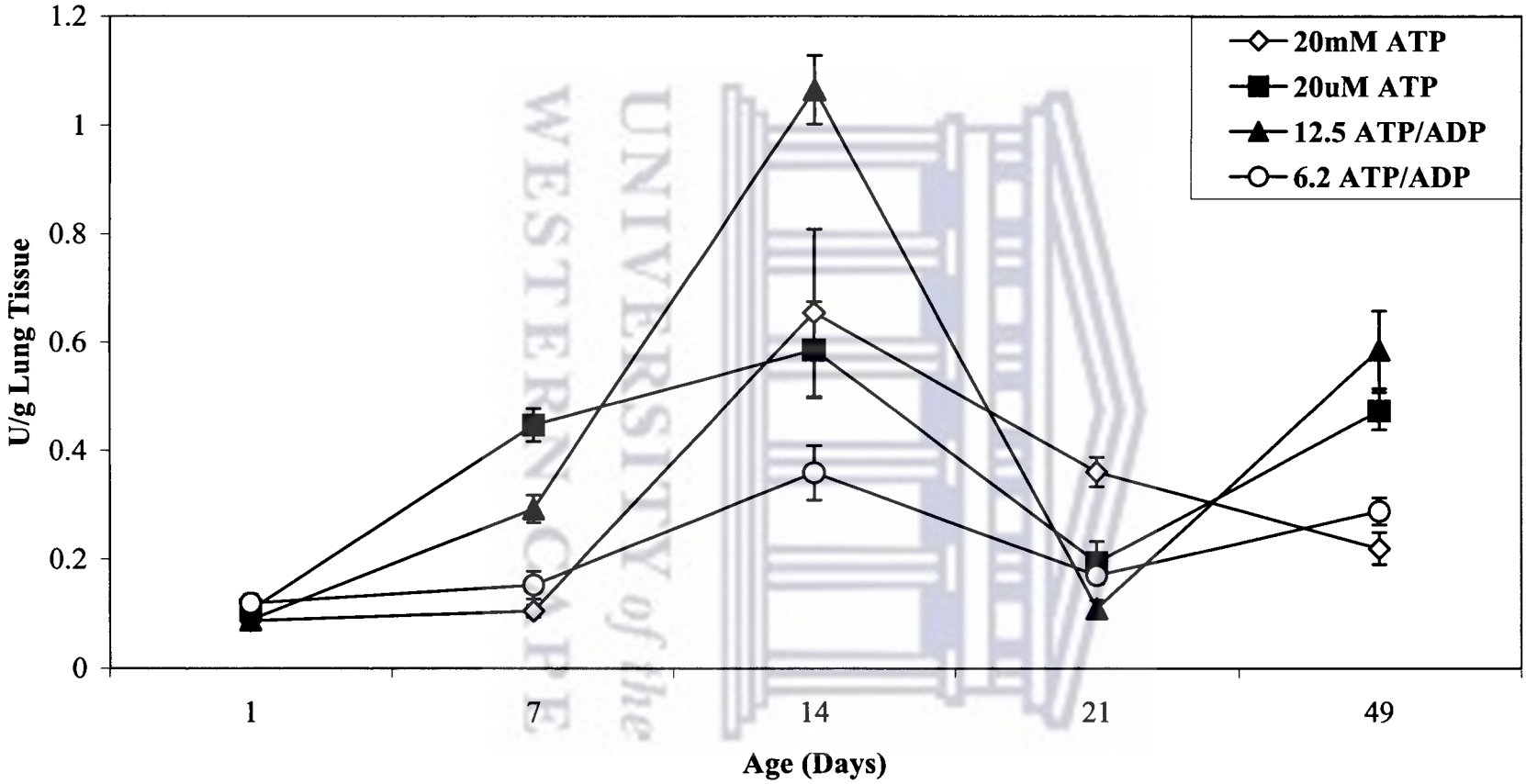

Figure 2.12: The effect of age on PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios: a comparison between age groups.

At an ATP concentration of 20*u*M, the PFK activity was at 0.44 ± 0.03 U/g lung tissue on postnatal day 7, 4.2 times higher ($P<0.001$) than the lung tissue of postnatal day 1 pups at 0.10 ± 0.008 U/g lung tissue. No significant difference ($P>0.10$) in PFK activity between the 7 and 14 postnatal day groups was observed. Between postnatal days 14 and 21 the enzyme activity decreased to 0.19 ± 0.03 U/g lung tissue which was the same as on day 1 after birth ($P>0.10$). Due to an 2.4 fold increase in PFK activity between postnatal days 21 and 49, the PFK activity which was at 0.47 ± 0.03 U/g lung tissue on postnatal day 49, 4.4 times higher ($P<0.001$) than the enzyme activity at day 1 after birth.

The activity of PFK at an ATP/ADP ratio of 12.5 increased gradually between postnatal days 1 and 14. Consequently, the activity of PFK was 3.3 times higher ($P<0.001$) on postnatal day 7 and 12 times higher ($P<0.001$) on postnatal day 14 than on postnatal day 1. The rate at which the activity of PFK increased between days 1 and 7 after birth and between days 7 and 14 after birth was the same. Between postnatal days 14 and 21, the PFK activity decreased to a level equal to that of postnatal day 1 ($P>0.10$). However, after postnatal day 21 the PFK activity again increased ($P<0.001$) 5.3 fold so that the enzyme activity at postnatal day 49, was at 0.58 ± 0.07 U/g lung tissue, 6.6 times higher ($P<0.001$) than the enzyme activity at postnatal day 1.

At an ATP/ADP ratio of 6.2, the PFK activity of the lung tissue of day 1 old rat pups after birth was the same ($P>0.05$) as for lung tissue of postnatal day 7 rat pups. On postnatal day 14 the PFK activity at 0.35 ± 0.05 U/g lung tissue was 3 times higher ($P<0.001$) than that of day 1 after birth. As for the previous experiments, the PFK

activity decreased after postnatal day 14, to reach a significant lower (P<0.001) level on postnatal day 21 but was still 1.4 times higher (P<0.05) than on postnatal day1. On postnatal day 49 the activity of the PFK in the lung tissue was at 0.28 ± 0.02 U/g lung tissue, 2.4 times more (P<0.001) active than the activity of the enzyme in postnatal day 1 lung tissue but significantly lower (P<0.001) than on postnatal day 14.

2.3.2.4 A comparison of PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group

The data in table 2.9 and figure 2.13 displays the influence of the change in the ATP concentrations and the ATP/ADP ratios on the PFK activity for the nicotine exposed rat pups. An analysis of the data show that the PFK activity on day 1 after birth at an ATP concentration of 20mM was not significantly different from the enzyme activity at an ATP concentration of 20*u*M and an ATP/ADP ratio of 12.5 and 6.2 (P>0.10). Thus the PFK activity of the lung tissue of 1 day old nicotine exposed rat pups was not sensitive for the changes in ATP concentration or the changes in the ATP/ADP ratio. From the findings it was clear that control of PFK activity by the adenine nucleotides become active after postnatal day 1. The ATP/ADP ratio of 12.5 appears to play a prominent role after postnatal day 14. A detailed description of the data follows below.

The activity of PFK of lungs of 1 and 7 day old rat pups was not affected by a concentration of 20mM ATP. However, in contrast to the above observation, the PFK activity increased (P<0.001) 4.3 fold between postnatal days 1 and 7 in the presence of 20*u*M ATP. In the presence of an ATP/ADP ratio of 12.5, the PFK activity was at 0.29 ± 0.02 U/g lung tissue, 1.5 times lower (P<0.001) than at 20*u*M ATP but 2.8 times

Table 2.9: A comparison of PFK activity (U/g lung tissue ± SEM) of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group.

ATP Concentrations	Day 1	Day 7	Day 14	Day 21	Day 49
20mM	0.08 ± 0.01 (n = 10)	0.10 ± 0.01 (n = 8)	0.65 ± 0.15 (n = 8)	0.36 ± 0.02 (n = 10)	0.21 ± 0.02 (n = 8)
20*u*M	0.10 ± 0.008 (n = 10) P>0.10	0.44 ± 0.03 (n = 7) P<0.001	0.58 ± 0.08 (n = 8) P>0.10	0.19 ± 0.03 (n = 10) P<0.01	0.47 ± 0.03 (n = 10) P<0.001
ATP/ADP					
12.5	0.08 ± 0.01 (n = 8) P>0.10	0.29 ± 0.02 (n = 6) P<0.001	1.06 ± 0.06 (n = 7) P<0.01	0.10 ±0.01 (n = 10) P<0.001	0.58 ± 0.07 (n = 8) P<0.001
6.2	0.11 ± 0.01 (n = 10) P>0.10	0.15 ± 0.02 (n = 9) P>0.10	0.35 ± 0.05 (n = 7) P<0.001	0.16 ± 0.01 (n = 9) P<0.001	0.28 ± 0.02 (n = 9) P>0.10

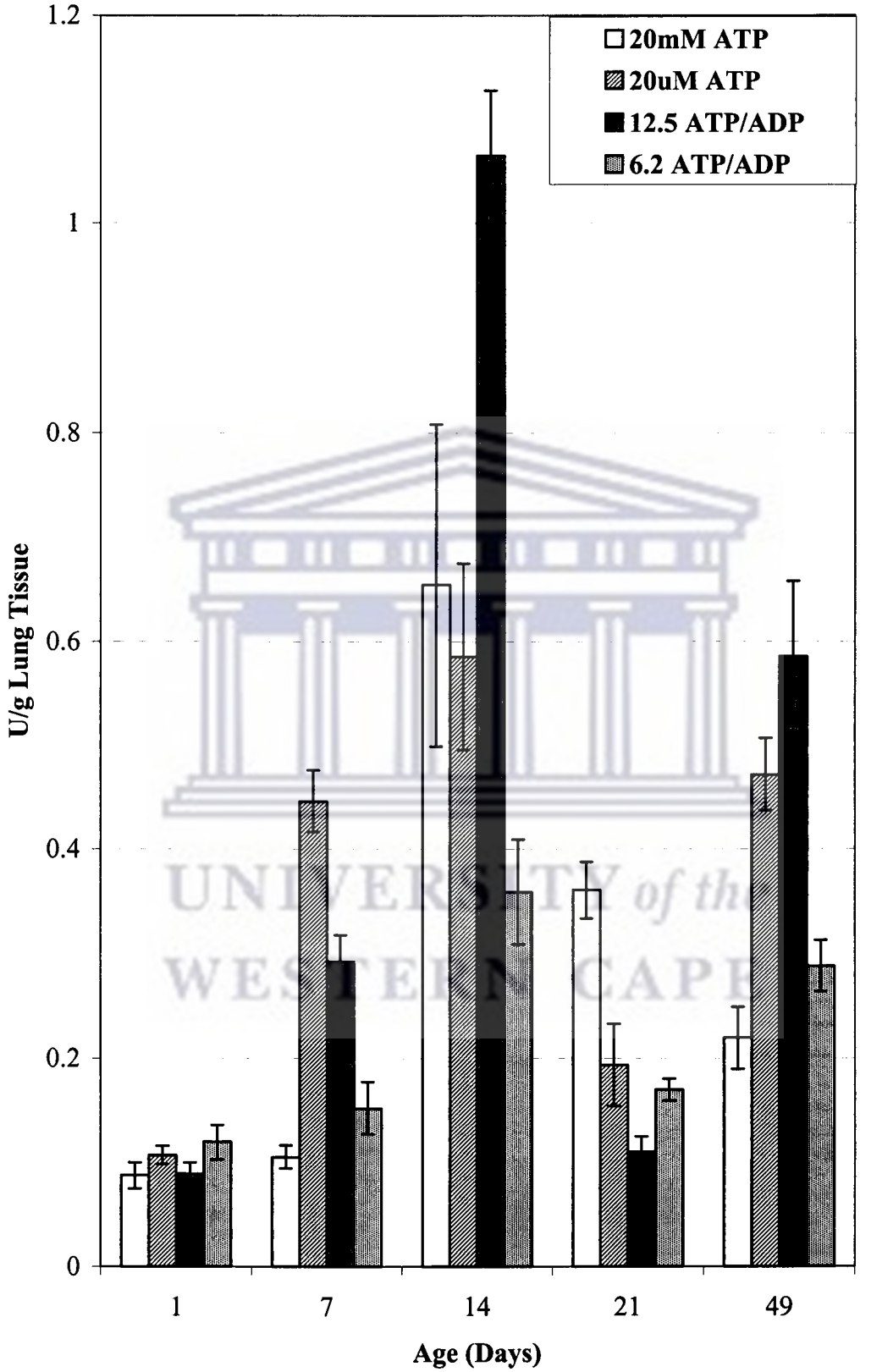

Figure 2.13: A comparison of PFK activity of lung tissue of nicotine exposed rats at different ATP concentrations and ATP/ADP ratios within each age group.

higher (P<0.001) than at 20mM ATP. The PFK activity at an ATP/ADP ratio of 6.2 was the same as at 20mM ATP (P>0.05).

The PFK activity on postnatal day 14 at an ATP concentration of 20mM was not significantly different from the enzyme activity at a concentration of 20*u*M ATP (P>0.10). However, the enzyme activity was at an ATP/ADP ratio of 12.5 at 1.06 ± 0.06 U/g lung tissue, significantly higher (P<0.01) than the enzyme activity at 20mM ATP and thus also at 20*u*M ATP. At a ratio of 6.2, the activity of PFK was 3 times lower (P<0.001) at 0.35 ± 0.05 U/g lung tissue than at an ATP/ADP ratio of 12.5 and 1.9 times lower (P<0.001) than at an ATP concentration of 20mM.

The activity of PFK on postnatal day 21 at an ATP concentration of 20mM was 0.36 ± 0.02 U/g lung tissue. The PFK activity decreased (P<0.01) to 0.19 ± 0.03 U/g lung tissue when the concentration was reduced to 20*u*M ATP. At an ATP/ADP ratio of 12.5, the PFK activity was the same as the enzyme activity at a concentration of 20*u*M ATP (P>0.10), but 3.3 times lower (P<0.001) than the enzyme activity at a concentration of 20mM ATP. At an ATP/ADP ratio of 6.2, the PFK activity was 1.6 fold higher (P<0.01) than at a ratio of 12.5.

On postnatal day 49 the PFK activity had increased (P<0.001) 2.2 fold from 0.21 ± 0.02 U/g lung tissue at 20mM ATP to 0.47 ± 0.03 U/g lung tissue when the ATP concentration was lowered to 20*u*M. At an ATP/ADP ratio of 12.5, the PFK activity was not significantly different from the activity at 20*u*M ATP (P>0.10) however, it was 2.7 times higher (P<0.001) than the enzyme activity at a concentration of 20mM ATP.

At a ratio of 6.2 the PFK activity was 2 times lower ($P<0.001$) at 0.28 ± 0.02 U/g lung tissue from the ATP/ADP ratio of 12.5 however, the enzyme activity was not significantly different ($P>0.10$) from that of a concentration of 20mM ATP.

2.3.2.5 The effect of maternal nicotine exposure on PFK activity: a comparison with the PFK activity of lungs of control rats

The data in figures 2.14 to 2.17 clearly shows that the PFK activity on day 14 after birth was consistently higher in the control animals than in the lung tissue of the nicotine exposed animals. However, at 20*u*M ATP, the PFK activity reached maximal activity on postnatal day 7 instead of postnatal day 14 as in the control animals.

At 20mM ATP (figure 2.14) the activity of PFK of lung tissue of 1 and 7 day old control animals was not significantly different ($P>0.10$) from the enzyme activity of the nicotine exposed animals. However, on postnatal day 14 the PFK activity of lung tissue was higher ($P<0.001$) in the control animals than in the lung tissue of nicotine exposed animals. For both the control animals and the nicotine exposed animals the PFK activity decreased between postnatal days 14 and 21. The PFK activity increased between postnatal days 21 and 49 in the lungs of the control animals, but the enzyme activity continued to decrease in the lungs of the nicotine exposed animals. Thus, on postnatal day 49, the PFK activity was significantly higher ($P<0.001$) in the lungs of the control animals.

