THE EFFECT OF NICOTINE EXPOSURE ON ASPECTS OF LIVER CARBOHYDRATE METABOLISM

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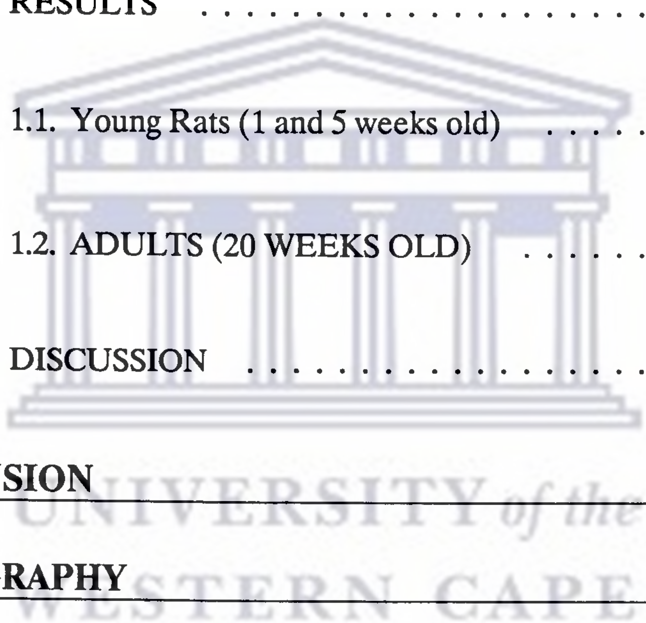
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ABSTRACT

The liver plays an important role in maintaining the blood glucose concentration. In this respect glycogenolysis and gluconeogenesis play an important role. Interference with these pathways may therefore have an effect on the ability of this organ to maintain blood glucose levels. In this study the effect of *in vivo* and *in vitro* nicotine exposure was investigated to establish whether nicotine exposure:

i. influences glycogenolysis and in gluconeogenesis,

ii. have the same effect on fasted animals and

iii. have the same effect on neonates exposed to nicotine via mother's milk.

Experimental animals were Wistar rats of : 1 day, 1 week, 2 weeks, 5 weeks, and 20 weeks old. Animals were exposed to nicotine (1 mg/kg/day) i.p./s.c. and were killed by means of decapitation on predetermined days, 1 and 24 hours after the final dose. Control animals received 0,9% saline instead of nicotine. The volume depended upon the animal's body mass. Parameters such as *in vivo* blood glucose, blood urea, *in vitro* lactate, glucose and urea production, and glycogen and oxygen utilization were investigated.

Maternal exposure to nicotine had no effect on blood glucose levels and the liver glycogen content of 1 and 7 day old suckling rat pups. However in 2 and 5 week old and adult rats (20 weeks) a decrease in liver glycogen content and a increase in blood glucose concentration was recorded. All groups showed a marked increase in blood urea level. Nicotine had no effect on the *in vitro* oxygen utilization of rat liver tissue. Under the experimental conditions, the *in vitro* glycogen utilisation and lactate production of liver tissue slices of 1 and 5 week'old rats (maternally treated during gestation and after weaning) were surpressed whereas liver of fed adult rats showed an increase in glycogen utilization ($p < 0,05$) *in vitro*. Nicotine decreased

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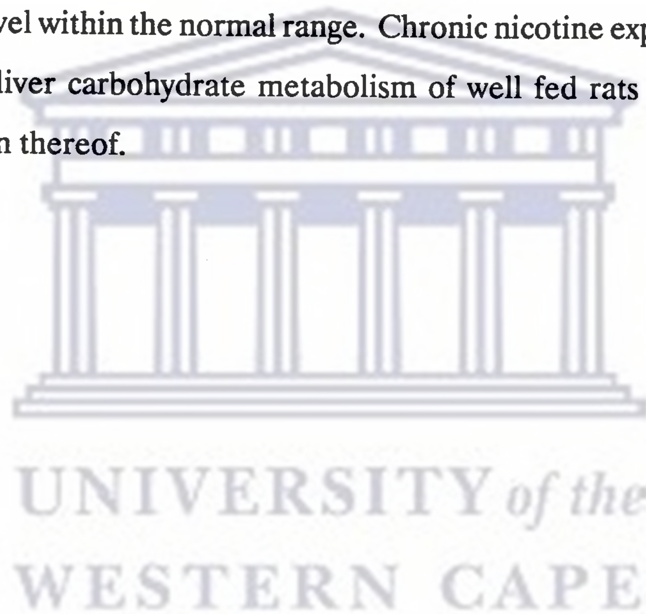
the rate of *in vitro* glucose release in fasted female rat liver tissue. Chronic nicotine treatment for 30 days had no significant effect on the blood glucose and urea concentrations and liver glycogen content.

It is impossible at this stage to pinpoint the exact mechanism of nicotine's action on the glycogen stores, but it is clear that nicotine in some way or other influences the maturational aspect of some liver enzymes. Nicotine had no effect on the oxygen utilization and therefore probably did not affect mitochondrial function. From the investigation it is clear that acute nicotine exposure stimulates glycogenolysis and probably gluconeogenesis resulting in an elevated blood glucose level within the normal range. Chronic nicotine exposure had no apparent effect on liver carbohydrate metabolism of well fed rats probably due to rapid breakdown thereof.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OPSOMMING

Die lewer speel n belangrike rol in die onderhoud van die bloedglukose konsentrasie. In hierdie geval speel glikogenolise en glukoneogenese 'n belangrike rol. Inmenging met hierdie paaie kan moontlik die vermoe van hierdie orgaan om die bloedglukose konsentrasie te onderhou beinvloed. In hierdie studie was *in vivo* en *in vitro* nikotien blootstelling van lewer ondersoek om vas te stel of dit:

i. glikogenolise en glukoneogenese beinvloed,

ii. dieselfde effek op vastende diere het nie en

iii. dieselfde effek op pasgebore rotte het wat aan aan nikotien via moedersmelk blootgestel is.

Een dag, 1 week, 2 weke, 5 weke en 20 weke oue Wistar rotte is as eksperimentele diere gebruik. Nikotien (1 mg/kg liggaam massa per dag) is of intraperitoneal of subkutaan toegedien. Kontrole rotte het 0,9% NaCl oplossing in plaas van nikotien ontvang. Die volume NaCl is deur die massa van die rot bepaal. Die rotte is gedood deur middel van dekapitasie op voorafbeplande dae, 1 en 24 uur na toediening van die laaste dossering. Parameters wat ondersoek is, was die *in vivo* bloedglukose- en bloedureumkonsentrasie , asook *in vitro* laktaat-, glukose- en ureumproduksie, en glikogeen- en suurstofverbruik.

Moederlike blootstelling aan nikotien het geen effek op bloedglukose vlakke en lewerglikogeen inhoud van pasgebore suigelingrotte van 1 en 7 dae oud gehad nie. n Afname in hierdie twee parameters is egter waargeneem in jong rotte van 2 en 5 weke oud. Alle groepe het 'n merkbare toename in bloed ureumvlakke getoon. Nikotien het geen effek op die *in vitro* suurstofverbruik van rot lewerweefsel gehad nie. Glikogeenverbruik en laktaatproduksie is onderdruk in 1 en 5 weke oue rotte , maar volwasse rotte het n toename in glikogeenverbruik *in vitro* getoon. Nikotien het die tempo van *in vitro* glukose vrystelling in vastende wyfierotte verlaag.

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Kroniese blootstelling van nikotien vir 30 dae het geen merkbare effek op bloedglukose en ureumkonsentrasies of lewer glikogeeninhoud gehad nie.