At 20*u*M ATP (figure 2.15) no significant difference ($P>0.10$) was observed in the PFK activity of lung tissue of day 1 old control and nicotine exposed animals. Between

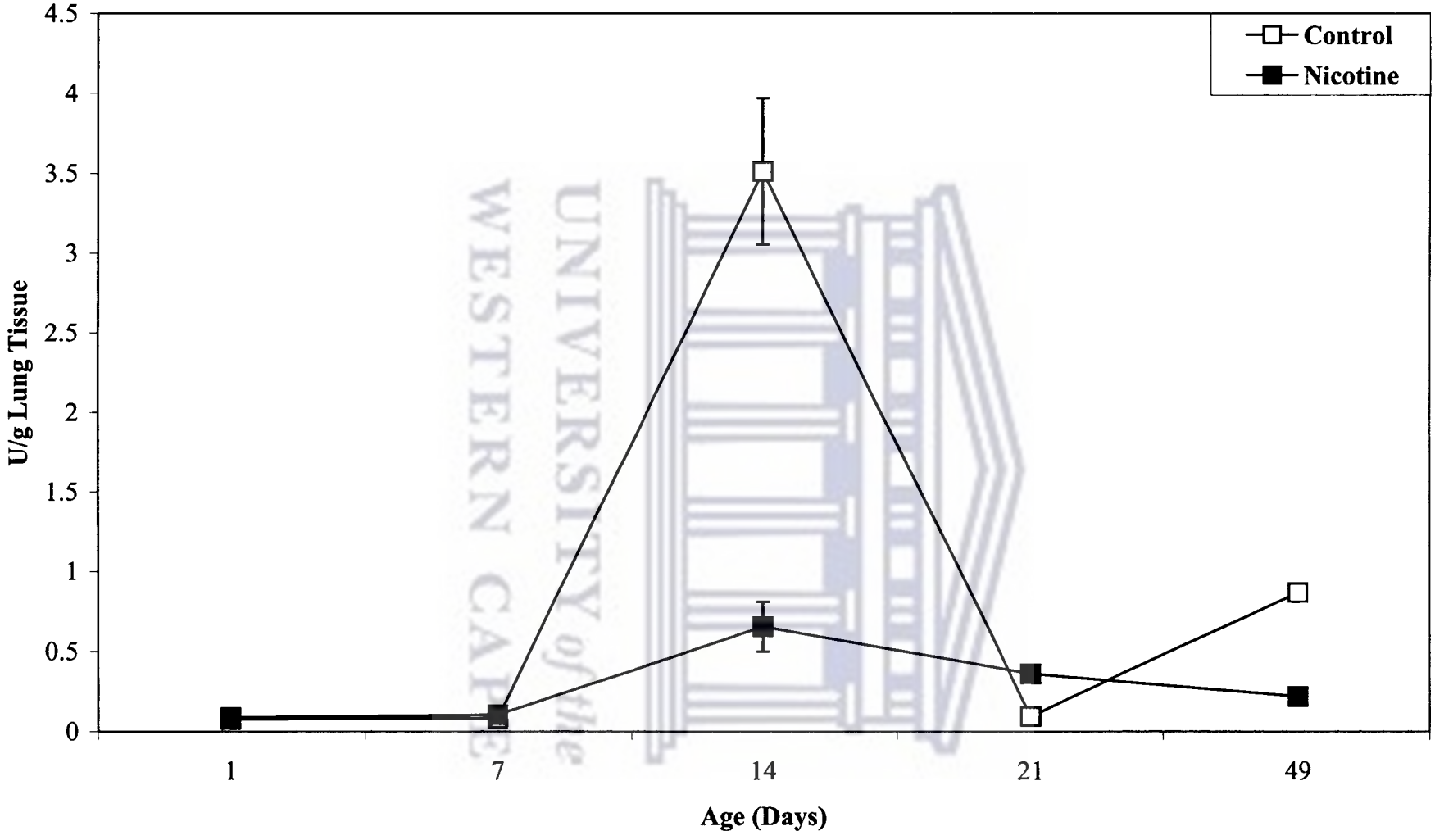

Figure 2.14: The influence of maternal nicotine exposure on the PFK activity at an ATP concentration of 20mM.

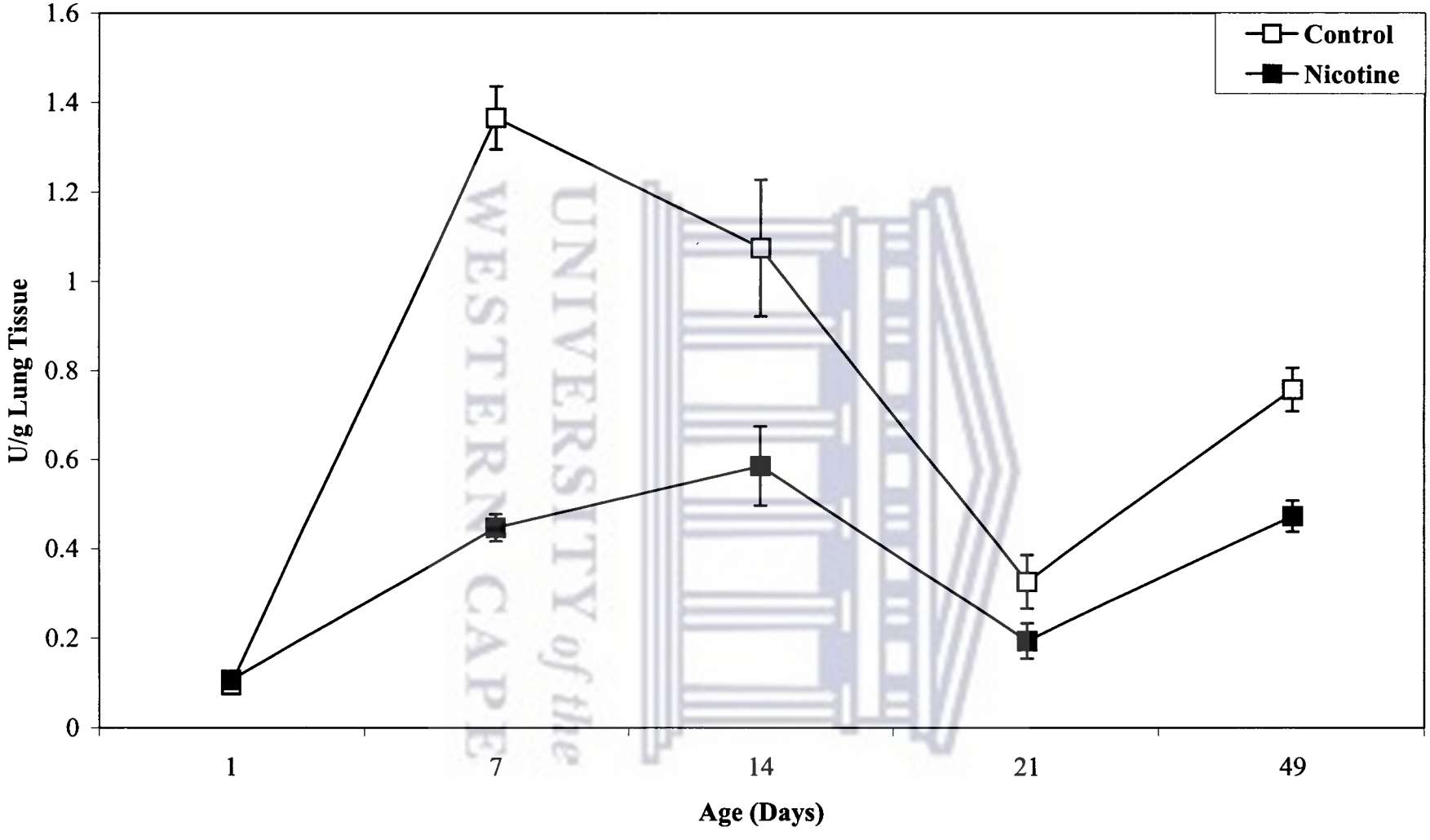

Figure 2.15: The influence of maternal nicotine exposure on the PFK activity at an ATP concentration of 20uM.

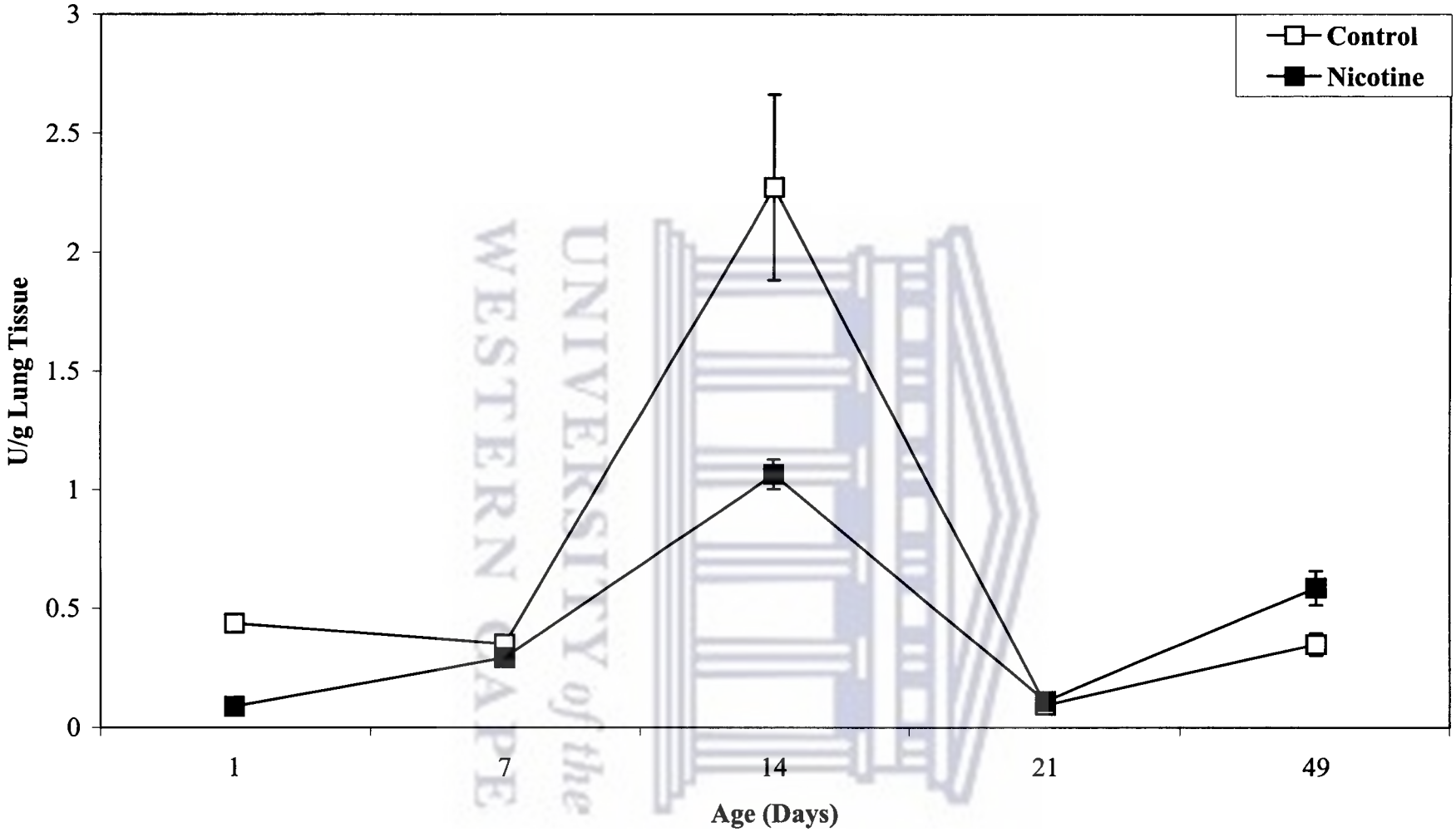

Figure 2.16: The influence of maternal nicotine exposure on the PFK activity at an ATP/ADP ratio of 12.5.

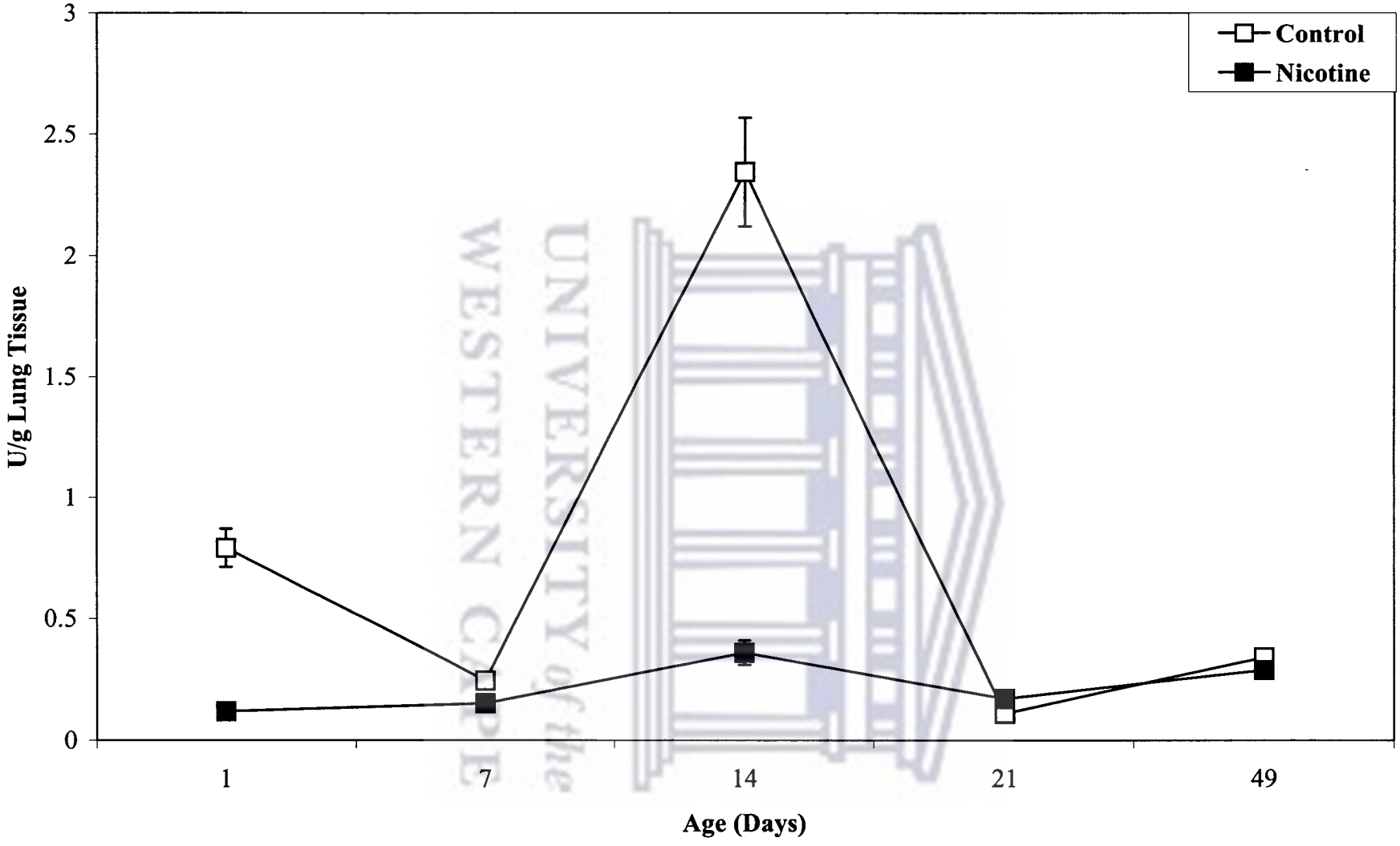

Figure 2.17: The effect of maternal nicotine exposure on the PFK activity at an ATP/ADP ratio of 6.2.

postnatal days 1 and 7, the PFK of lung tissue of control animals increased at a faster rate and reached maximum activity on postnatal day 7. This level of activity was maintained up to postnatal day 14, whereas the nicotine exposed animals reached maximum enzyme activity only on postnatal day 14. The PFK activity in the lungs of the control and the nicotine exposed animals, decreased between postnatal days14 and 21 whereafter it increased again to postnatal day 49. However, although the PFK activity of lung tissue of control and nicotine exposed animals followed the same developmental trend, the PFK activity of the control lungs were significantly higher than the enzyme activity of nicotine exposed animals from postnatal day 7 to postnatal day 49.