Alhoewel dit onmoontlik is op hierdie stadium om die presiese meganisme van nikotien se reaksie op die glikogeenstore vas te stel, is dit duidelik dat dit die ontwikkelingsaspek van sommige lewerensieme beinvloed. Nikotien het geen invloed op die suurstofverbruik nie en het daarom mitochondriale funksie nie beinvloed nie. Van die ondersoek is dit duidelik dat akute nikotien blootstelling glikogenolise en moontlik glukoneogenese stimuleer wat weer oorsprong gee aan. verhoogde bloedglukose vlakke binne die normale grenswaardes. Kroniese blootstelling aan nikotien het geen ooglopende effek op koolhidraatmetabolisme van gevoede rotte gehad nie en dit kan moontlik toegeskryf word aan die vinnige afbreek van nikotien.

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LIST OF ABBREVIATIONS

g-6-p:	Glucose-6-phosphate
g-1-p:	Glucose-1-phosphate
UDPG:	Uridine diphosphate glucose
UTP:	Uridine triphosphate glucose
POD:	Peroxidase
ABTS:	Di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
GOD:	Glucose oxidase
HMP:	Hexose mono-phosphate
s.c.:	Subcutaneous
i.p.:	Intraperitoneal

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CHAPTER 1

REVIEW OF LITERATURE

1. INTRODUCTION

The liver contains all the enzymes for the synthesis and catabolism of glycogen, glucose and fat in many species (Martin *et al,* 1981; p 167).

Glycogen is the main storage polysaccharide of animal cells. In times of glucose surplus glucose units are stored by undergoing enzymatic linkage to the ends of glycogen chains. In times of metabolic need they are released enzymatically for use as fuel. Glycogen is especially abundant in the liver, where it may attain up to 10 percent of the liver wet mass (Hers, 1976).

The importance of the liver in the glucose homeostasis has been recognised since the time of Claud Bernard over a 100 years ago (Martin *et al*, 1985; p 91). Glycogen is of little use for the liver, which mostly consumes fatty acids, but stores glucose as glycogen when it is abundant and liberates it for the benefit of other tissues, especially the brain and the erythrocytes, during fasting (Hers, 1976). The changes in the rate of glycogen synthesis and catabolism in the liver are tightly controlled by various mechanisms to maintain the blood glucose concentration.

Since this project deals with the effect of nicotine on liver carbohydrate metabolism in rats, the enzymes, hormones and other factors controlling gluconeogenesis and glycogen metabolism in the liver will be discussed briefly in this chapter.

2. GLYCOGEN METABOLISM IN FETAL AND POSTNATAL RAT LIVER

Glycogen accumulates in the rat liver during the final stages of gestation and is rapidly converted to glucose immediately after birth to supply energy to the

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newborn (Schwartz and Rall,1973; Watts and Gain, 1976; Walker *et al*, 1974). These changes in glycogen levels occur as a result of changes in the activity of many hepatic enzymes. Many of these changes occur as a result of adaptation of the liver enzymes in response to the major changes in dietary status occurring at birth and weaning in suckling rats (Watts and Gain, 1976).

Khandelwal (1982) examined glycogen deposition and its mobilization in the last six days of gestation and the first 36 hours after parturition. He determined the activities of glycogen synthase, glycogen phosphorylase, phosphorylase kinase, protein kinase and phosphoprotein phosphatase. Both active (I-form) and total (I + D form) glycogen synthase activities gradually increased through the fetal period up to birth and then decreased slightly in the first 12 post-natal hours. The data produced by this researcher indicated a close relationship between the synthesis of glycogen and the development pattern of glycogen synthase and was in agreement with that of other workers (Watts and Gain, 1976; Devos and Hers, 1974). Schwartz and Rall (1973), proposed that changes in glycogen metabolism during perinatal development are consistent with changes in the glycogen synthetase activity.

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Gain and Watts (1986) found that the fetus contributes to its own blood glucose pool by using its liver glycogen. They came to this conclusion after they determined the glycogen concentration in the livers of fetal and newborn rats during the last 4 days of gestation and the first few hours after birth, by using rats with a glycogen storage disorder, rendering them unable to mobilise liver glycogen and healthy control fetuses. Fetuses with a glycogen storage disorder had a significantly higher glycogen concentration at all ages. The consequence of this was a reduction in the fetal blood glucose in the abnormal animals. However, in the control animals a lower liver glycogen content were recorded but these animals maintained their blood glucose concentrations at higher levels than their abnormal littermates. As the fetal rat lacks the capability for gluconeogenesis (Gain and Watts, 1986) from lactate and most of the amino

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acids, the metabolic products from an active placental glycolytic pathway, such as trioses may be exported by the placenta (Newgard *et al*, 1983) and act as precursors of fetal liver glycogen. This may explain why the fetus can utilise its liver glycogen to maintain glucose homeostasis and still show a nett glycogen deposition at days 20 and 21 of gestation (Gain and Watts, 1986). The data produced by several of the researchers thus prove that liver glycogen metabolism already plays an important role in glucose homeostasis in early fetal and neonatal life .

3. GLYCOGEN AND GLUCOSE METABOLISM IN ADULT RAT LIVER

The rate limiting step in glycogen degradation is the glycogen phosphorylase-mediated reaction (Nuttall *et a*l 1983). This enzyme is present in a phosphorylated and a nonphosphorylated form of which only the former, phosphorylase a, is catalytically active *in vivo* (Tan and Nuttall, 1975). The a and the b forms of this enzyme are interconvertible through phosphorylation by kinases and dephosphorylation by phosphatase as indicated in fig. 1. Experimental data

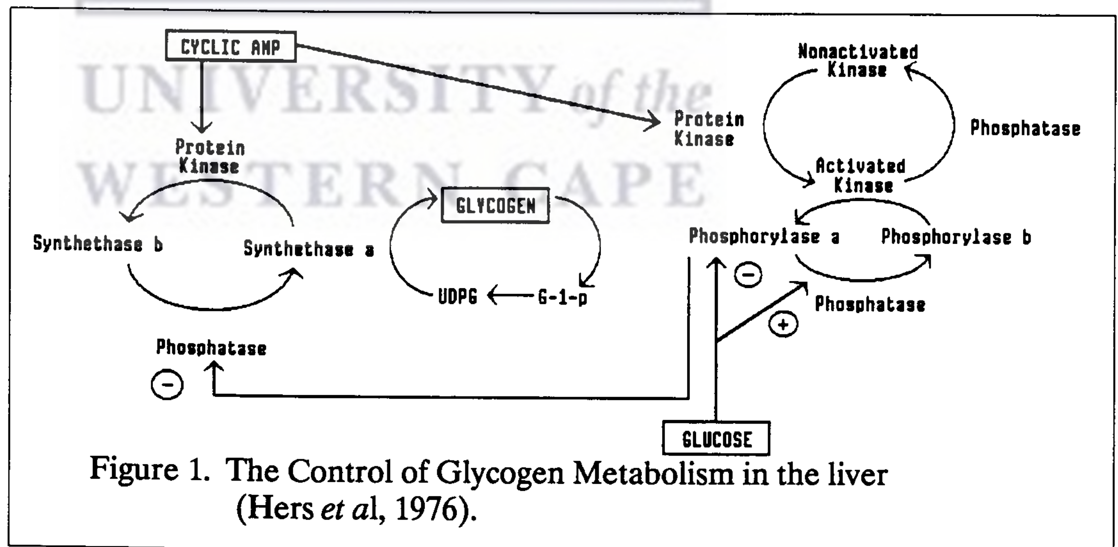

Figure 1. The Control of Glycogen Metabolism in the liver (Hers *et a*l, 1976).

indicate that phosphorylase systems *in vivo* respond primarily to a rise in plasma glucose (Mulmed *et al* 1979). Phosphorylase a is inhibited by glucose and stimulated by cAMP. Nuttall *et al* (1983) showed that glucose causes a rapid decrease in the active phosphorylase in both fed and fasted rats. Not only

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does this enzyme control the rate-limiting step in glycogenolysis, but it also inhibits the activity of synthase phosphatase and thereby controls glycogen synthesis (Martin *et al*, 1985; p 172). This mechanism prevents glycogen synthase from being activated when phosphorylase is active (Hers, 1976). Phosphorylase is activated by a rise in the concentration of cAMP and at the same time glycogen synthase is converted to the inactive form (Martin *et al*, 1981; p 169). Thus, inhibition of glycogenolysis is accompanied by net glycogenesis, and inhibition of glycogenesis is accompanied by net glycogenolysis. This means that the two oppositely directed pathways cannot have the same activity at the same time.