Figures 2.16 and 2.17 illustrate the PFK activity of lung tissue of control and nicotine exposed animals of various age groups and at ATP/ADP ratios of 12.5 and 6.2. The PFK activity (figures 2.16 and 2.17) in the lungs of the control animals decreased between postnatal days 1 and 7 at both the ATP/ADP ratios whereafter it reached maximum activity on postnatal day 14, whereas the PFK activity in the nicotine exposed lungs had gradually increased from postnatal day 1 to postnatal day 14. For both the control animals as well as the nicotine exposed animals, the PFK activity decreased between postnatal days 14 and 21, whereafter the enzyme activity increased to postnatal day 49. At an ATP/ADP ratio of 12.5 (figure 2.16), the activity of PFK was not significantly different ($P>0.05$) between the control and nicotine exposed animals on postnatal days 7 and 21. On postnatal day 49 the PFK activity was however, higher ($P<0.05$), in the nicotine exposed animals than in the control animals (figure 2.16). However, at an ATP/ADP ratio of 6.2 (figure 2.17) on postnatal day 7, the control animals had a higher

(P<0.10) PFK activity than the nicotine exposed animals and on postnatal day 21 the nicotine exposed animals had a higher (P<0.10) PFK activity than the control animals. On postnatal day 49 however, no signiffigant difference (P>0.05) was observed in the PFK activity of the control and the nicotine exposed lung tissue.

2.4 Discussion

Carbohydrates play an important role in the structural (Tierney and Levy, 1976) and functional development and maturation of fetal and neonatal lung tissue (Bourbon and Jost, 1982; Gilden *et al*, 1977; Maniscalco *et al*, 1978). Interference with carbohydrate metabolism during late gestation result in retarded fetal lung growth (Rhoades and Ryder, 1981) and maturation (Maniscalco *et al*, 1979). The process of lung development and maturation involves cellular multiplication and growth as well as cell differentiation which depends on ATP energy derived from carbohydrate metabolism. Interference with energy metabolism may therefore interfere with lung growth and maturation. Research conducted by Maritz (1988) shows that maternal nicotine exposure during pregnancy and lactation results in the suppression of glycogenolysis and an irreversible inhibition of glycolysis in the lungs of the offspring (Maritz, 1987). Glycogenolysis plays an important role in preparing the fetal lung for birth, in that it supplies the precursors for surfactant synthesis. It also supplies glucose for ATP production (Thibeault and Gregory, 1986). Glycolysis plays a crucial role in the maintenance of lung cell integrity and growth of the lung. Inhibition of glycolysis indeed result in a reduced rate of lung growth (Sorokin, 1961). The type I cell, which constitutes 96% of the gas exchange surface area of the lung, where the surface area in the human is equal to 60 to 80m^2 (Thibeault and Gregory, 1986), depends on glycolysis for its energy (Massaro *et al*, 1975). It can therefore be expected that inhibition of glycolysis will adversely affect the integrity of this cell and also the development of the fetal and neonatal lung.

Lung tissue is composed of at least 48 different types of cells, that, like cells in other

tissues, actively carry out metabolic processes and require energy for their synthetic and other life sustaining reactions (Fisher *et al*, 1974). The whole process of cell growth and development depends on ATP (Maritz and Najaar, 1995). A lack of ATP due to inhibition of ATP hydrolysis or reduced production will suppress cell function and thus interfere with normal development. The ATP in the lung is produced via glycolysis (Sayeed and Murthy, 1981). Glycolysis is one of the major metabolic pathways that supplies the cell with energy as mentioned earlier in the text and at the same time with material for synthesis (Boiteux and Hess, 1981). The transformation of energy is carried out by enzyme molecules (Stryer, 1975). In other words, collectively, enzymes are responsible for carrying out all the chemical reactions of the living cell, and as such, they represent the life force of the cell (Dressler and Potter, 1991). Therefore growth of cells and the development of their specialized functions are under the control of enzymes, but so too are the physiological processes that characterize the organism as a whole (Dressler and Potter, 1991). The enzyme is conceptually the trump which allows the cell to exist and can be seen as fundamental to life, providing a way of keeping ahead of the universal tendency towards disorder (Ferdinand, 1976).

In the cells, the rate of glycolysis is limited by the rate at which ATP is used. Thus the glycolytic flux depends on the cellular levels of ATP and ADP (Fisher, 1984). The overall rate of glycolysis is therefore determined by the availability of substrate, utilization of ATP and the concentrations of the various enzymes (White *et al*, 1973). An inhibition of glycogenolysis due to suppressed enzyme activity will thus result in less glucose-6-phosphate for glycolytic activity which again will have an impact on the ability of the pathway to supply energy for lung development as well as its ability to

supply precursors for surfactant synthesis.

The catalytic activities of many enzymes vary in response to the concentrations of substances other than their substrates. The mechanisms of these regulatory processes include allosteric control, covalent modification of enzymes and variation of the amounts of enzymes synthesized (Voet and Voet, 1995). Research has found that nicotine has an effect on the metabolic and structural development of the lung cells (Maritz and Thomas, 1994 and 1995) and since growth of cells and the development of their specialized functions are under the control of enzymes (Dressler and Potter, 1991), it could be that nicotine might be responsible for having an adverse effect on the regulation of the enzyme activity. In order to understand the effect of maternal nicotine exposure on the development of HK and PFK as a function of age, the change in the activity of these enzymes in lung tissue of control rats will be discussed first.

2.4.1 Hexokinase

2.4.1.1 Change in the hexokinase activity in the lung tissue of control animals as a function of age

During the period just before birth as well as after birth, the lungs undergo phases of rapid development (Burri *et al*, 1974) to prepare the lungs for birth and also to ensure the development of this organ into an efficient gas exchanger (Harding and Hooper, 1993). During the phase of rapid alveolarization which occurs between postnatal days 4 and 13 in neonatal rats, rapid cell proliferation also occurs (Burri *et al*, 1974; Kauffman *et al*, 1974) which requires ATP, energy which must be derived from carbohydrates (Fisher, 1984). The accompanying synthetic process requires additional

energy and materials, such as NADPH, to support this phase of lung development. Since the developing lung depends on glucose for its energy, it is evident that glycolysis plays a central role in lung development (Girard and Ferre, 1982). It is therefore also clear that control of the glucose flux through the glycolytic pathway is important to ensure a sufficient supply of energy for this phase of lung development.

Lung glycogen is an important source of phosphorylated glucose before birth (Farrell and Bourbon, 1986). The importance of glycogen before birth for lung development and preparation of the lung to act as a gas exchanger after birth is demonstrated by the rapid decrease in lung glycogen content just before birth (Maritz, 1987). However, after birth the glycogen stores of the neonatal lung is very low and will not supply sufficient quantities of glucose-6-phosphate to support the rapid growth and development of the lung. Exogenous glucose is therefore important to sustain growth and development. However, before glucose can supply the precursors for lung development and before it can be oxidized for energy purposes, it must be converted to glucose-6-phosphate by HK (Griffin *et al*, 1992). HK thus plays an important role in lung development. It is therefore conceivable that inhibition of this enzyme will suppress glucose turnover in the lung and have an adverse effect on lung growth and development. Apart from this, it also plays a role in protecting the lung against oxidants in that glucose-6-phosphate is the substrate for the production of NADPH which plays a role in protecting the lung (Fisher, 1984). HK occupies a unique metabolic position as the only enzymatic step that is required for all pathways of glucose utilization (figure 2.18) (Robert *et al*, 1977).

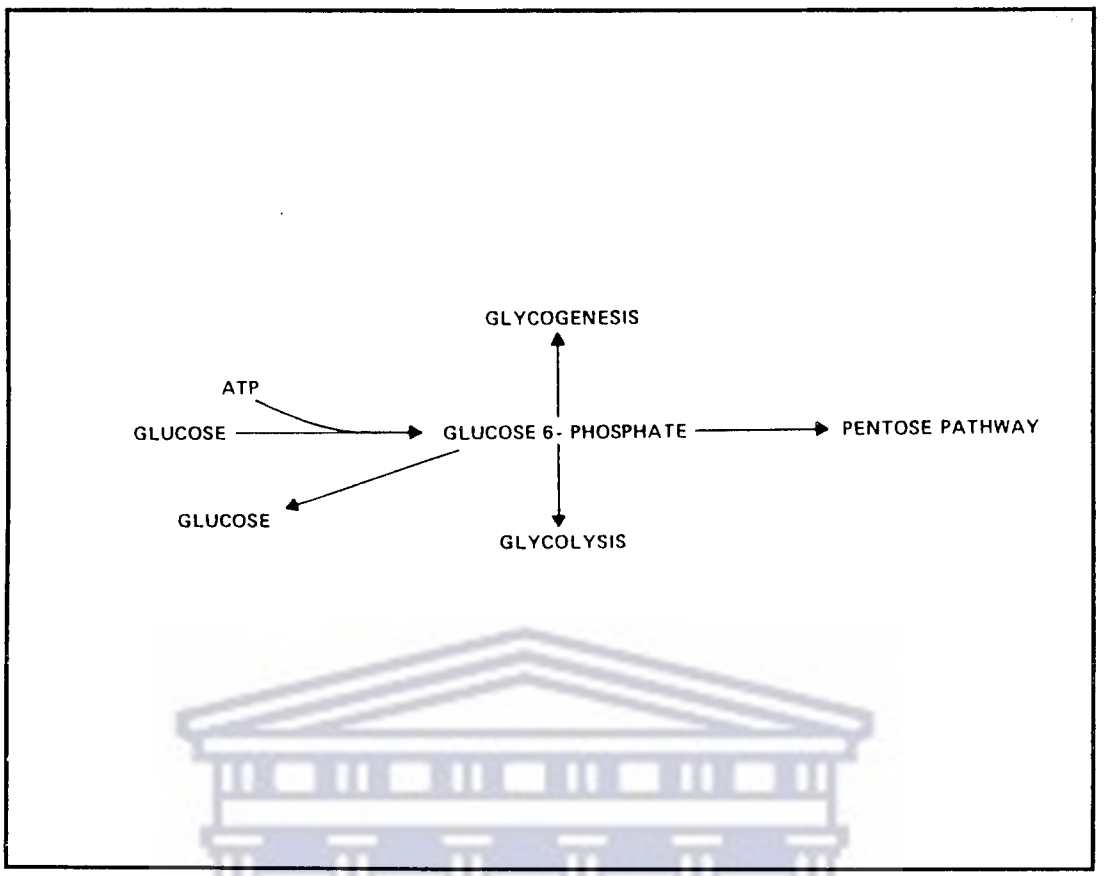

Figure 2.18: The alternative pathways for glucose utilization (Crystal, 1976).

In a study by Maritz (1987), it was shown that maternal nicotine exposure during pregnancy resulted in an irreversible inhibition of glycolysis in lung tissue of the offspring. Maritz and Burger (1992) also found that the ATP concentration of the lung tissue of the offspring increased and suggested that it may result in inhibition of the allosteric enzymes of glycolysis, namely HK and PFK. The data presented in this study (table 2.2 and figure 2.2) shows the developmental pattern of HK activity for the control rat pups. The present study indicated that the activity of the neonatal lung HK increased from day one after birth to postnatal day 14 which coincides with the phase of tissue proliferation and rapid alveolarization between postnatal days 4 to13. This phase of lung development is characterised by a marked increase in tissue mass and surface area

(Burri *et al*, 1974). During this period, rapid cell production and outgrowth of secondary alveolar septa characterized by differential cell proliferation on septal buds and capillary surface area occur (Kauffman *et al*, 1974). Thus this period is seen as the period where most of the alveoli are formed (Burri *et al*, 1974). An adequate supply of energy is therefore required to ensure optimal growth of the lung. However, since the rate of lung alveolarization increases between postnatal days 4 and 13, the energy supply must also increase to satisfy the increased demand associated with synthesis of new cells and cellular materials. This implies that the rate of glucose utilization must increase. This can only be achieved by an increase in the activity of HK. It is therefore plausible that the increase in HK during the phase of rapid lung development is to ensure an adequate energy supply to support the increased rate of tissue growth and development. It is therefore also plausible that a low HK activity during the phase of rapid lung growth and development, such as during alveolarization, will suppress the development of the lung into an effective gas exchanger. From postnatal day 14 to postnatal day 21 (the phase of equilibrated growth), the HK activity decreased (table 2.2 and figure 2.2). During this period a rapid decline in cell production and a concomitant decrease in the energy requirement for synthetic processes occurred. The lower HK activity after postnatal day 14 is thus a reflection of the reduced energy demand related to the reduced rate of cell formation.

Glycogen is stored in the alveolar type II cell prior to the initiation of surfactant synthesis (Heesbeen *et al*, 1989). Both glycogen and glucose are associated with surfactant synthesis during late gestation (Hamosh *et al*, 1978) and in the mature lung (Salisbury-Murphy *et al*, 1966). Glucose and glycogen can supply sufficient amounts

of precursors such as dihydroxyacetone phosphate [an intermediary product of glycolysis which is the first acceptor of acyls in the synthesis of phospholipids in lung (Agranoff and Hajra, 1971)] for phospholipid synthesis, which is an important component of surfactant (Thibeault and Gregory, 1986). The glycogen content increases from day 1 after birth to postnatal day 7 (Maritz, 1986). This finding corresponds with the data in this study where the HK activity increased from day 1 after birth to postnatal day 7. However, Maritz (1986) showed that after postnatal day 7, the glycogen content of the lungs decreased gradually till postnatal day 21, whereas the HK activity in this study, gradually increased up to postnatal day 14 and then gradually decreased from postnatal day 14 to postnatal day 49. Since the lung glycogen content decrease to adult levels after postnatal day 7, the increased HK activity will ensure that the glucose-6-phosphate supply is adequate to satisfy the demands of the maturing lung. The decrease in HK activity from postnatal day 14 to postnatal day 49 could be due to an alteration in metabolism, possibly due to changes in nutrient availability or it could be due to the fact that rapid lung growth has come to an end and energy is only required for lung tissue maintenance and surfactant synthesis.

At birth the newborn undergoes several major alterations in metabolism due to changes in nutrient availability (Sperling, 1988). For the first few hours of life the neonate is entirely dependent upon endogenous glucose (Griffin *et al*, 1992). However, one of the few behaviours of the rat that is fully developed at birth, is suckling. Around the 16th or 17th postnatal day, the pups begin to nibble on solid matter including bedding, feaces (Henning, 1981) and Epol cubes in the case of this study. During the first two postnatal weeks, up to postnatal day 14, mother's milk is the only food ingested by the neonates,

therefore their diet change from a high fat and low carbohydrate diet to one that is low in fat and high in carbohydrates, and from one that has lactose and its derivates as sole carbohydrates to one that has starch and sucrose as principal carbohydrate (Henning, 1981). Although dietary carbohydrates is not necessary to bring about increase in HK activity, carbohydrate is the most important dietary constituent in regulating the enzyme (Bernstein *et al*, 1977). However, since the rate of cell proliferation is reduced after postnatal day 14, the energy demand of the lung is reduced to a level which is sufficient to maintain lung cell integrity. It is therefore more likely that the reduced HK activity after postnatal day 14 is a reflection of the glucose demand of the lung.

Since HK catalyses the reaction which involves the transfer of a phosphate to glucose to form glucose-6-phosphate in the presence of ATP (Griffin *et al*, 1992), it could be assumed that a high concentration of ATP as well as the presence of product can cause the decrease in enzyme activity from postnatal day 14 to postnatal day 49. In the lung tissue of adult rats, three forms of HK exist, HK I, II and III. HK I has a higher affinity for glucose than the other two isoforms and is the primary isoenzyme involved in phosphorylation of glucose in adult lung. However, the Km of glucose turnover for the suckling rat lung is higher than that of adults and has a lower affinity for glucose and therefore, possibly, a different isoenzyme pattern than in the adult lung (Salotra and Singh,1982). However, further investigation is needed to identify the HK isoenzyme development expression, since these isoforms are involved in the process of organ development and in regulation of glucose metabolism at multiple levels during development (Coerver *et al*, 1998).

2.4.1.2 Change in the hexokinase activity in the lung tissue of nicotine exposed animals as a function of age

Table 2.4 and figure 2.4 displayed the data presenting the development of HK as a function of age of rat pups exposed to nicotine via the placenta and mothers milk. The results clearly show that the enzyme activity pattern for the nicotine exposed rat pups (table 2.4 and figure 2.4) was the same as for control pups (table 2.2 and figure 2.2). In the present study it was found that maternal nicotine exposure during pregnancy and lactation resulted in an increase in the HK activity of neonatal rat lung (figures 2.6 to 2.9). This implies that sufficient quantities of glucose can be phosphorylated to provide in the energy and NADPH needs of the developing lung. In previous studies (Maritz and Burger, 1992), it was shown that maternal nicotine exposure also resulted in an increase in glucose utilization by the lung of the offspring. This was attributed to an increase in AMP activity in order to supply sufficient quantities of NADPH to detoxify nicotine.

The type II epithelial cells proliferate and differentiate into alveolar type I cells which cover the newly formed alveolar surfaces (Kauffman *et al*, 1974). In normal lung development the period between postnatal days 4 and 13 is where most alveoli are formed (Burri *et al*, 1974), thus increasing type I cells and reducing type II cells. However, the type I cell is more susceptible to damage than the type II cell and a study by Maritz and Thomas (1994) indicated that maternal nicotine exposure during gestation and lactation decreased the type 1:type II cell ratio. Due to type I cell injury in lungs of nicotine exposed rat pups, type II cell proliferation thus occur which is followed by differentiation of one of the daughter cells to form a type I cell. This process also require energy derived from glucose as a source of energy for mitosis (Bullough, 1952).

From the above it is thus clear that the reserve capacity of HK of lung tissue of nicotine exposed rat pups will ensure that the rate of phosphorylation of glucose is sufficient to supply in the needs of the growing lung, and for maintenance of the type I cell integrity, as well as for detoxification. Inhibition of the glucose flux through glycolysis is therefore not due to an inhibition of HK and will thus not have an adverse effect on the phase of rapid alveolarization.

It is interesting to note that the maximum stimulatory effect of nicotine was achieved during the phase of rapid alveolarization. It is furthermore important to note that maximum activity was already achieved on postnatal day 7 and maintained through to postnatal day 14 in contrast to the lungs of the control animals, where maximal activity was achieved on postnatal day 14. After postnatal day 14, the HK activity of the lung tissue of nicotine exposed rats decreased to levels which resembles the HK activity of lung tissue of control rats, except for the activity of HK of lung tissue of nicotine exposed rats at an ATP concentration of 20*u*M. This implies that glucose turnover will be maintained after day 14 at least at the same level as for control animals. Furthermore, since HK activity was highest at 20mM ATP, it means that a higher tissue ATP concentration may even play a protective role in the lung, and also support growth and development.

The data obtained in the study clearly show that the HK activity of neonatal rat lung changes as the animals matures. It also shows that the highest activity was achieved at 20mM ATP. Another interesting observation is that at an ATP/ADP ratio of 12.5 (20*u*M ATP/1.6*u*M ADP), the activity increased to a level significantly higher than at 20*u*M

ATP. This is an indication that at the low ATP concentrations, ADP plays a role in the control of HK activity. However, the activity of HK of lung tissue of nicotine exposed rat pups was not affected by the ATP/ADP ratio which is an indication of a change in the binding site on the enzyme for ADP as positive modulator.

Studies showed that postnatal days 4 to 13 are characterized by rapid cell proliferation and growth of rat lung. During this phase the alveolar sacs are converted to alveoli and outgrowth of secondary septa result in more alveoli (Burri *et al*, 1974). This implies that the energy demand of the lung during this phase of lung development will increase due to the ATP requirements related to this various synthetic processes. It is therefore important that the energy supply meets the energy demands of this phase of lung growth and development, the implication being that a lower energy supply due to a reduced rate of glucose utilization will interfere with this phase of lung development. Therefore, if glycolysis is suppressed due to a reduced rate of phosphorylation due to lower HK activity, or due to inhibition of another enzyme, flux of glucose through the glycolytic pathway will be slower, negatively affecting the energy supply. This then will suppress cell proliferation and eventually interfere with the functional development of the lung. The findings in this study and the observations by Maritz (1987) therefore indicate that inhibition of glycolysis in neonatal lung was not due to a lower HK activity. It however, appears that maternal nicotine exposure during gestation and lactation resulted in raising HK activity of neonatal lung to reach higher levels of activity on postnatal day 7 and postnatal day 14 than in control animals, except at an ATP/ADP ratio of 12.5. These findings support the observations of Maritz (1987) namely that maternal nicotine exposure during pregnancy and lactation results in an increase in glucose turnover by

86.4% compared to the control animals.

The inhibition of glycolysis in the lungs of nicotine exposed rat pups was therefor due to inhibition at a site below the HK catalysed reaction. The mechanism of inhibition of glycolysis is however, not clear. Since it was suggested that the elevated ATP content of lung tissue of nicotine exposed rats result in an inhibition of glycolysis by suppressing HK and PFK, and since HK was not affected by nicotine exposure, it was necessary to also investigate the activity of PFK the key regulatory enzyme of glycolysis (Heesbeen *et al*, 1989).

2.4.2 Phosphofructokinase

2.4.2.1 Change in the phosphofructokinase activity in lung tissue of control animals as a function of age

As mentioned above, PFK is a key regulatory enzyme of glycolysis (Heesbeen *et al*, 1989). This enzyme catalyses the phosphorylation of fructose-6-phosphate to yield fructose-1,6-bisphosphate (Dunaway, 1983). This enzyme is important in regulating surfactant synthesis in the rat lung during the period of glycogen breakdown (Heesbeen *et al*, 1989). Inhibition of this enzyme will lead to an increase in the glycogen stores and thereby suppress the synthesis of precursors for surfactant. Since PFK is the only regulatory enzyme between glycogen breakdown and formation of dihydroxyacetone phosphate, which is a precursor of surfactant lipids, an increase in its activity appears to be correlated with the onset of surfactant synthesis (Heesbeen *et al*, 1989).

In addition to its role in surfactant synthesis, PFK also control the rate of glucose

oxidation to satisfy the energy requirements of the cell (Voet and Voet, 1995). It is therefore conceivable that PFK activity will increase during phases of rapid cell proliferation such as during the phase of rapid alveolarization between days 4 and 13 in neonatal rat lung. It can therefore be expected that the phases of structural development of the fetal and neonatal lung will be accompanied by developmental changes in PFK to satisfy the changing energy and precursor requirements of the maturing lung. If this is true, it has the implication that interference with the normal development of PFK will be reflected in changes in structural development of the lung. Thus, an inhibition of PFK during early lung development may result in delayed maturation of the lung.

The data presented in this study (table 2.6 and figure 2.10) shows the developmental pattern of PFK activity for the control rat pups. Even though there was a shift in the developmental pattern at the different concentrations of ATP and ATP/ADP ratios, maximum activity of PFK was achieved on postnatal day 14 with the exception of 20*u*M ATP which reached maximum activity on postnatal day 7. This phenomenon corresponds with the phase of tissue proliferation which occur between postnatal days 4 and 13. This period could be seen as the period where most of the alveoli are formed as mentioned before (Burri *et al*, 1974). Since energy is needed for cell production, it is not surprising that PFK reached maximum activity during the phase of rapid alveolarization and cell proliferation. In all instances the PFK activity decreased between postnatal days 14 and 21. The decrease in activity from postnatal day 14 to 21 corresponds with the phase of equilibrated growth. During this period, from postnatal day 13, cell production still occur and the alveolar and capillary surface area continue

to increase. However, the rate of cell proliferation is slower (Kauffman *et al*, 1974). From postnatal day 21 to 49, the PFK activity increased again but to a lesser extend than during the phase of rapid alveolarization. This increase in the enzyme activity might be related to the tissue mass increase from postnatal day 21.

In a study by Maritz (1986) it was shown that the glycogen content in the lungs of neonatal rats increased between postnatal days 1 and 7 and these findings correspond to the data in the present study (table 2.6). After postnatal day 7 the lung glycogen content decreased to very low levels. This implies that between postnatal days 1 and 7, the use of glycogen for surfactant synthesis and for energy supply, was lower than after postnatal day 7. Less PFK is therefore required to phosphorylate fructose-6-phosphate. The lungs of these neonates are also more dependent on the stored glycogen, since the carbohydrate content of the mother's milk is low and the fat content high (Sperling, 1981). It can therefore be expected for PFK activity to be higher until postnatal day 14 to cope with the increased rate of glucose-6-phosphate release from the glycogen stores and to satisfy the demands of the developing lung.

2.4.2.2 Change in the phosphofructokinase activity in lung tissue of nicotine exposed animals as a function of age

Table 2.8 and figure 2.12 displays the data presenting the development of PFK as a function of age of rat pups exposed to maternal nicotine. The results clearly shows that the enzyme activity pattern for the nicotine exposed rat pups (table 2.8 and figure 2.12) was the same as for control pups (table 2.6 and figure 2.10). An exception was that the PFK activity in the lung of the control animals decreased between postnatal days 1 and

7 at both the ATP/ADP ratios whereafter it reached maximum activity on postnatal day 14. The PFK activity in the nicotine exposed lung, on the other hand, increased between postnatal days 1 and 14. It was furthermore found that for both the control and the nicotine exposed lungs (figures 2.14 to 2.17), the PFK activity decreased between postnatal days 14 and 21 whereafter it again increased between postnatal days 21 and 49. The only exception was at a concentration of 20mM ATP for nicotine exposed lungs where the activity of PFK actually decreased even further. Further investigation is needed to determine the reason for this response. The activity of PFK on day 14 after birth was however, consistently higher in the control animals than in the lung tissue of the nicotine exposed rat pups. The mechanism of inhibition is however, not known. This decrease in activity may contribute to the lower glycolytic activity in lungs of rat pups exposed to nicotine via the placenta and mothers milk (Maritz, 1987). Compared to the control animals, the decrease in PFK activity also indicated that less surfactant will be produced provided that glycogen and glucose are the sole sources of precursors for surfactant synthesis. It was speculated that fatty acids could also supply precursors for surfactant synthesis (Maritz, 1986). The fact that maternal nicotine exposure is not suppressing surfactant synthesis in the offspring (Maritz and Thomas, 1994 and 1995) indicates that it is unlikely that the reduced PFK activity will influence this facet of lung development. It is possible that the degree of inhibition of PFK is not sufficient to affect the need for precursors to synthesize surfactant. The requirement for precursors is probably low since breakdown products of surfactant is absorbed by the type II cells for surfactant resynthesis (Maritz and Thomas, 1994 and 1995). It is also possible that fatty acids supply precursors as suggested by Maritz (1986). This will reduce the glycogen requirement for this purpose.

Since PFK is the regulatory enzyme of glycolysis (Heesbeen *et al*, 1989) and the results indicated a decrease in PFK activity in nicotine exposed rats, it could result in the inhibition of glycolysis observed in an earlier study (Maritz, 1987 and 1988). Since this inhibition of glycolysis is irreversible, this implies that inhibition of PFK must also be irreversible. This was indeed found at 20*u*M ATP.

From this data it was found that nicotine suppresses PFK at almost al ages under investigation, except at postnatal day 21. The mechanism of inhibition is however, not clear. It is possible that maternal nicotine exposure during pregnancy and lactation may interfere with PFK formation at DNA level. If this is so it might explain why glycolysis is irreversibly inhibited in lung tissue of rat pups exposed to nicotine via the placenta. This is in line with the Barker hypothesis which states that interference with "programming" at a critical phase of development, for example PFK development, will induce permanent deviations from normal (Barker, 1997). It is also possible that the high ATP/ADP ratio of lung tissue of nicotine exposed rat pups will result in a lower PFK activity. It might be a combination of the two above mechanisms. However, since HK was not affected by maternal nicotine exposure, it is unlikely that maternal nicotine exposure influenced PFK structure post-translational and thus its function as enzyme and the control of PFK activity.

The major pathways of glucose utilization include the glycolytic and pentose phosphate pathways (Fisher, 1984). Figure 2.18 indicates the alternative pathways of glucose utilization. Glycolysis produces the high energy compound ATP which is of physiological importance to the lung (Tortora and Grabowski, 1993). The pentose

pathway is the source of NADPH which is the major biological reductant for a wide variety of biosynthetic and detoxification reactions (Fisher, 1984). In this study, (figures 2.6 to 2.9 and figures 2.14 to 2.17) the HK activity was higher in the lungs of nicotine exposed pups, whereas the PFK activity was suppressed in the lungs of the nicotine exposed pups. This data correlates with the findings of Maritz (1987), who indicated that the total glucose turnover of lung tissue of suckling rats receiving nicotine via mother's milk was higher than the control animals. However, lactate production was significantly inhibited in the lungs of the nicotine exposed pups. The decrease in PFK activity in this study, in the lungs of the nicotine exposed pups, could be the explanation for the decrease in lactate production. The increase of total glucose turnover corresponds with the increase in HK activity in this study in the lungs of nicotine exposed pups. However, since the glycolytic pathway is inhibited, the increase in HK activity and glucose turnover could be an indication that the glucose utilization has shifted to the hexose monophosphate shunt. This could be attributed to an increase in the activity of certain microsomal enzymes involved in the inactivation of nicotine, which could lead to a decrease in intracellular NADPH. The decrease in NADPH could eventually enhance the hexose monophosphate shunt activity (Maritz, 1983) and therefore the shift in glucose towards this pathway.

2.4.2.3 Response of PFK to changes in the ATP concentration and the ATP/ADP ratio

PFK is an allosteric enzyme and plays a major role in the control of glucose flux through the glycolytic pathway. The nucleotides ATP and ADP are respectively negative and positive modulators of this enzyme (Boitex and Hess, 1981; Tortora and

Grabowski, 1993). It is therefore to be expected that an increase in the ATP concentration will result in an inhibition of PFK. An increase in the ATP/ADP ratio, such as during a low demand for ATP, will also suppress PFK activity. Conversely, a low ATP/ADP ratio due to an increase in the demand for ATP will result in an increased PFK activity and thus the rate of glucose flow through the glycolytic pathway.

In the present study, it was illustrated that the response of PFK of lung tissue of neonatal rats changes in the ATP concentration and the ATP/ADP ratio, depends on the age of the animal. The PFK activity of 1 and 49 day old control rat pups was not affected by an increase in the ATP concentration from 20*u*M to 20mM. However, on postnatal days 7 and 21, the PFK activity of lung tissue of control rat pups was inhibited by an increase in ATP concentration. This implies that ATP plays a role in the control of glycolysis at PFK level in the lungs of postnatal days 7 and 21 rat pups. In contrast to the above findings, and against all expectations, an increase in the ATP concentration resulted in a pronounced increase in PFK activity of lung tissue of 14 day old rat pups. This implies that ATP acted as a positive modulator for PFK in the lung of 14 day old control rat pups and as a negative modulator on postnatal days 7 and 21. It is however, unlikely that the ATP concentration in lung tissue of rats will increase to this level and will therefore not affect the control of glycolysis in the lung tissue of these animals.

As expected, the ATP/ADP ratios of 12.5 and 6.2 increased the activity of PFK of lung tissue of 1 and 14 day old rat pups. On the other hand on postnatal days 7, 21 and 49, the activity of PFK was lower at both the ATP ratios than at 20*u*M ATP. It is interesting to note that the highest PFK activity was achieved during the phase of rapid cell

proliferation which occurs between postnatal days 4 and 13. Between days 7 and 14, the response of PFK to changes in ATP concentration and ATP/ADP ratios was also maximal. It is therefore plausible that the development of PFK is synchronized with structural development to ensure that the energy supply is sufficient to satisfy demands of the maturing lung.