Phosphorylation itself is catalysed by a group of enzymes known as protein kinases of which at least three classes exist. The first class is dependant upon the presence of cAMP for activity while the second class is cyclic Guanosine monophosphate dependant (O'Riordan *et al*, 1982). Studies on the liver by Exton (1981), has indicated a third class of calcium ion-dependant protein kinases which is regulated by alterations in the intracellular concentration of calcium. The importance of this will be discussed below under hormonal control of glycogen metabolism.

The synthesis of glycogen is a slow process in contrast to the degradation of glycogen. It is estimated that the maximum rate of synthesis is approximately one percent of that of glycogenolysis (Hers, 1976). In glycogenesis glucose is first phosphorylated by glucokinase to yield glucose-6-phosphate. Glucose-6-phosphate is then converted to g-1-P, by phosphoglucomutase. Uridine triphosphate (UTP) then reacts with g-1-P to form the active nucleotide uridine diphosphate glucose (UDPG), a reaction catalyzed by UDPG pyrophosphorylase. The glucosyl unit of UDPG is then transferred to glycogen by UDPG glycogen synthase (Martin *et al*, 1985; p 181). The activity of UDPG glycogen synthase can be regulated by hormonal and non-hormonal factors.

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3.1. Hormonal Factors

The following hormones : insulin, glucagon, glucocorticoids, and the catecholamines play key roles in liver glycogen metabolism. The beta cells of the pancreatic islands release insulin when the blood glucose concentration rises and the alpha cells of the islands release glucagon when the blood glucose concentration falls. Insulin and glucagon have reciprocal effects on glycogen synthesis. Whereas insulin promotes energy storage by stimulating glycogenesis, glucagon causes the rapid mobilization of glycogen into glucose by stimulating glycogenolysis. Glucagon activates adenylate cyclase to produce cAMP from ATP. The cAMP activates a protein kinase which converts inactive phosphorylase b to active phosphorylase a. By a cascade effect with three successive enzymes, the activity is so amplified that the production of g-6- P from glycogen can be accelerated a thousandfold. Insulin promotes glycogenesis by inhibiting the cAMP-independent protein kinase in a mechanism that involves Ca^{++} (Exton, 1981). The source of this calcium may be the extracellular fluid or it may arise from the mobilization of intracellular, tissue-bound calcium. Protein hormones increase the uptake of extracellular calcium, whereas cAMP primarily mobilizes tissue-bound calcium. Intracellular calcium is now believed to act by binding to a heat labile acidic protein, calmodulin. The latter is found associated with cellular membranes and many enzymes. Thus calcium binding to calmodulin can result in conformational changes leading to rapid changes in enzymatic and membrane activity (Martin *et al*, 1981; p 465) .

Glucocorticoids increase glycogen deposition in the liver of fasted or fed animals by promoting the conversion of glycogen synthase from its inactive to active form, possibly by activating a phosphatase that enhances this conversion (Martin and Henning, 1985; Laloux, 1983).

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Catecholamines are secreted by the chromaffin granules in the adrenal medulla and act through 2 major classes of receptors designated, alpha-adrenergic and beta-adrenergic, which are each again subdivided into 2 subclasses. Three of these adrenergic receptor subgroups are coupled to the adenylate cyclase system. Activation of phosphoproteins by cAMP-dependent protein kinase accounts for many of the biochemical effects of epinephrine. In muscle and to a lesser extent in liver, epinephrine stimulates glycogenolysis through the alpha receptor in a cAMP-independent process that apparently involves changes in calcium flux, phosphatidylinositide metabolism or both. Conversely, phosphorylation of glycogen synthase decreases glycogen synthesis (Hers, 1976).

3.2. Non-hormonal Factors

Mulmed (1979), proposed that the synthase and phosphorylase systems *in vivo* respond primarily to a rise in plasma glucose. Glucose thus regulates its own storage as glycogen in the liver. Goldstein and Curnov (1978), reported that the effect of prolonged fasting on the basal activities of hepatic glycogen synthase and phosphorylase showed striking changes in the levels of these enzymes. The liver glycogen synthase a showed increases and the phosphorylase showed decreases in concentration. Fasting (48-72h) also caused decreased insulin and glucagon concentrations. Nuttall *et al* (1983), also found that fasting periods of 24 and 48h considerably increased the percentage synthase I in rat liver, while little change was found in the percentage phosphorylase a. The mechanism of this is not well understood, but it was considered possible that an increase in liver glucose concentration due to the rapid conversion of gluconeogenic substrates to glucose could explain the increase in percentage synthase a observed (Nuttall *et al*, 1983).

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Inside the cell there are four isoenzymic forms of hexokinase and of these, three types have a low Km for glucose and are markedly inhibited by g-6-P. The fourth type also called glucokinase is found almost exclusively in the liver (Bhagavan, 1974; Martin *et al*, 1985; p 163), where it is particularly important in the regulation of blood glucose. This enzyme may account for 80% of the normal capacity of liver for phosphorylation of glucose. The kinetic properties of glucokinase are important in glucose homeostasis; it has a high Km for glucose of approximately 10 mM, i.e. twice the normal blood glucose concentration. The high Km ensures that the enzyme is fully active only at high glucose concentrations. Glucokinase will therefore respond to the glucose concentration of the hepatic portal system after a meal. The activity of the enzyme at normal blood glucose concentrations of 3,5 - 5,5 mmol/l is only a fraction of its maximum possible activity. Unlike other hexokinases, glucokinase is not inhibited by its product, g-6-P (O'Riordan *et al*, 1982).

This highlights the importance of glucokinase in the storage of glycogen in the liver and in the homeostasis and regulation of the blood glucose concentration. Glucokinase will therefore respond to the glucose concentration of the hepatic portal system after a meal.

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3.3. Gluconeogenesis in the Liver

This is the formation of glucose from noncarbohydrate precursors in the liver and kidney and it involves a series of enzymatic steps, many of which are stimulated by glucagon, by glucocorticoids and to a lesser degree by catecholamines (Exton and Park, 1968). Insulin inhibits gluconeogenesis. The key gluconeogenic enzyme in the liver is phosphoenolpyruvate carboxykinase (PEPCK), which converts oxaloacetate to phosphoenolpyruvate (Hers and Hue, 1983). Insulin decreases the amount of this enzyme by selectively inhibiting transcription of the gene

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that codes for phosphoenolpyruvate carboxykinase mRNA (Martin *et al*, 1985; p 480).

The various compounds that can undergo gluconeogenesis fall into two categories. According to Martin *et al*, (1985; p 251) the one category include those which involve a direct net conversion to glucose such as amino acids and propionate. A second category include those which are the products of the partial metabolism of glucose in certain tissues and which are conveyed to the kidney and liver where they are resynthesized to glucose (Peters, 1984; Bhagavan, 1974). Glycerol and alanine are relatively poor precursors whereas the rate of gluconeogenesis is about 50% more for lactate (Hers *et al*, 1983; Remesy and Demigue, 1983). Gluconeogenesis is essentially a reversal of glycolysis. This can explain why the glycolytic activity of liver and kidney is low when there is active gluconeogenesis.