From the data obtained in this study it is clear that maternal nicotine exposure interfere with the development of PFK in neonatal lung tissue since, the PFK activity of lung tissue of nicotine exposed rat pups was lower than that of the control rat pups. A comparison of the data regarding the response of PFK of lung tissue of control and nicotine exposed rat pups (figures 2.14 to 2.17) with an increase in the ATP concentration and changes in the ATP/ADP ratios, show that there were in general no significant differences. It therefore appears that maternal nicotine exposure during pregnancy and lactation only reduce the activity of PFK in lung tissue without affecting the control of the enzyme. This supports the previous suggestion that maternal nicotine exposure was not affecting the enzyme directly.

This finding explains the inhibition of glycolysis in lung tissue of rats exposed to nicotine via the placenta and mothers milk. The fact that the activity of PFK of lung tissue of nicotine exposed animals remains significantly lower than that of the control animals, even after 4 weeks of withdrawal, implies that the decreased activity is irreversible and this also explains the observation by Maritz (1987) that maternal nicotine exposure resulted in irreversible inhibition of glycolysis.

In conclusion, HK and PFK activity is age dependent. The structural development of the neonatal lung correlates with the activities of HK and PFK for both control and nicotine exposed animals. Thus exposure of the rats to nicotine via the placenta and mothers milk had no effect on the developmental pattern of these enzymes. The variation in ATP concentrations and ATP/ADP ratios did influence the activity of the HK and PFK by either increasing or decreasing the enzyme's activity. Since between postnatal days 7 and 14 most of the alveoli are being formed, it was interesting to find that nicotine increases HK activity between postnatal days 7 and 14 whereas it suppresses PFK activity between postnatal days 7 and 14, thereby suppressing the flux of glucose through the glycolytic pathway. Even though nicotine exposure via the placenta and mothers milk had no effect on the enzymes developmental pattern, it had an adverse effect on the glycolytic pathway. By suppressing the PFK activity, it contributes to the inhibition of glycolysis and therefore the decreased formation of alveoli in lungs of neonates who were exposed to maternal nicotine (Dennis, 1998).

Further investigation is however, needed since total HK and PFK activities were investigated and because isoforms are involved in the process of organ development and regulation of glucose metabolism at multiple levels during development. However, allosteric effectors also play an important role in regulating the activity of the enzyme either by inhibiting or enhancing the activity of the enzyme. ATP is one of the most important regulators of the allosteric enzymes and is indispensable to the life of the cell as the "energy currency" of living systems (Tortora and Grabowski, 1993). Inhibition of PFK and thus of glycolysis will reduce the capacity of this pathway to produce ATP to sustain the integrity of type I epithelial cells that are dependent on glycolysis.

Therefore, the influence of maternal nicotine exposure during gestation and lactation on the adenine nucleotides and adenosine was investigated in chapter III.

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CHAPTER III

Influence of maternal nicotine exposure on the adenine nucleotides (ATP, ADP and AMP) and adenosine

CHAPTER III

Influence of maternal nicotine exposure on the adenine nucleotides (ATP, ADP and AMP) and adenosine

3.1 Introduction

In addition to its function as an organ for passive gas exchange, the lung is known to have metabolic functions essential to both the lung and whole organism (Heinemann and Fishman, 1969). Energy is required for these functions, for development and maintenance of lung integrity. Research showed that carbohydrates have an essential role in the functional and structural development and maturation of fetal and neonatal lung tissue (Bourbon and Jost, 1982; Maniscalco *et al*, 1978) and that glucose is a major energy source of the lung (Felts, 1964). The catabolism of glucose results in synthesis of high energy compounds, such as adenosine triphosphate (ATP). The nett ATP gain from glycolysis is relatively small but may be physiologically important (Fisher, 1984). The lung tissue *per se* is a relative modest consumer of energy in comparison to muscle or other working tissue (Fisher, 1976). However, the lung has energy dependant functions (Fisher, 1984). Some of these energy requiring functions include lung clearance (phagocytosis and ciliary activity), bronchial gland secretion, contraction of tracheobronchial smooth muscle (Fisher, 1984), surfactant synthesis and secretion (Chander *et al*, 1995), and the maintenance of normal transcellular ion gradients (Fisher, 1984).

The ATP molecule is an intracellular, high energy phosphate compound that is

intricately involved in numerous reactions of intermediary metabolism (Fox, 1985), such as donating a phosphate group in a phosphorylation reaction and being a substrate of ATPases (El-Moatassim *et al*, 1992) or being the second substrate for the allosteric enzymes in stage I of the glycolytic pathway (figure 2.1; p48) (Voet and Voet, 1995). Metabolic control of the glycolytic pathway can be analysed from agents that exert regulation on the enzymes involved in glucose uptake (Fisher, 1984). Several enzymes are involved in energy-yielding sequences that catalyse reactions at metabolic branch points and their activity are regulated by important reactants and modifiers. The allosteric enzymes are specifically regulated by means of the regulatory effectors such as the adenine nucleotides (Fisher, 1984; Maritz and Burger, 1992). An important glycolytic regulator is the cellular ATP concentration (Fisher, 1984), which may either enhance or reduce the enzyme's ability to act as a catalyst (Ferdinand, 1976). ATP is the "energy currency" of a living cell (Tortora and Grabowski, 1993) and is a critical compound for maintaining cellular homeostasis. Any alterations in ATP levels in cells can lead to disordered cell function. Therefore, the metabolic basis of specific disorders involves ATP degradation (Fox, 1985). Figure 3.1 indicates the relationship of ATP degradation to uric acid formation.

Changes in energy metabolism may therefore interfere with lung growth and maturation (Maritz and Burger, 1992) and any alterations in ATP levels may lead to disordered morphological integrity (Farber, 1973) and cell function (Farber, 1973; Fox, 1985). Efficient functioning of the above mentioned energy dependant systems as well as lung growth, development and maturation requires the maintenance of tissue energy stores (Edelman *et al*, 1986; Fisher, 1984). The energy stores are therefore most likely to be

altered *in utero* and throughout early life when the bulk of alveoli are added (Langston *et al*, 1984).

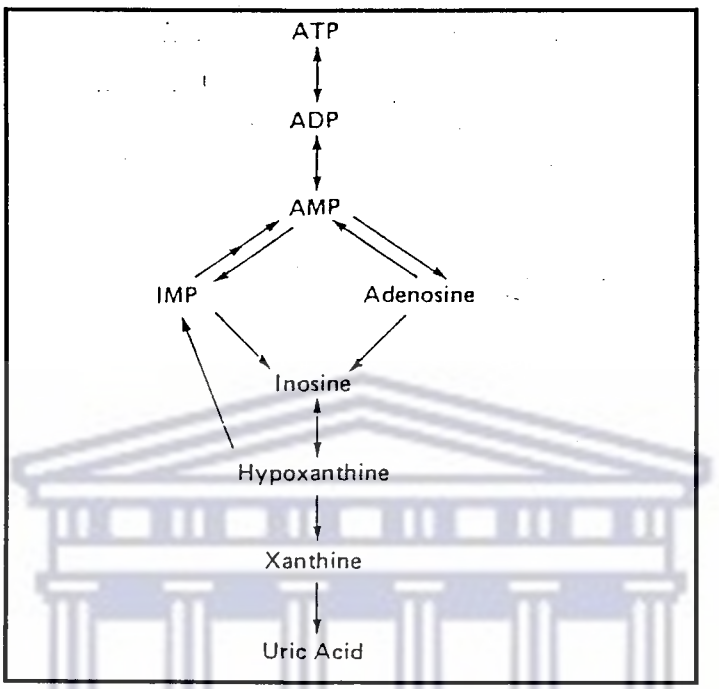


Figure 3.1: ATP degradation to uric acid (Fox, 1985).

Since nicotine interferes with carbohydrate metabolism in the adult rat lung (Maritz, 1983) and suppresses neonatal lung glycolysis (Maritz, 1987), the possibility exists for it to have a negative effect on the normal functional development and maturation of the fetal and neonatal lung. Maritz and Thomas (1994) indeed found that maternal nicotine exposure cause the type I:type II cell ratio to decrease as a result of type II cell proliferation. They (1995) furthermore found that the type II cell mitochondria of lung tissue of nicotine exposed rat pups were swollen and no microvilli occurred on the alveolar surface of the type II cells. These changes are associated with a loss of type II cell function.

It is known that the type I pneumocyte depends on glycolysis for its integrity (Massaro *et al*, 1975). In our findings in the study in chapter III it was found that maternal nicotine exposure enhances HK activity but suppresses PFK activity during the crucial point of lung development between postnatal days 7 and 14. Other studies in our laboratory showed that maternal nicotine exposure suppress glycolysis thus having an impact on lung alveolar integrity and perhaps gas-exchange since about 96% of the gas exchange area of the lung is composed of type I pneumocytes.

In the previous chapter it was illustrated that HK and PFK activity changed as a function of age. It was also shown that the activity of HK and PFK reached maximal activity during the phase of lung development (phase of tissue proliferation) which is associated with rapid cell proliferation and alveolarization. From the results it was also clear that these enzymes are more sensitive to the effect of the adenine nucleotides on their activity during alveolarization than after alveolarization. It is therefore the aim of this chapter to: (a) determine the effect of age on the nucleotide pool and adenosine of the neonatal lung and the impact of maternal nicotine exposure on it. This might give some insight on the effect of the nucleotides on the activities of these enzymes and whether nicotine influence it. (b) propose a mechanism whereby nicotine induce an increase in lung ATP content and (c) to determine whether the increased ATP/ADP ratio indeed result in inhibition of glycolysis.

3.2 Materials and Methods

3.2.1 Sample selection and management

In order to ensure that all the experimental conditions were the same, the sample selection for this section of the study, was exactly as described in chapter II, page 57.

3.2.2 Sampling of lung tissue

The sampling of the lung tissue were the same, as described in chapter II, page 58.

3.2.3 The high performance liquid chromatographic system and mobile phase

The high performance liquid chromatographic system consisted of two Beckman 110B solvent delivery module pumps, a Beckman System Gold Analog interface module 340, a Beckman autosampler module 501 with a 100*ul* loop, a programmable Beckman variable ultra violet (UV) wavelength detector module 166 and an IBM computer. A Panasonic KX-1081 printer was connected to the system. A reversed phase column 15cm x 4.6mm and guard column 5cm x 4.6mm was used. The stationary phase for both the column and guard column consisted of spherical 5*u*m particles on silicon core with C-18 coating.

The mobile phase contained 0.25M pro-analytic potassium dihydrogen phosphate (KH_2PO_4), tetrabutylammonium hydrogen sulphate (TBAHS) and 1.25% HPLC grade methanol. Thus in 2000ml distilled water, 70g KH_2PO_4, 0.8g TBAHS and 25ml of methanol was dissolved. The pH was adjusted to 4 with phosphoric acid and the mobile phase was filtered through a 0.45*u*m nylon filter and degassed before use. The chromatography was performed at ambient temperature and the detector was set at a

wavelength of 254nm. The flow rate was maintained at 1ml/min.

3.2.4 Preparation of standards

Nucleotide and nucleoside standards were of the highest degree of purity. Four standards, ATP 380*u*mol/*l*, ADP 180*u*mol/*l*, AMP 80*u*mol/*l* and adenosine 180*u*mol/*l* were prepared. The following formula was used to prepare a standard:

$$\frac{\text{Concentration x molecular weight (MW)}}{1}$$

$$= \text{Xg/l}$$

$$= \frac{\text{Xg/l}}{1} \times \frac{10\text{ml}}{1000\text{ml}}$$

$$= \text{Yg/10ml}$$

$$= (\text{Y}) \times 10\text{ml}$$

$$= \text{Zg}$$

Therefore Z grams were dissolved in a 100ml of mobile phase. This solution was known as the stock solution. A further 1 in 10 solution was prepared. Therefore 1ml of stock was added to 9ml of the mobile phase. The dilution was filtered through a 0.45*u*m syringe nylon filter. From the filtered dilution, 25*ul* was injected into the HPLC system.

Table 3.1: Nucleotide and nucleoside standard information.

Standards	**Concentration (*umol/l*)**	**Grams dissolved in a 100ml of mobile phase**
ATP	380	0.0230
ADP	180	0.0085
AMP	80	0.0040
Adenosine	180	0.0048

3.2.5 Preparation of lung tissue

This assay was based on the protocol as described by Sellevold and colleagues (1986). The stored lung sample was removed from the -80^{0}C freezer. The frozen lung tissue was then emerged in liquid nitrogen and placed in a test tube and homogenized with a Heidolph RZR 50 homogenizer. About 100mg (0.1g) of homogenized tissue was weighed out into an eppendorf, 500*ul* of 0.42M perchloric acid was then added to dissolve the tissue. The extract was then stirred intermittently for 10 minutes with a vortex mixer model K-550-Genie. All samples and chemical solutions were kept on ice between manipulations. The extract was precipitated with 180*ul* 1M potassium hydroxide (KOH). After precipitation the extract was centrifuged at 10 000g (gravity units) for 6 minutes in a chilled (4^{0}C) bench top Eppindorf centrifuge. The supernatant was filtered through a 0.45*u*m syringe nylon filter and kept on ice until 25*ul* was injected into the HPLC system.

3.2.6 Maintenance and running of the HPLC system

The present system employs a mobile phase containing a high KH_2PO_4 concentration. Therefore, to eliminate salt deposits in the column on storage, adequate rinsing was necessary. The apparatus in running status, was switched on and the detector was switched on, to make sure that the lamp warmed up. The rinsing of the column was done by placing the feed line in a water bottle, and the pump as well as the columns were rinsed for a minimum time of an hour. When the rinsing program was completed the feed line was placed in the mobile phase.

An isocratic program was followed. The flow rate was 1ml/min and auto zero was done

at 0.10 minutes and a single run was done in 30 minutes. The pump received a 100% mobile phase. Before calibration or injection of samples, the column was stabilised for a minimum of an hour in the mobile phase. The wavelength for nucleotide absorbance was set to 254nm. After the running of the samples, the pump and column were rinsed with distilled water for at least an hour. Thereafter, the feed line of the pump was placed in 70% methanol (HPLC grade) to prevent bacterial growth. However, if the column would not be used for a long period of time, the column would be flushed with the specified shipping solvent and stored. To summarize the running and rinsing was done as followed:

buffer ⇆ water ⇆ 70% methanol

All solutions were filtered and degassed and all samples were filtered before injecting into the system.

3.2.7 Quantitative analysis of ATP, ADP, AMP and adenosine by means of HPLC

The separations were achieved over a time period of 30 minutes, and peak areas were automatically determined by the Beckman Gold System. Table 3.2 indicates the average area under the curve of three runs of the standards as well as the known concentrations of the standards. Figure 3.2 shows the chromatogram of the separation of the standard nucleotides and adenosine in the mobile phase on the Beckman Gold System with absorbance detected at 254nm.