Lactate is first transformed to pyruvate which enters the mitochondria by a carrier-mediated process where it is carboxylated to oxaloacetate by pyruvate carboxylase (Hers and Hue, 1983). Oxaloacetate is then transported to the cytosol where the enzyme phosphoenolpyruvate carboxykinase is located and it is converted to phosphoenolpyruvate which will join the glycolysis pathway. Two other key enzymes that play a role namely fructose 1,6-biphosphatase and glucose-6-phosphatase in converting fructose 1,6-biphosphate and glucose-6-phosphate to fructose-6-phosphate and glucose respectively (Hers and Hue, 1983) .

Glucagon is thought to activate membrane-bound adenyl cyclase, resulting in accumulation of intracellular cAMP and thereby stimulate gluconeogenesis (Peters, 1984).

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4. THE INTRACELLULAR METABOLISM OF NICOTINE

4.1. The Absorption of Nicotine

Nicotine is one of the components of tobacco smoke. It is highly lipid soluble and therefore have a significant influence on biological systems (Meyer *et al*, 1971).

According to Armitage *et al*, (1974), smokers inhale between 1,83 and 2,62 mg nicotine by smoking only one cigarette. The nicotine concentration of arterial blood alternate between 30 and 40 ng/ml (195 - 247 nM), during the smoking of a cigarette. The nicotine concentration of venous blood varies between 13 and 46 mg/ml (79 - 281 nM) when a cigar is smoked and between 1,0 and 8,0 mg/ml (6,2 - 49,3 nM) in pipe smokers (McCusker *et al*, 1983). The lower nicotine concentration can probably be ascribed to the fact that cigar and pipe smoke are not inhaled. The pH of the tobacco smoke determines the rate of nicotine absorption in the oral cavity and airways. In cigar smoke the pH is 8,5 and in cigarette smoke it is 5,0. Nicotine in cigar smoke is absorbed faster than that of cigarette smoke as result of the high pH. At high pH values, nicotine is in a non-ionised state and this facilitates absorption (Armitage and Turner, 1970). About 15 to 20 percent of the nicotine in cigar smoke is absorbed in the oral cavity while 80 to 85 percent is absorbed through the airways and lungs (Larson *et al*, 1975; p 124).

4.2. The Distribution of Nicotine in the Tissues

The concentration of nicotine in plasma increase rapidly during smoking. The half-life of nicotine in the plasma of smokers varies from 20 (Isaac and Rand, 1972) to 119 minutes (Benowitz *et al*, 1984), whilst that of the products of nicotine metabolism, such as cotinine varies from one to two days (Shen *et al*, 1977b).

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In experiments on pregnant mice, Schmiterlow and Hansson (1962) showed that nicotine crosses the placental barrier. The distribution of nicotine in mother and fetus was determined by Mosier and Jansons (1972) after intravenous and subcutaneous administration of methyl-^{14}C nicotine to pregnant mice. Five minutes after the administration the concentration of nicotine was the highest in the blood, brain and liver of the mother. Thirty minutes after administration of nicotine the concentration of nicotine was still high in the placenta and fetus, but the highest concentrations were found in the lung, trachea, larynx, adrenal gland, kidney and gastrointestinal tract of the fetus. The nicotine concentration remained high in the placenta and fetus until four hours after the nicotine injection and then it decreased. Mosier and Jansons (1972), suggested that the high activity recorded after thirty minutes was due to the breakdown products of nicotine. The radio-activity of the fetal plasma was higher than that of the mother's plasma, 30 minutes to 20 hours after the administration of nicotine.

4.3. Metabolism of Nicotine

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Different breakdown products are produced during metabolism of nicotine of which cotinine is the most important (Poole and Urwin, 1976). The metabolism of nicotine occurs at a much faster rate in the tissue of smokers compared to that of non-smokers. This is due to an induction of the microsomal enzymes that play a role in the metabolism of nicotine (Beckett and Triggs, 1967). Liver, kidney and lung were found to metabolize nicotine while brain, diaphragm, spleen, stomach, small intestine, and adrenal glands did not (Schmiterlow and Hansson, 1962). Booth and Boyland (1971) reported that nicotine was oxidized *in vitro* by NADPH- and oxygen dependant oxidases in guinea-pig tissues to two optically active stereoisomers of nicotine-1'-oxide and to cotinine when the soluble fraction was also present. Of the tissues examined liver was

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the most active in synthesizing these three metabolites, although there was some activity in lung and kidney (Larson *et al,* 1975; p9). According to Benowitz and Jacob (1984), nicotine is metabolized faster in males than in females.

The main route of nicotine detoxification in the body of adult animals is by enzymatic oxidation which occurs in the microsomal fraction of the liver (Hucker, 1960). It was shown that the fetal liver one day before delivery has a much lower ability than the liver of the mother to metabolize nicotine to cotinine (Tjalve *et a*l, 1968). The cotinine formation starts to increase after birth and the metabolism of nicotine rapidly increases with age (Stalhandske *et al*, 1969). The increase in nicotine metabolism observed after birth and the gradual metabolic increase with age is in agreement with the observations made for some drugs by various investigators (Peters *et al*, 1963; Fouts, 1961). Of particular interest is the steep increase in nicotine metabolism in the three to four week old pups. This period of life coincides with the cessation of weaning and the general expansion of the physical activity in the mouse (Stalhandske *et al*, 1969). After the third week a higher amount of other metabolites in addition to cotinine appears, suggesting the appearance of further pathways in nicotine metabolism (McKennis *et al*, 1962); Bowman *et al*, 1964).

With the exception of the neonates, mice show a decreasing sensitivity to the acute toxic effect of nicotine with increasing age. The difference between various age groups might depend on differences in absorption, distribution, excretion and metabolism of nicotine or differences in the sensitivity of the physiological receptors. Investigations by Stalhandske *et al* (1969) show that toxicity seems to vary directly with the developement of enzymatic metabolic activity in animals from 2 to 8 weeks old.

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4.4. Excretion of nicotine

Large quantities of nicotine and products of nicotine metabolism are excreted in urine (Schmiterlow and Hansson, 1962). The rate of renal excretion of nicotine is influenced by urinary pH (Feyerabend *et al*, 1985). The excretion is higher during urine acidification (Benowitz and Jacob, 1985) and decreases with an increase in pH since the latter results in an increased reabsorption in the wall of the bladder. Chromatographic separations showed that at least 8 metabolites of nicotine occurs in the urine (Larson *et al*, 1975; p 9).

The cotinine concentration in the blood of smokers is higher (650 ng/ml) than the nicotine concentration (60 - 70 ng/ml) after subjects smoked one to two cigarettes with a nicotine content of 0,5 to 2 mg/cigarette (Shen *et al*, 1977a). This is probably due to a more rapid excretion of nicotine in the urine (Dumas *et al*, 1975).

Nicotine is also excreted in the faeces via the gall, (Schmiterlow and Hansson, 1962) the saliva, digestive juices (Larson *et al*, 1968; p 7) and during lactation (Larson *et al*, 1968; p 206). The average concentration of nicotine in human milk of moderate smokers were found to be 91 ppb and ranged from less than 20 ppb to 512 ppb (Ferguson *et al*, 1976).

5. EFFECT OF NICOTINE AND SMOKING ON METABOLISM

Only a few studies were performed to investigate the effect of smoking and that of its most important alkaloid, nicotine (Larson *et al*, 1975), on the blood glucose concentration and the ability of the liver to maintain the blood glucose concentration within normal limits.