The calculation of the concentration of the unknown were done by the following formula 1:

$$\text{Concentration of unknown} = \frac{\text{Area of test}}{\text{Area of standard}} \times \frac{\text{Concentration of standard}}{1}$$

However, since the final data was expressed in *u*g/g wet lung tissue the following calculations were done to convert the above mentioned answer (calculated from formula 1) using formula 2:

$$\text{mass} = M \times MW \times L$$

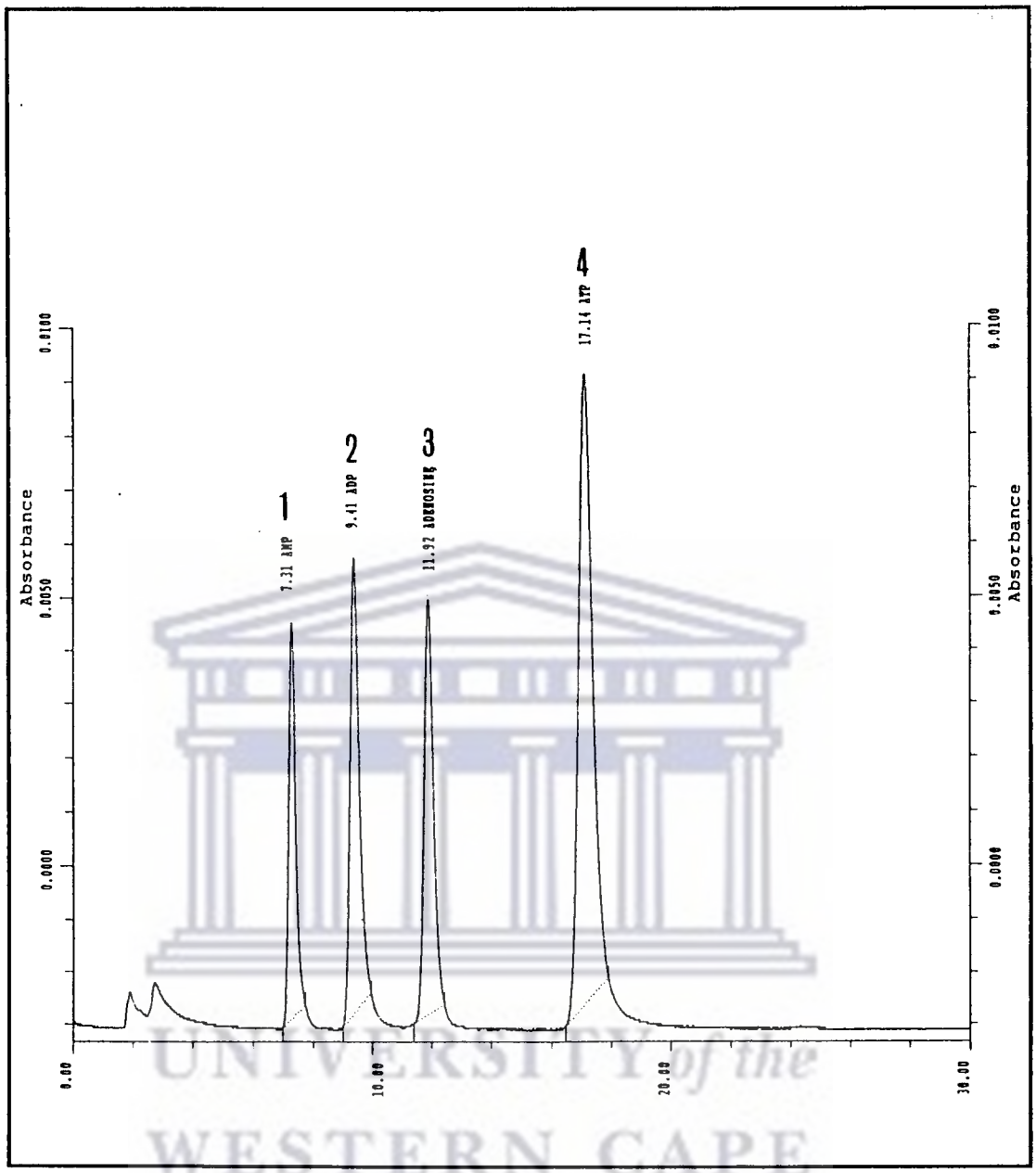
where: M = concentration of the unknown sample, from formula 1

MW = molecular weight of the specific standard

L = volume of fluid used to extract the nucleotides or nucleoside from the tissue

Table 3.2: The known average area of the adenine nucleotides and adenosine standard and known concentrations of the standards used in the calculations of the unknown.

Nucleotides and Nucleoside	**Average area of standards**	**Concentration of standards (*u*mol/l)**
ATP	6.65241	380
ADP	4.82481	180
AMP	1.91559	80
Adenosine	3.04873	180



Key: **1** - AMP

2 - ADP

3 - Adenosine

4 - ATP

Figure 3.2: The chromatogram of the separated standard nucleotides and adenosine, in mobile phase on the Beckman Gold System with absorption detected at 254nm.

3.2.8 Statistical analysis

Statistical analysis was performed using Medcalc (Schoonjans *et al*, 1995). Results were recorded throughout as the mean ± standard error of the mean. For statistical evaluation the unpaired Wilcoxon Rank t-test was employed. The probability level of $P < 0.05$ was designated as being statistically significant between the mean values of control and experimental (nicotine) groups.

3.3 Results

3.3.1 Influence of maternal nicotine exposure on the ATP content of the lung tissue of the neonates

The data in table 3.3 and figure 3.3 displays the ATP content in the lungs of control and nicotine exposed neonates. Comparison of the lung ATP content of control animals to the nicotine exposed animals at postnatal days 1 and 7 respectively showed no significant changes. The ATP content for the control animals was however, at 2.00 ± 0.48 *u*g/g tissue on postnatal day 14. On postnatal day 21 the ATP content was at 4.92 ± 0.41 *u*g/g tissue 64% higher ($P<0.001$) within the nicotine exposed lungs than that of the controls at 3.00 ± 0.23 *u*g/g tissue. After 4 weeks of nicotine withdrawal, at the age of 49 days the ATP content of the nicotine exposed lungs was at 6.32 ± 0.26 *u*g/g tissue, 110.7% higher ($P<0.001$) than that of the controls at 3.00 ± 0.23 *u*g/g tissue.

No significant ($P>0.05$) change in the lung ATP content was observed with increase in age in the control animals. However, the ATP content in the nicotine exposed lung was constant from postnatal day 1 to postnatal day 7. On postnatal day 14, the ATP content was to low to be detected. The ATP content increased to 4.92 ± 0.41 *u*g/g tissue on postnatal day 21 in the nicotine exposed lung. Between postnatal days 21 and 49 the ATP content, increased further by 28.5% to 6.32 ± 0.26 *u*g/g tissue on postnatal day 49 for nicotine exposed animals.

Table 3.3: Influence of maternal nicotine exposure on the ATP content of the lung tissue of the neonates.

ATP (*ug*/g lung tissue)			
Age (days)	**Control**	**Nicotine**	**P - values**
1	2.43 ± 0.62 (n = 7)	1.40 ± 0.43 (n = 10)	> 0.05
7	1.50 ± 1.36 (n = 8)	1.13 ± 0.18 (n = 8)	> 0.05
14	2.00 ± 0.48 (n = 9)	-	-
21	3.00 ± 0.23 (n = 9)	4.92 ± 0.41 (n = 9)	< 0.001
49	3.00 ± 0.23 (n = 9)	6.32 ± 0.26 (n = 9)	< 0.001

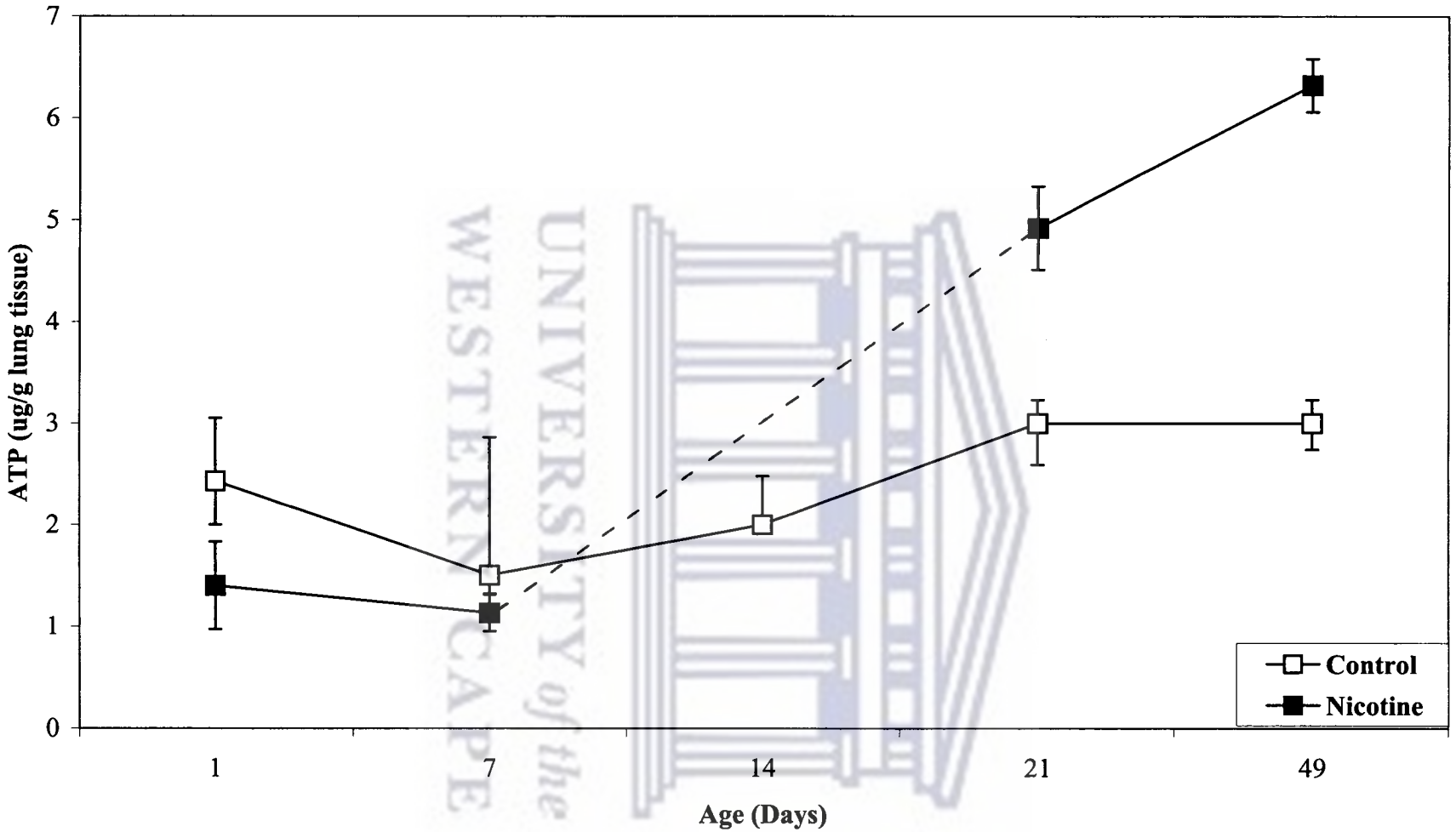


Figure 3.3: The influence of maternal nicotine exposure on the ATP content of the lung tissue of the offsprings.

3.3.2 Influence of maternal nicotine exposure on the ADP content of the lung tissue of the neonates

The data in table 3.4 and figure 3.4 represents the ADP content in the lungs of control and nicotine exposed neonates. Maternal nicotine exposure resulted in a higher ($P<0.05$) ADP content of 32% at 5.32 ± 0.38 *u*g/g tissue in the nicotine exposed lung compared to that of the control lung at 4.03 ± 0.31 *u*g/g tissue of postnatal day 1 pups. No significant ($P>0.05$) difference was observed in the ADP content of the lungs of the control and the nicotine exposed pups on postnatal day 7. After 14 days, the ADP content of lung tissue of nicotine exposed rat pups, was at 3.48 ± 0.28 *u*g/g tissue, 21.1% lower ($P<0.05$) than that of the control lung at 4.41 ± 0.23 *u*g/g tissue. The ADP content of the lung of the neonates exposed to nicotine on postnatal day 21 was at 4.52 ± 0.40 *u*g/g tissue, 270.5% higher ($P<0.001$) than that of the control lung at 1.22 ± 0.37 *u*g/g tissue. In contrast to results obtained for ATP, no significant difference was observed between the ADP content of the lungs exposed to nicotine and control lungs of the neonates on postnatal day 49.

The ADP content in the lungs of the control animals decreased ($P<0.01$) by 31.5% between postnatal days 1 and 7 from 4.03 ± 0.31 *u*g/g tissue to 2.76 ± 0.58 *u*g/g tissue. Between postnatal days 1 and 7 the nicotine exposed animals ADP content decreased ($P<0.001$) by 47.2% from 5.32 ± 0.38 *u*g/g tissue to 2.81 ± 0.11 *u*g/g tissue. Between postnatal days 7 and 14 the ADP content of the control animals increased ($P<0.05$) by 59.8% to 4.41 ± 0.23 *u*g/g tissue and increased ($P<0.05$) by 67% to 3.48 ± 0.28 *u*g/g tissue for those pups exposed to nicotine. The ADP content in the lung for control animals decreased ($P<0.001$) by 72.3% between postnatal days 14 and 21 to 1.22 ± 0.37

Table 3.4: Influence of maternal nicotine exposure on the ADP content of the lung tissue of the neonates.

ADP (*ug*/g lung tissue)			
Age (days)	**Control**	**Nicotine**	**P - values**
1	4.03 ± 0.31 (n = 9)	5.32 ± 0.38 (n = 9)	< 0.05
7	2.76 ± 0.58 (n = 10)	2.81 ± 0.11 (n = 9)	> 0.05
14	4.41 ± 0.23 (n = 7)	3.48 ± 0.28 (n = 10)	< 0.05
21	1.22 ± 0.37 (n = 10)	4.52 ± 0.40 (n = 10)	< 0.001
49	6.28 ± 0.23 (n = 9)	6.40 ± 0.45 (n = 8)	> 0.05

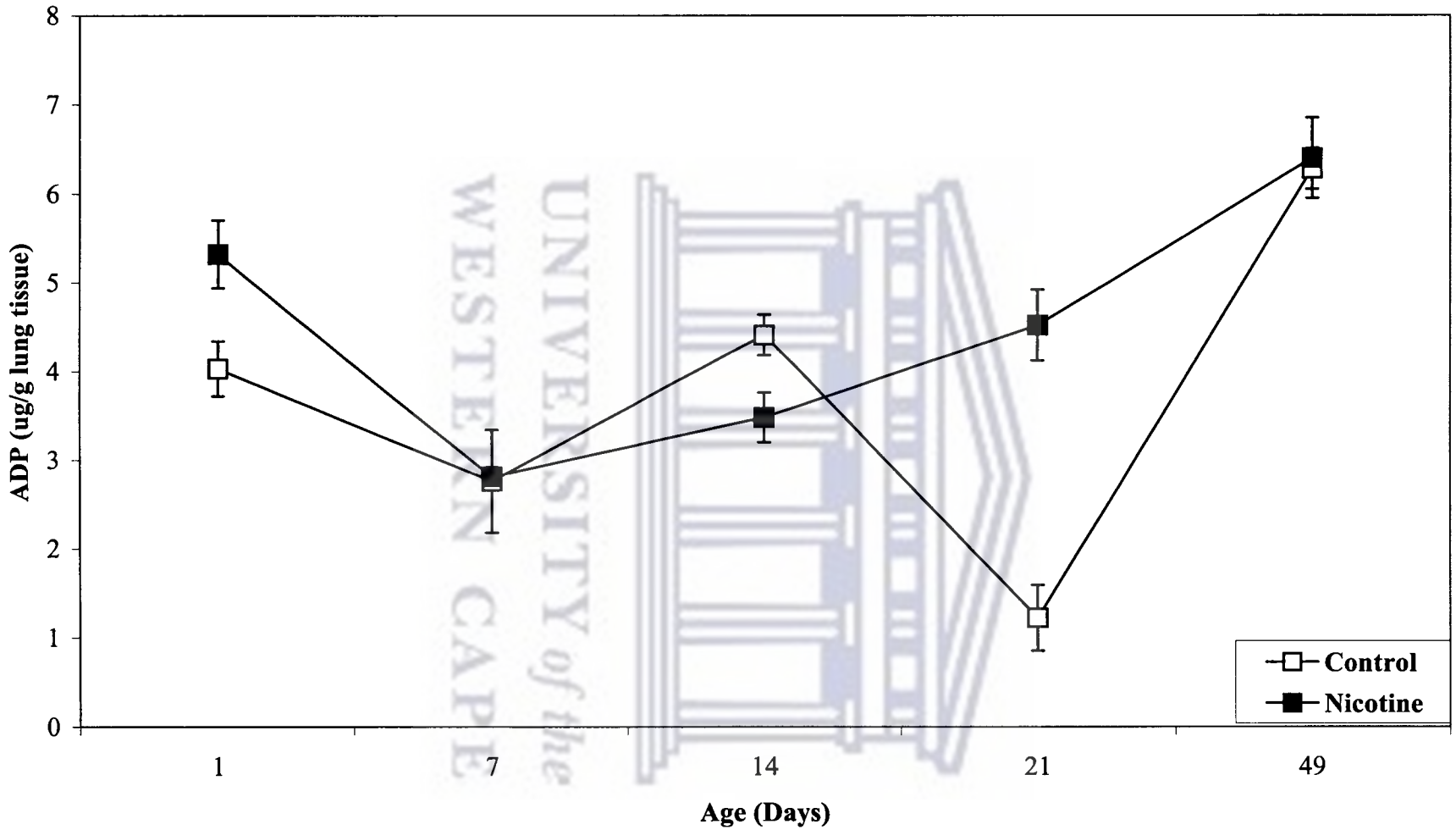


Figure 3.4: The influence of maternal nicotine exposure on the ADP content of the lung tissue of the offsprings.

*u*g/g tissue, however, the nicotine exposed pups ADP content increased (P<0.01) by 29.9% to 4.52 ± 0.40 *u*g/g tissue between postnatal days 14 and 21. Between postnatal days 21 and 49 the ADP content increased (P<0.001) by 414.8% to 6.28 ± 0.23 *u*g/g tissue for the control animals and increased (P<0.01) by 41.6% to 6.40 ± 0.45 for the nicotine exposed animals.

3.3.3 Influence of maternal nicotine exposure on the AMP content of the lung tissue of the neonates

The data in table 3.5 and figure 3.5 displays the AMP content in the lungs of control and nicotine exposed neonates. Maternal nicotine exposure resulted in a higher (P<0.05) AMP content of 27% at 0.94 ± 0.07 *u*g/g tissue in the nicotine exposed lung compared to that of the control lung at 0.74 ± 0.05 *u*g/g tissue of postnatal day 1 pups. No significant difference (P>0.05) was observed in the AMP content of the lungs of the control and the nicotine exposed pups on postnatal day 7. On postnatal day 14 maternal nicotine exposure had no significant (P>0.05) effect on the AMP content in the lungs. However, in contrast to the significant increase in ADP content observed in the 21 day old pups, the AMP content on postnatal day 21 of lung tissue of nicotine exposed rat pups was at 0.35 ± 0.02 *u*g/g tissue, 66.7% lower (P<0.05) than that of the control lung at 0.21 ± 0.04 *u*g/g tissue. On postnatal day 49 the AMP lung content was significantly lower (P<0.001), by 35.8% for those animals exposed to nicotine at 0.34 ± 0.008 *u*g/g tissue compared to that of the control animals at 0.53 ± 0.04 *u*g/g tissue.

The AMP content in the lungs of the control animals decreased (P<0.001) by 71.6% between postnatal days 1 and 21 from 0.74 ± 0.05 *u*g/g tissue to 0.21 ± 0.04 *u*g/g tissue.

Table 3.5: Influence of maternal nicotine exposure on the AMP content of the lung tissue of the neonates.

AMP (*ug*/g lung tissue)			
Age (days)	**Control**	**Nicotine**	**P - values**
1	0.74 ± 0.05 (n = 10)	0.94 ± 0.07 (n = 10)	< 0.05
7	0.40 ± 0.15 (n = 10)	0.53 ± 0.006 (n = 8)	> 0.05
14	0.46 ± 0.10 (n = 10)	0.48 ± 0.04 (n = 10)	> 0.05
21	0.21 ± 0.04 (n = 10)	0.35 ± 0.02 (n = 10)	< 0.05
49	0.53 ± 0.04 (n = 9)	0.34 ± 0.008 (n = 8)	< 0.001

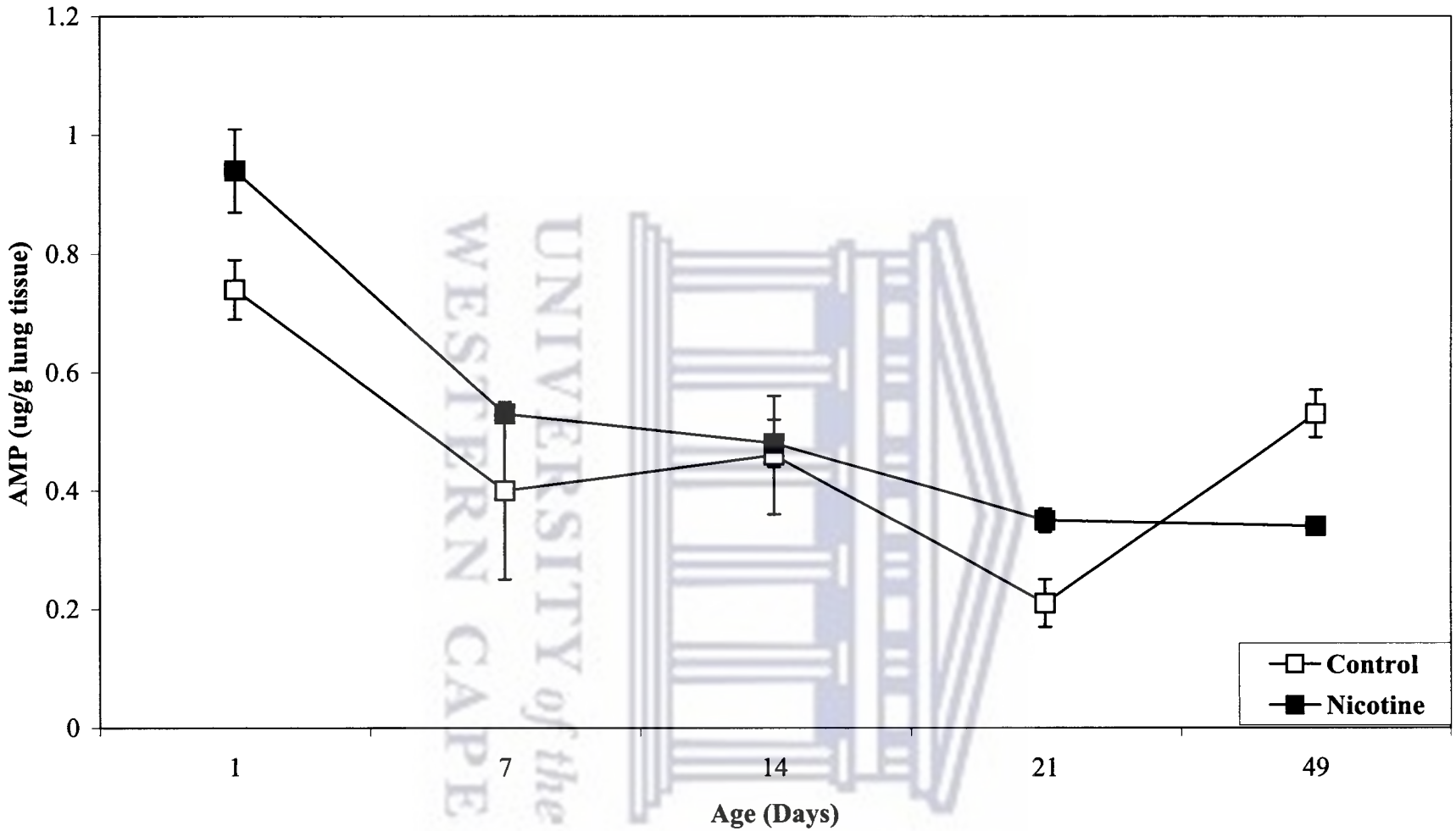


Figure 3.5: The influence of maternal nicotine exposure on the AMP content of the lung tissue of the offsprings.

From postnatal day 21 to 49 the AMP content of lung tissue of control rats increased ($P<0.001$) by 152.4%. Due to this increase the difference in AMP content of lung tissue of 1 day old rats and 49 day old rats was 28.4% ($P<0.05$). Unlike the control lungs, the AMP content of the lung tissue of nicotine exposed animals continuously decreased ($P<0.001$) as the lungs mature with 63.8% between postnatal days 1 and 49.

3.3.4 Influence of maternal nicotine exposure on the ADO content of the lung tissue of the neonates

The data summarized in table 3.6 and illustrated in figure 3.6 shows the ADO content of control lung increased 3.7 fold ($P<0.05$) between postnatal days 1 and 7. After postnatal day 7 the ADO content remained constant ($P>0.05$). The ADO content of the nicotine exposed lungs increased 2 fold from 0.32 ± 0.13 *u*g/g tissue on postnatal day 1 to 0.64 ± 00.04 *u*g/g tissue on postnatal day 49. This means that although ADO increased in lung tissue of both the control and nicotine exposed animals, the increase in the latter group was less profound. After postnatal day 7, the high ADO content was maintained in both the control and nicotine exposed animals. It is important to note that the ADO content of lung tissue of control rats were always significantly higher ($P<0.05$) than that of the nicotine exposed animals.

Furthermore, maternal nicotine exposure resulted in a lower ($P<0.05$) ADO content of 77.6% in the lung at 0.32 ± 0.13 *u*g/g tissue in the nicotine exposed lung, compared to that of the control lung at 1.43 ± 0.15 *u*g/g tissue on postnatal day 1. On postnatal day 7 the nicotine exposed animals ADO lung content was at 0.64 ± 0.04 *u*g/g tissue significantly lower ($P<0.05$), by 87.9% compared to that of the control animals at 5.30

Table 3.6: Influence of maternal nicotine exposure on the ADO content of the lung tissue of the neonates.

ADO (*u*g/g lung tissue)			
Age (days)	**Control**	**Nicotine**	**P - values**
1	1.43 ± 0.15 (n = 8)	0.32 ± 0.13 (n = 10)	< 0.005
7	5.30 ± 2.88 (n = 10)	0.64 ± 0.04 (n = 9)	< 0.05
14	5.80 ± 1.40 (n = 10)	0.47 ± 0.08 (n = 9)	< 0.01
21	5.12 ± 2.03 (n = 10)	-	-
49	5.11 ± 0.77 (n = 9)	0.51 ± 0.14 (n = 7)	< 0.001

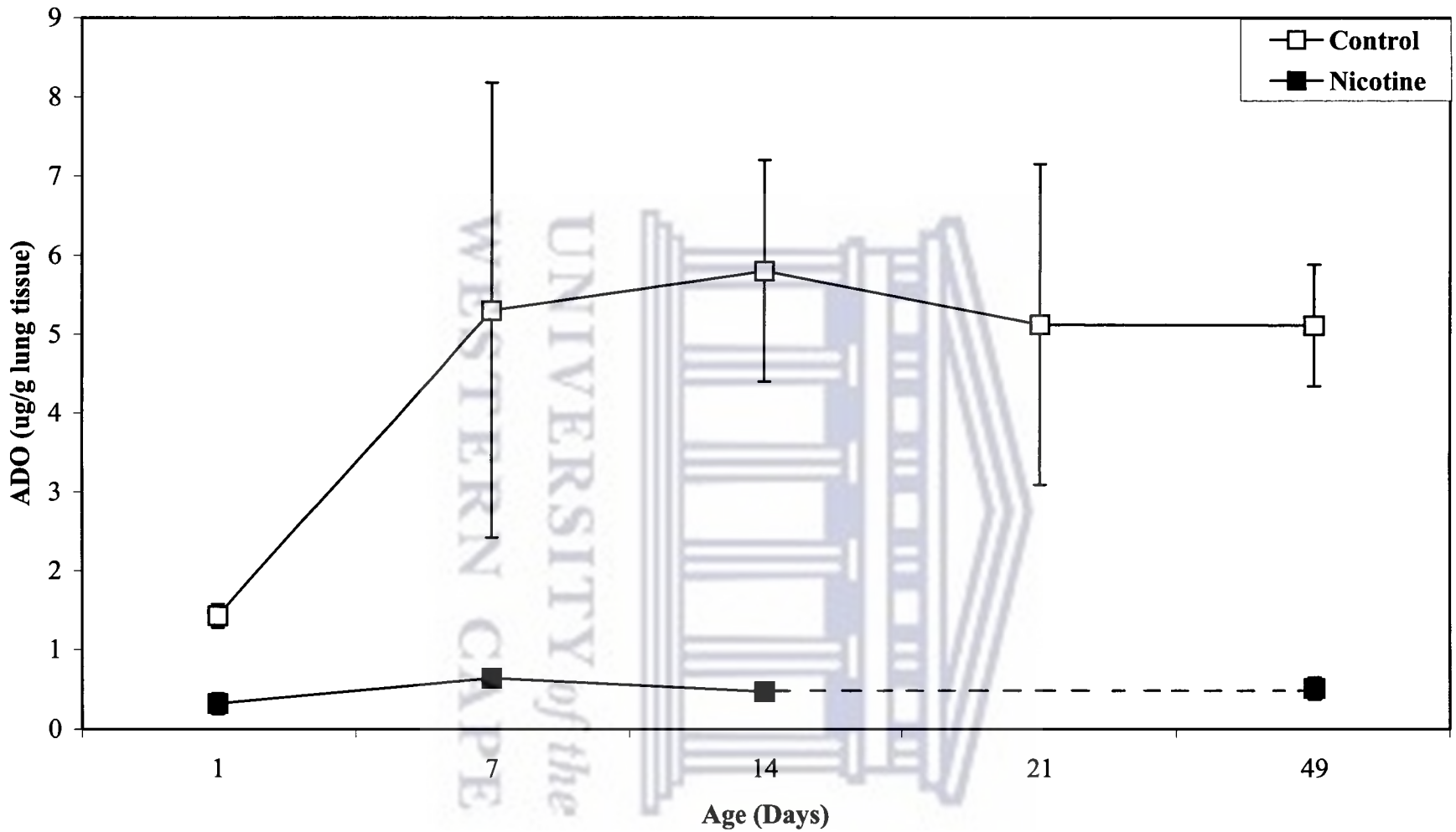


Figure 3.6: The influence of maternal nicotine exposure on the ADO content of the lung tissue of the offsprings.

± 2.88 *u*g/g tissue. On postnatal day 14 the nicotine exposed animals ADO content of the lung was at 0.47 ± 0.08 *u*g/g tissue, significantly lower (P<0.01) by 91.9% compared to that of the control animals at 5.80 ± 1.40 *u*g/g. However, on postnatal day 21 no ADO could be detected in the lungs of the nicotine exposed animals, while in lung tissue of control animals of the same age it was 5.12 ± 2.03 *u*g/g tissue. The ADO content of the lung at postnatal day 49 was significantly lower (P<0.001) by 90% at 0.51 ± 0.14 *u*g/g tissue for those animals exposed to nicotine compared to the control animals at 5.11 ± 0.77 *u*g/g tissue.

3.3.5 Energy stores of the lung

The data in table 3.7 and figure 3.7 displays the adenine nucleotide pool and ratios of the lung tissue of the offsprings of the control animals. Calculation of the adenine nucleotide pool from (table 3.7), shows that the pool fluctuates with higher levels on postnatal day 1 (7.20 *u*g/g tissue) and 14 (6.87 *u*g/g tissue) than on postnatal days 7 (4.66 *u*g/g tissue) and 21 (4.43 *u*g/g tissue). On postnatal day 49, the adenine nucleotide pool was 9.81 *u*g/g. In comparison the above data on the adenine nucleotide pool of lung tissue of nicotine exposed rat pups (table 3.8), it shows that it gradually decreased from 7.66 *u*g/g tissue, which resembles that of the control animals, on postnatal day 1 to 3.96 *u*g/g tissue on postnatal day 14. However, after postnatal day 14 the adenine nucleotide pool of these rat pups gradually increased to 9.79 *u*g/g tissue on postnatal day 21 and 13.06 *u*g/g tissue on postnatal day 49. On postnatal days 21 and 49 the pool size of lung tissue of nicotine exposed rats were respectively 2.2 and 1.3 fold higher than in lungs of control rats. This was primarily due to the high ATP content of the lungs of the nicotine exposed animals.

Table 3.7: The adenine nucleotide pool of the lung tissue of the control offsprings.

	Age (Days)				
Adenine nucleotide pool	**1**	**7**	**14**	**21**	**49**
ATP + ADP + AMP	7.20	4.66	6.87	4.43	9.81
ATP/ADP	0.60	0.54	0.45	2.46	0.48
ATP/ADP + AMP	1.34	0.94	0.91	2.67	1.01
$\frac{\text{ATP + ½ADP}}{\text{ATP + ADP + AMP}}$	0.62	0.62	0.61	0.81	0.6

Table 3.8: The adenine nucleotide pool of the lung tissue of nicotine exposed offsprings.