In 1970 Stalhandske reported that chronic nicotine administration causes a 71% decrease in hepatic glycogen content in adult mouse liver tissue *in vitro*. This was confirmed by the studies of Bizzi *et al* (1972). They also found that

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smoking as well as nicotine not only increase the blood glucose concentration of humans but also the blood free fatty acid concentration. According to them it was due to the release of catecholamines from the adrenal glands.

Smoking is also associated with lower human fetal and neonatal body mass (Bosley *et al*, 1981). However, it is unlikely that this could be due to a lack of substrate because in experiments by Younoszai *et al*, (1968) it was found that maternal smoking had no effect on the blood glucose concentration of infants. Maritz (1988), showed that nicotine (0,25 and 1,0 mg/kg/day) administered subcutaneously to pregnant rats from day 7 of gestation until weaning, resulted in neonatal lungs that were about 15% smaller on postnatal day 8. The lower lung mass was attributed to the smaller cell size. Nicotine (0,25 and 1,0 mg/kg/day) inhibits glycolysis and this is reflected in a 42 percent reduction in lactate production (Maritz,1988). It was proposed by him that the consequence of this inhibition could probably result in Type 1 cell injury in lung and enhanced cell proliferation resulting in bigger cell numbers. The cells however are smaller than in the controls. The above studies confirmed previous work done by Maritz (1986), where he suggested that although nicotine will have no effect on the incidence of respiratory distress syndrome due to a lack of lecithin, it may have a detrimental effect on the functional development of the lung as a result of it's inhibitory effect on glucose oxidation via the glycolytic pathway.

In studies by Maritz (1983), it was found that high concentrations of nicotine (10 mM) suppress the *in vitro* glucose turnover of rat lung tissue. On the other hand a relatively low nicotine concentration (10 nM) stimulates the total glucose turnover in rat lung tissue despite the lower rate of glucose turnover via the glycolytic pathway. This increase in the total glucose turnover was due to a huge increase in HMP activity (Maritz, 1983). From this it is clear that nicotine has an effect on glucose metabolism on tissue level which may also affect the blood glucose concentration.

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Apart from its effect on the glycolytic pathway and citric acid cycle, nicotine also affects oxygen utilisation. In 1965 Adachi et al found that high concentrations of nicotine (0,06%) suppressed the *in vitro* oxygen consumption of cardiac tissue. They also found that hypercholesterolaemia increased the sensitivity of cardiac muscle due to the inhibitory effect of nicotine (Adachi *et al* , 1965). According to them there might be a synergistic action between nicotine and hypercholesterolaemia since the latter condition also resulted in a reduced uptake of oxygen by cardiac muscle. Bhagat (1972) found that exposure of rats to 1 mg nicotine / kg body mass 5 times per day, resulted in a reduced oxygen utilisation by the animals.

Contrary to the above findings Brachfeld and Oran (1966) found that 2,0 to 20,0 μg nicotine/ml Krebs Ringer perfusion medium, containing 5,5 mM glucose, resulted in an increased *in vitro* oxygen uptake by cardiac tissue. At the same time lactic acid production increased by 263% and glucose uptake by 52%. They suggested that this was probably due to an increase in the rate of anaerobic oxidation of glucose.

6. PURPOSE OF THIS INVESTIGATION

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The main purpose of this investigation was to establish the effect of nicotine on aspects of liver carbohydrate metabolism and thus its ability to maintain the blood glucose within the normal range.

The following specific metabolic parameters and problems were therefore addressed:

i. the effect of nicotine on the *in vitro* glucose utilisation, oxygen utilisation, urea and lactic acid production of rat liver tissue, and

ii. to establish whether nicotine exposure influence the ability of the liver to maintain a normal blood glucose concentration via glycogenolysis and

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gluconeogenesis by using a dose that corresponds with that of individuals smoking 10 to 40 cigarettes per day.

In order to study these aspects, the following experiments were performed:

i. Diurnal variations in the liver glycogen content of male and female suckling, sexually immature and mature adult rats.

ii. The effect of *in vivo* nicotine exposure on suckling and adult rat liver glycogen content.

iii. The effect of *in vivo* nicotine exposure on the blood glucose and urea concentration of postnatal, fed and fasted adults rats.

iv. The effect of intraperitoneal nicotine exposure on the *in vitro* urea production and glucose release of fed and fasted adult rats treated for 7 and 30 days.

v. The effect of nicotine on *in vitro* oxygen consumption, glycogen utilisation and lactate production of rat liver tissue slices in a KRP-medium.

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CHAPTER 2

MATERIALS AND METHODS

1. GENERAL

1.1. Chemicals

All chemicals and reagents utilised were obtained from Merck and were of *pro analysi* and Analar grades.

1.2. Biochemical Tests

All test kits used were supplied by Boehringer Mannheim.

1.3. Statistical Analysis

Statistical analysis were performed using the Student t-test for unpaired data. Results were given as: mean ± standard error of the mean and a probability level of $p < 0,05$ was designated as significant.

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2. EXPERIMENTAL ANIMALS AND TISSUE PREPARATION

2.1. Experimental Animals

Healthy male and female Wistar rats, bred in the laboratory animal unit of the Department of Physiological Sciences, University of the Western Cape, were used. Rats were fed *ad libitum* on a stock diet (Epol rat cubes) and tapwater except where otherwise indicated. The room temperature (25°C) and the 12 hour day-night cycle (7h00-19h00 day cycle) was regulated. Only healthy animals were used.

Animals were treated for *in vitro* work by means of intraperitoneal or subcutaneous injections of 1 mg nicotine per kg body mass per day. Rats were injected at the same time every day i.e. at 08h00.

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In a study by Benowitz and Jacobs (1984), it was found that the daily nicotine intake of male and female smokers vary between 10,5 and 78,6 mg. Thus, the nicotine intake of a 60 kg female will be between 0,16 and 1,18 mg/kg body mass per day (Maritz, 1986), assuming that about 90 percent of the nicotine is absorbed on inhalation (Gleason *et al*, 1963). The dose of 1,0 mg nicotine/kg body mass per day used in this experimental series compares with the intake of habitual smokers (Maritz, 1986)).

Animals were mated over night and were afterwards randomly assigned to control and experimental groups. The occurence of mating was determined by the appearance of mating plugs and sperm in vaginal smears. The day of appearance of the vaginal plug was designated day 0 of gestation. Nicotine exposure commenced on day 7 of gestation to avoid the interference of nicotine with blastocyte implantation and initial embryonic growth and was continued until weaning, 3 weeks after birth. The dams received single daily doses subcutaneously until birth of the litter, whereafter nicotine was given intraperitoneally. This procedure was followed to ensure that nicotine reached the fetus or suckling rats only after its absorption into the blood.

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Animals were killed by decapitation on predetermined days. The livers were then quickly removed and placed in ice cold saline for further processing.

2.2. Tissue Preparation

Rats were sacrificed by means of decapitation and blood samples were taken immediately, while the rats bled freely. The blood was placed on ice to prevent enzymatic degradation of glucose. The left liver lobe was immediately excised and placed on ice to reduce enzymatic activity to the minimum. Liver slices, 0,7 mm thick, were prepared by means of a McIllwain tissue chopper. Slices (± 40 mg/flask) were weighed immedi-

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ately on a Mettler AE163 balance and placed in the Krebs-Ringer-Phospate incubation medium which was kept cold on ice. Corrin and Aterman (1968) investigated the pattern of glycogen distribution in the liver in a combined chemical and histochemical study. They found that the distribution of glycogen in the liver was sufficiently uniform for a sample to be considered representative of the whole. For this reason the left liver lobe was used in order to facilitate experimental uniformity.

3. INCUBATION MEDIUM

3.1. Krebs-Ringer-phoshate (KRP) Medium

Composition: 100 ml 0,154 M NaCl

4 ml 0,154 M KCl

3 ml 0,110 M $CaCl_2$

1 ml 0,154 M $MgSO_4.7H_20$

Buffer: 12 ml 0,154 M $Na_2HPO_4.12H_20$. The pH of buffer was adjusted to 7,4 with 1,0 M HCl using a Beckman zeromatic pH meter.

The osmolarity of the medium was 301 ± 6,0 mOsmol and was determined using a Fiske OM osmometer.

3.2. Substrates:

3.2.1. 30,0 mM glucose. After addition of glucose to the incubation medium its concentration was 3,0 mM. This concentration cor-

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responds to normal rat blood glucose concentration in the post absortive phase.

3.2.2. 0,5 mM Alanine was added to the medium for the determination of *in vitro* urea production since the liver is capable of using alanine for gluconeogenesis.

4. METHODS

4.1. Determination of oxygen utilisation

4.1.1. Apparatus

The Gilson respirometer model G.R.20, a constant pressure instrument was used. It consists of a thermostatted waterbath in which 20 active flasks are immersed. Each reaction flask is connected by means of a flexible capillary Tygon tube to a differential manometer equipped with a direct reading volumeter (reading range:0-500 μl). The second arm of each differential manometer is connected to a single reference flask.

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The use of a reference flask permits operation at a standard pressure and with independence from barometric pressure variations and minimal temperatures in the bath. The reaction vessels consist of Pyrex glass with a main compartment,off-centre well, one sidesac, one venting plug and has a volume of 15 ml.

4.1.2. Procedure for the preparation of Apparatus

The waterbath was switched on approximately one hour before the start of the experiment and set at 37°C.

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The reaction vessels were divided into two groups namely experimental and control. The top of the centre well was greased with petroleum jelly in order to prevent the alkali from creeping over the edge of the centre well into the incubation medium. All ground glass joints between vessel holders and reaction vessels and side arm plugs were greased with silicone grease to make the vessels airtight and waterproof. Filter paper strips (1,5 x 2 cm) were placed in the centre wells. 0,2m1 20% KOH was pipetted into each well in order to absorb the CO_2 released during respiration. 2,7 ml KRP-medium was pipetted into the main chambers of control and test vessels. To each control vessel, 0,3 ml saline was added. To each test vessel 0,3 ml nicotine was added. All the vessels were kept on ice during preparation to minimize the rate of enzyme catalyzed reactions. Liver slices were prepared as described above and approximately 40 to 50 mg tissue was placed in the main chambers of all vessels.

The vessels were attached to the vessel holders by means of rotation, secured with springs and then lowered into the waterbath.

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The shaking mechanism of the apparatus was set to 120 oscillations per minute started. Vessels were allowed to equilibrate for 15 minutes. The manometer valves were closed and the fluid level was adjusted to the black index mark by rotating the micrometers. This was taken as the zero reading for the experiment. Shaking was resumed and oxygen uptake was recorded at regular intervals of sixty minutes by re-adjusting the respirometer fluid to the index mark.

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4.2.3. Procedure

Reagent	Blank ml	Standard ml	Sample ml
Distilled H_2O	0,2	-	-
*Solution 1	-	0,2	-
Supernatant	-	-	0,2
**Solution 2	5,0	5,0	5,0

*Solution 1: Standard: 0,505 mmol glucose/l

**Solution 2: Phosphate Buffer: 100 mM (pH 7,0)

POD: ≥ 0,80 U/ml

GOD: ≥10 U/ml

ABTS: 1,0 mg/ml

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The volumes of reagents were added as indicated above, mixed and incubated at 25°C for 30 minutes to allow reaction to take place. The absorption of the standard and sample was determined with a Beckman SP25 spectrophotometer set at 620 nm against the blank.

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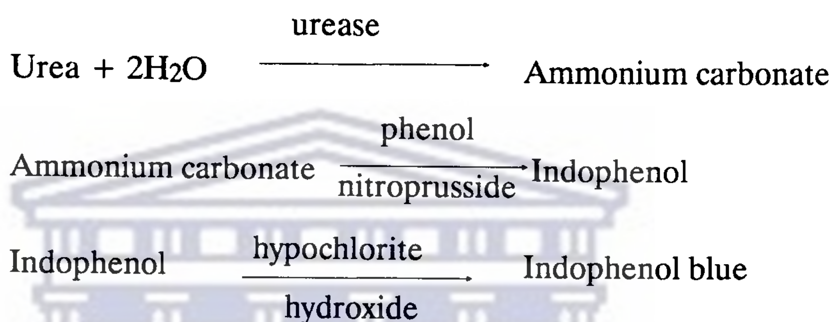
4.2.4. Calculation

$$c = 5{,}55 \times \frac{\text{A Sample}}{\text{A STD}} \text{ mmol/l}$$

The 5,55 is a dilution factor of the standard concentration of 0,505 mmol/l which was diluted eleven times.

4.3. Urea Determination (Fawcett and Scott, 1960).

4.3.1. Test principle



$$\text{Urea} + 2H_2O \xrightarrow{\text{urease}} \text{Ammonium carbonate}$$

$$\text{Ammonium carbonate} \xrightarrow[\text{nitroprusside}]{\text{phenol}} \text{Indophenol}$$

$$\text{Indophenol} \xrightarrow[\text{hydroxide}]{\text{hypochlorite}} \text{Indophenol blue}$$

The colour intensity of the indophenol blue is proportional to the urea concentration.

4.3.2. Sample preparation

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4.3.3. The *in vitro* urea production was determined by using 0,02 ml of the incubation medium and blood urea; 0,02 ml serum was used.

4.3.4. Procedure

Reagent	Blank ml	Standard ml	Sample ml
* Solution 1	0,1	0,1	0,1
** Solution	-	0,2	-
Serum or medium	-	-	0,02

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The reagents were added as above, mixed and incubated for 10 minutes at 50-60°C with test tubes closed with parafilm. The procedure was continued as follows:

Reagent	Blank ml	Standard ml	Sample ml
***Solution 3	5,0	5,0	5,0
****Solution 4	5,0	5,0	5,0

The reagents were added as above, mixed and incubated at 50-60°C for 10-15 min. The absorbance of standard and sample was read at 550 nm on a Beckman SP25 spectrophotometer against the blank.

*Sol.1 :	Phosphate buffer	: 50 mmol/l
	Urease	: ≥10 U/ml
**Sol.2 :	Urea standard	: 0,5 mmol/l
***Sol.3 :	Phenol	: 0,106 mol/l
	Sodium nitroprusside	: 0,17 mmol/l
****Sol.4 :	Sodium hypochlorite	: 11 mmol/l
	Sodium hydroxide	: 0,125 N

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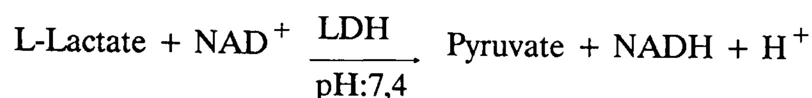
4.4. Lactate Determination

4.4.1. Test Principle



$$\text{L-Lactate} + NAD^{+} \xrightarrow[\text{pH:7,4}]{\text{LDH}} \text{Pyruvate} + NADH + H^{+}$$

The amount of lactate converted to pyruvate can be determined by reading the absorption changes due to reduction of NAD^{+} at 340 nm. The molar extinction coefficient for NADH at 340 nm and 25°C is 0,631 X 10^3 mmol/mm.

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4.4.2. Sample Preparation

0,5 ml of the incubation medium was added to 1,0 ml 10% TCA and centrifuged for 10 min at 1250 x g in a Beckman TJ6 centrifuge with a fixed angle rotor. The supernatant was used for lactate determinations.