	Age (Days)				
Adenine nucleotide pool	**1**	**7**	**14**	**21**	**49**
ATP + ADP + AMP	7.66	4.47	3.96	9.79	13.06
ATP/ADP	0.20	0.40	-	1.09	0.99
ATP/ADP + AMP	1.20	0.93	-	1.44	1.33
$\frac{\text{ATP + ½ADP}}{\text{ATP + ADP + AMP}}$	0.53	0.57	-	0.73	0.73

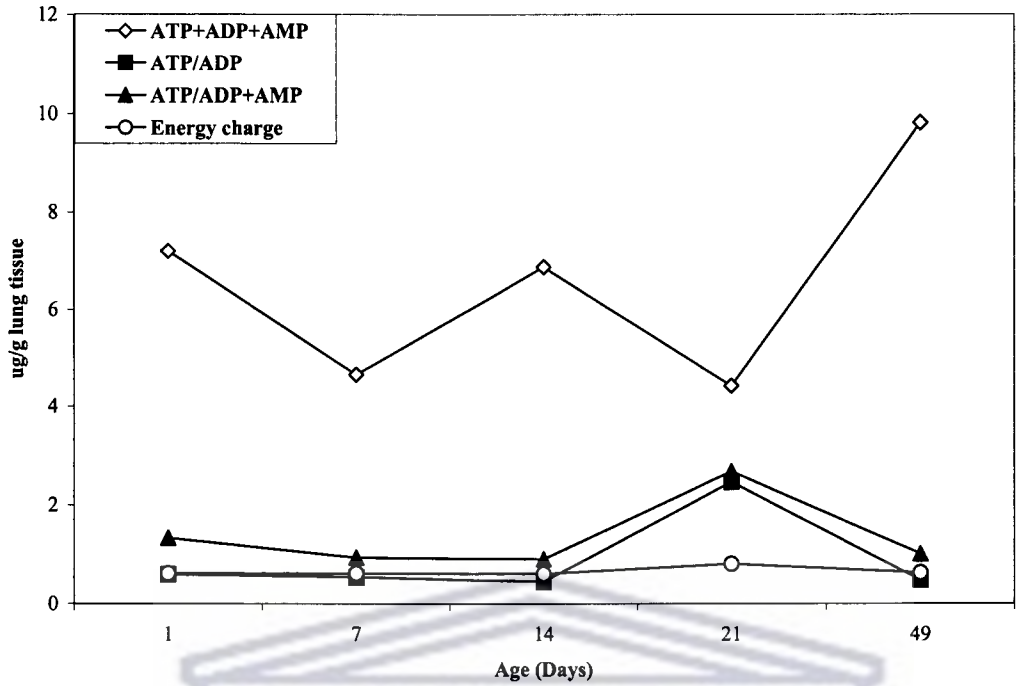


Figure 3.7: The energy stores of the lung tissue of the control offsprings.

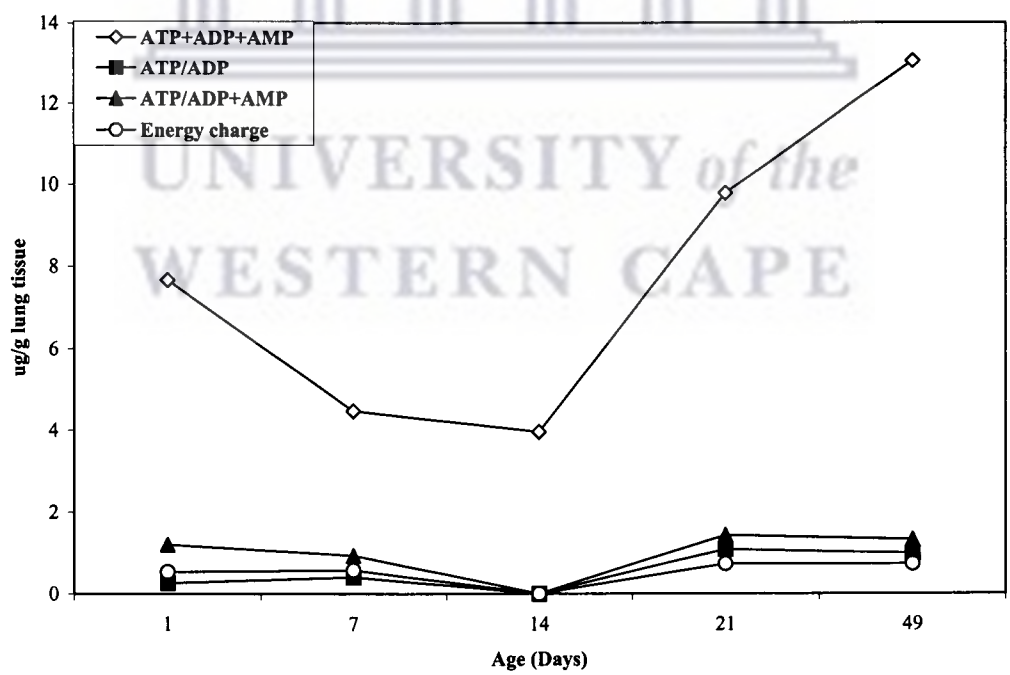


Figure 3.8: The energy stores of the lung tissue of nicotine exposed offsprings.

A comparison of the ATP/ADP ratio show that it was lower in the lungs of the 1, 7, 14 and 21 day old nicotine exposed animals. On postnatal day 49 it was however, 2.06 fold higher in the lungs of the nicotine exposed animals than in lungs of control animals. This was due to the fact that the ATP concentration in lung tissue of nicotine exposed rats was 3.3 fold higher than in lungs of control animals, despite the fact that the ADP content for control and nicotine exposed animals was the same. The same is true for the ATP/ADP + AMP ratio and the energy charge.

3.4 Discussion

From the data it is clear that the ATP content of the lung tissue of the control animals was maintained. This implies that the synthesis and degradation of ATP was controlled. This also implies that al the energy dependant processes are receiving adequate amounts of ATP-energy to ensure controlled growth and tissue maintenance. The accumulation of ATP in the lungs of the nicotine exposed animals implies that the control of ATP metabolism was impaired. It is unlikely that the accumulation of ATP in the lungs of these nicotine exposed animals was due to an increase in the rate of ATP production, since glycolysis was suppressed and therefore the flux of glucose through the glycolytic pathway and consequently ATP production via this pathway (Maritz, 1987). Nicotine exposure also have no influence on oxygen utilization and thus the synthesis of ATP at mitochondrial level. It is therefore plausible that the increase in lung ATP content was due to suppression of ATP degradation at a different site in the lung.

ATP degradation can be via the adenine nucleotide degradation pathway (figure 3.9) or it can be used in phosphorylation reactions, such as in the glycolytic pathway (Fox, 1985). It is unlikely that the latter mechanism of ATP utilization is involved in the accumulation of ATP, since the HK activity increased in the lungs of the nicotine exposed animals. In addition, Maritz and Burger (1992) showed that glucose turnover in lung tissue of nicotine exposed rats increased. This can only be achieved if the rate of glucose-phosphorylation under influence of HK increased.

It is therefore possible that inhibition of ATP degradation via the adenine nucleotide degradation pathway (figure 3.9) is affected by maternal nicotine exposure. According

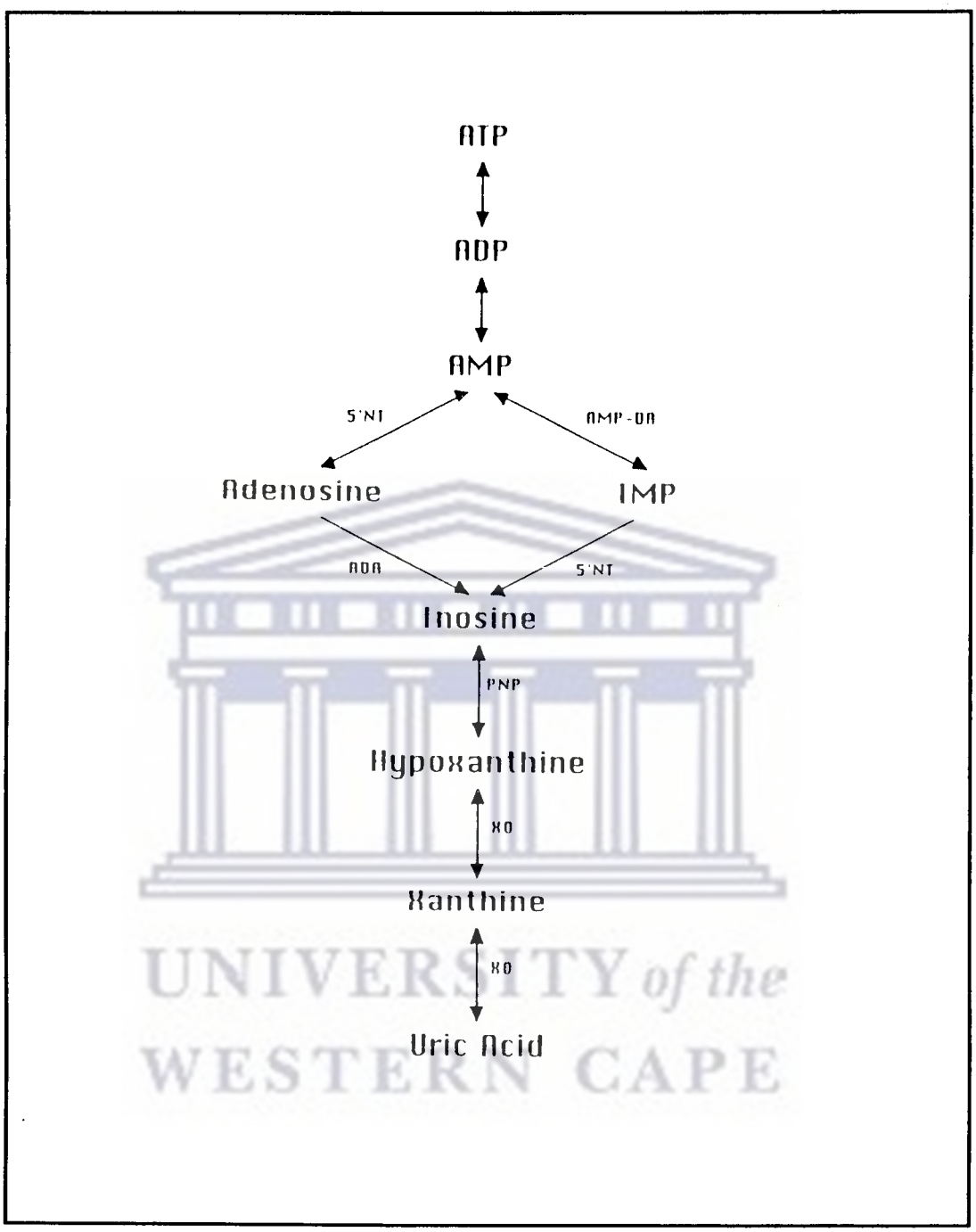


Figure 3.9: Adenine nucleotide degradation pathway. IMP (inosine monophosphate). Enzymes are as follows: 5' NT (5'-nucleotidase); AMP-DA (adenosine monophosphate deaminase); ADA (adenosine deaminase); PNP (purine nucleoside phosphorylase) and XO (xanthine oxidase) (De Leyn *et al*, 1993).

to this pathway ATP is converted to ADP and the latter to AMP. AMP is then converted to adenosine under influence of 5'-nucleotidase (5'NT) and to IMP by adenosine monophosphate deaminase (AMP-DA). Adenosine and IMP are both converted to inosine. The conversion of IMP to inosine is also catalysed by 5'NT. In the present investigation it was found that the AMP content of the nicotine exposed lungs was higher than in the lungs of the control animals. Furthermore, the adenosine content of lung tissue of nicotine exposed animals was markedly lower than in the lungs of their control counterparts. Based on these findings it is conceivable that the accumulation of ATP in lung tissue of nicotine exposed animals was due to an inhibition of 5'NT. If this is so it can be expected that the IMP content of the lungs of nicotine exposed animals will increase and that blood and urine uric acid content will decrease. It is however, important to recognize that the imbalance in the above pathway is not the only site of nicotine action, since nicotine also suppresses Na^+-K^+-ATPase (Meyer *et al*, 1971).

It was suggested by Maritz and Burger (1992) that the increase in ATP in lungs of nicotine exposed animals and consequently the ATP/ADP ratio, explains the inhibition of glycolysis in the lungs of these animals. However, the data in chapter II shows that this is unlikely since the HK activity and thus phosphorylation of glucose is increased in lungs of nicotine exposed animals. In addition, an increase in ATP actually increased the HK and PFK activities. This implies that the inhibition of the glucose flux via glycolysis was not due to an increase in the ATP/ADP ratio, but rather to the lower PFK activity. Furthermore, the change in HK and PFK activities was achieved at relatively high ATP concentrations which exceeded that of the lung. It therefore appears to be unlikely that a high ATP/ADP ratio is responsible for the inhibition of glycolysis in

lungs of nicotine exposed rat pups as suggested by Maritz and Burger (1992).

Since this study dealt with investigating whole lung nucleotides and adenosine further investigation is needed, to study the specific cell types such as the type I and type II cell nucleotide and adenosine composition and the energy charge levels of these cells. Also further investigation is needed in determining the uric acid in the blood of these animals exposed to nicotine and to determine the enzyme activity of 5'NT and AMP-DA.

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APPENDIX

APPENDIX A

List of Abbreviations

$\triangle$A..... change in absorbance

A.D...... Anno Domini

ADA..... adenosine deaminase

ADP..... adenosine diphosphate

ADO..... adenosine

AMP..... adenosine monophosphate

ATPases..... adenosinetriphosphatase

ATP..... adenosine triphosphate

C..... carbon

cAMP..... cyclic adenosine monophosphate

cm..... centimeter

CO..... carbon monoxide

EDTA..... ethylenediaminetetra-acetic acid disodium salt

FBP..... fructose-1,6-bisphosphate

F6P..... fructose-6-phosphate

FFA..... free fatty acid

g..... grams

GAPDH..... glyceraldehyde-3-phosphate dehydrogenase

G6PDH..... glucose-6-phosphate dehydrogenase

Glut..... glucose transporter

H^+..... hydrogen ion

HK..... hexokinase

HPLC..... high performance liquid chromatography

i.e..... that is

IMP..... inosine monophosphate

K..... potassium

K^+..... potassium ion

KCl..... potassium chloride

kg..... kilogram

KH_2PO_4..... potassium dihydrogen phosphate

Km..... Michaelis constant

KOH..... potassium hydroxide

l..... liter

M..... Molar

m^2..... square meter

Mg^{2+}..... magnesium ion

mg..... milligram

$MgCl_2.H_2O$... magnesium chloride with water

$MgSO_4$..... magnesium sulphate

min..... minute

ml..... milliliter

mM..... millimolar

mm..... millimeter

M.S..... main stream

MW..... molecular weight

n..... sample size

Na..... sodium

Na^+..... sodium ion

NAD^+..... nicotinamide adenine dinucleotide, oxidized form

NADH..... nicotinamide adenine dinucleotide, reduced form

NADP..... nicotinamide adenine dinucleotide phosphate

NADPH..... nicotinamide adenine dinucleotide phosphate, reduced form

nm..... nanometer

5'NT..... 5'-nucleotidase

OD..... optical density

OH..... hydroxyl

p..... page

P..... probability

PFK..... phosphofructokinase

P_i..... inorganic phosphate

PK..... pyruvatekinase

pp..... pages

SEM..... standard error of the mean

SGLT..... sodium-glucose cotransporter

TBAHS..... tetrabutylammonium hydrogen sulphate

TIM..... triose isomerase

Tris-HCl..... tris hydrochloride

U..... units

ug..... microgram

ul..... microliter

***u*M**..... micromolar

***u*m**..... micrometer

***u*mol**..... micromole

USA..... United States of America

UV..... ultra violet

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