4.4.3. Procedure

Reagent	Blank ml	Sample ml
*Solution 1	2	2
Supernatant	-	0,2
10% TCA	0,2	-
**Solution 2	0,2	0,2
***Solution 3	0,02	0,02

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The reagents were added as above, mixed and incubated for exactly one hour at 25°C. Absorbances were read on a LKB spectrophotometer at 340 nm.

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*Solution 1 :	Glycine buffer	: 0,5 mol/l; pH 9,0
	Hydrazine	: 0,4 mol/l
**Solution 2 :	NAD^+	: 27 mmol/l
***Solution 3 :	LDH	: ≥ 560 U/ml

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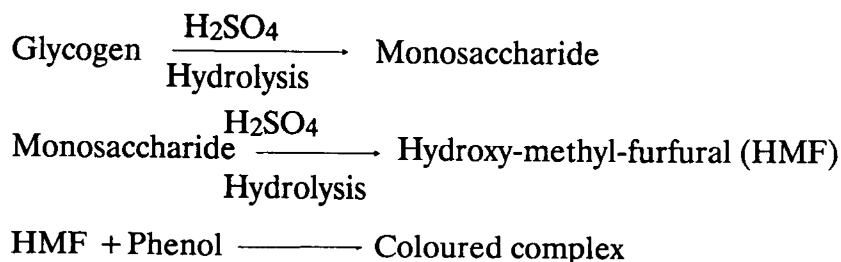
4.5. Glycogen Determination (Lo *et al*, 1977)

4.5.1. Test principle



The intensity of the coloured complex is measured and is proportionate to the amount of glycogen present in the sample.

4.5.2. Sample preparation

In the case of glycogen utilization tissue samples were prepared before and after incubation. Liver slices were accurately weighed with a AE 163 balance. Approximately 10-15 mg was added to 1 ml 30 percent KOH saturated with Na_2S0_4 on ice. The tubes were then placed in a boiling waterbath until the tissue was dissolved. The tubes were then cooled on ice and 2 ml 95 percent ethanol was added to each tube. The sample was kept on ice for a further 20-30 min in order to precipitate the glycogen.

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The samples were then centrifuged at 1250 x g for 20 min and the supernatant was carefully decanted. The glycogen precipitate of each tube was dissolved in 3 ml of distilled water. 1 ml of this solution was used to determine glycogen. Oyster glycogen obtained from Merck was used to prepare the standard. Experiments were performed three times where each determination was done in duplicate.

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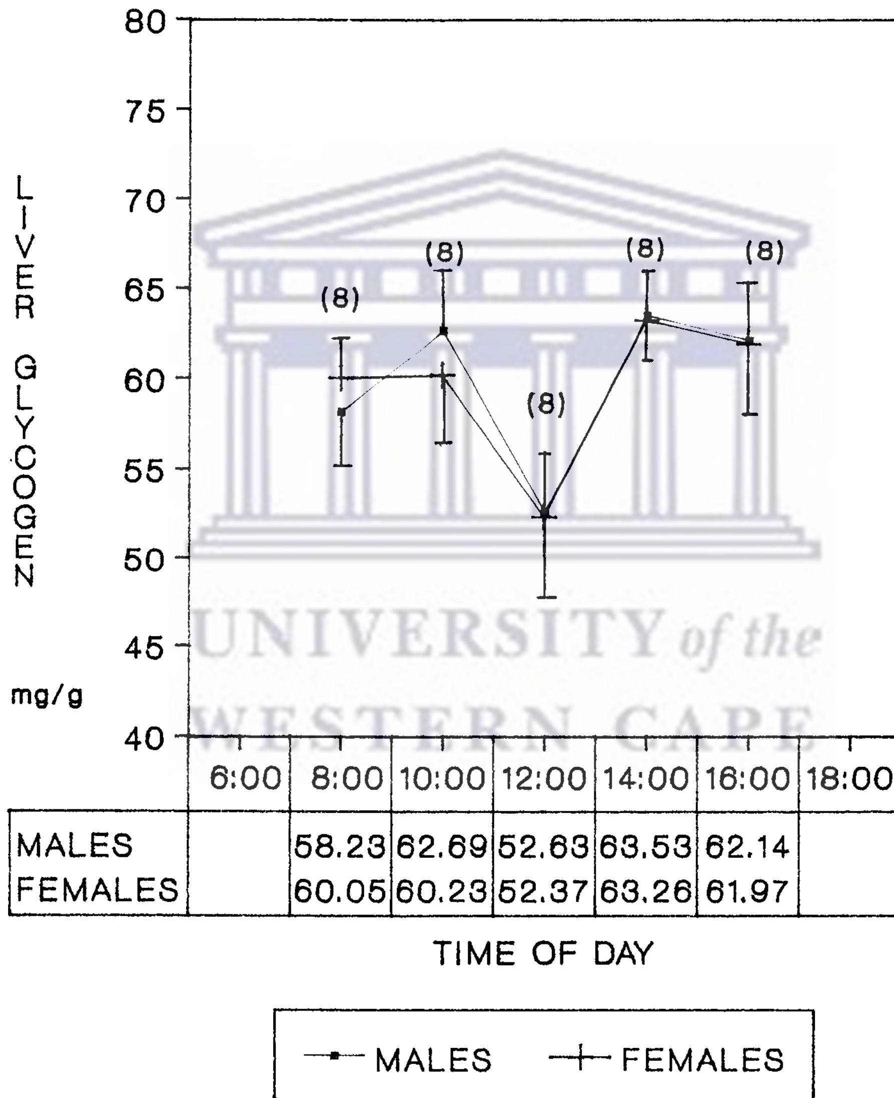

	6:00	8:00	10:00	12:00	14:00	16:00	18:00
MALES		58.23	62.69	52.63	63.53	62.14	
FEMALES		60.05	60.23	52.37	63.26	61.97	

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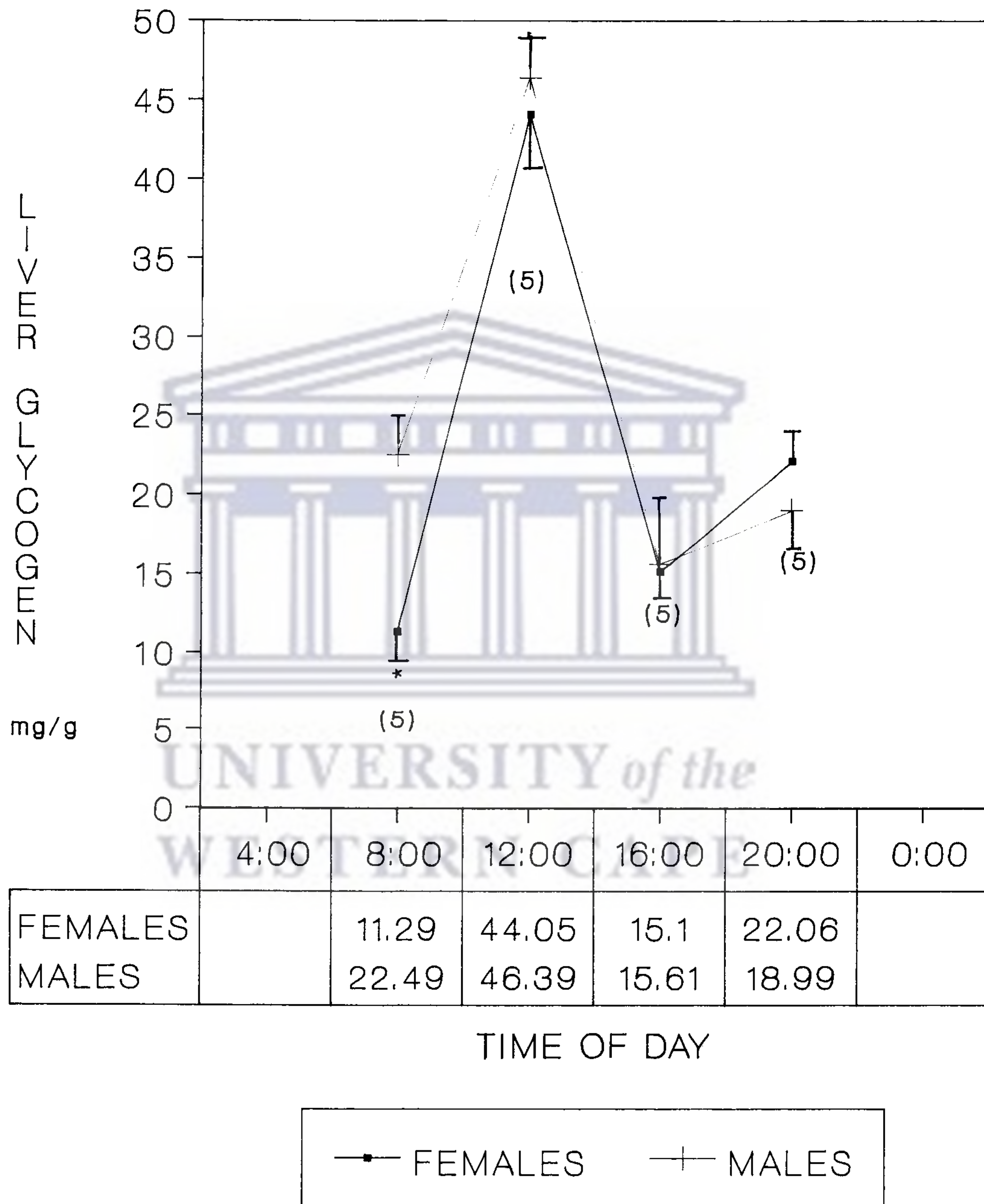
FIG.3 DIURNAL VARIATION: 5 WEEK OLD RATS

Rats were fed a balanced diet and the light-dark cycle was controlled between 08:00 and 20:00. Values are mean ± S.E.M

* : Value is significantly different ($p<0.05$).



	4:00	8:00	12:00	16:00	20:00	0:00
FEMALES		11.29	44.05	15.1	22.06	
MALES		22.49	46.39	15.61	18.99	

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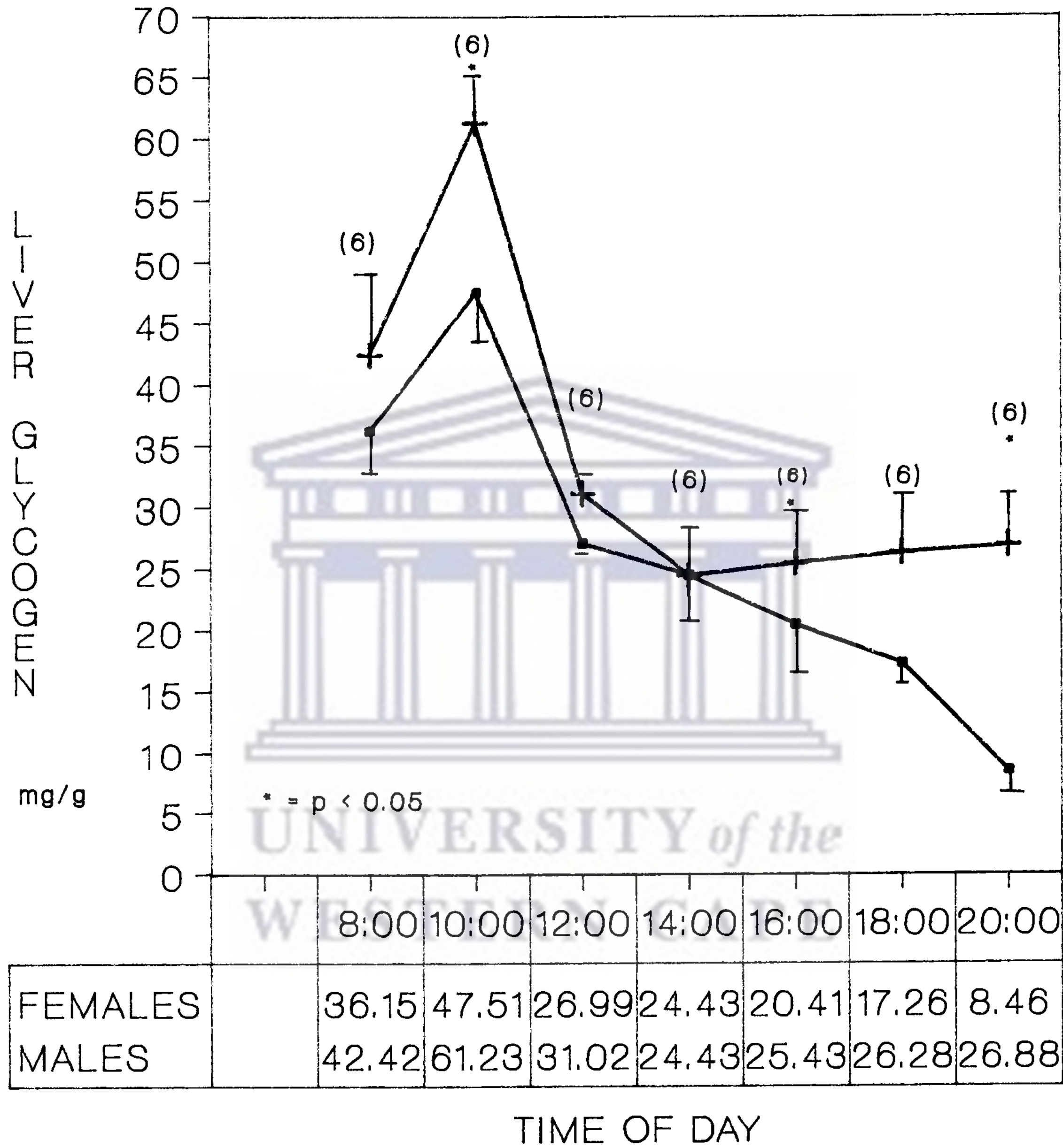
FIG 4. DIURNAL VARIATION:20 WEEKS OLD

The light-dark cycle was controlled between 08:00 and 20:00
Values are means ± S.E.M. * : Value is significantly different ($p<0.05$) from that of the opposite sex



	8:00	10:00	12:00	14:00	16:00	18:00	20:00
FEMALES	36.15	47.51	26.99	24.43	20.41	17.26	8.46
MALES	42.42	61.23	31.02	24.43	25.43	26.28	26.88

TIME OF DAY

FEMALES MALES

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1. RESULTS

In the two week old age group, no significant differences were observed in the liver glycogen content of male compared to female rats, during the period in which the diurnal rhythms were followed. The liver glycogen content remained constant between 08h00 and 10h00 as demonstrated in fig.2. However at 12h00 the liver glycogen content decreased significantly in both males and females by 16% ($p < 0,05$) but returned to the 10h00 level by 14h00. This indicates that at two weeks , a diurnal rhythm for liver glycogen content already exists. No differences occur at any point between males and females.

In contrast to the two week old rats, a significant difference ($p < 0,05$) in liver glycogen content between 5 week old females, 11,28 ± 0,89 mg glycogen/g liver tissue and males 22,49 ± 0,9 mg/g at 08h00, was recorded (fig 3). However, from 12h00 until 20h00 no sex differences were observed. The diurnal rhythm was more pronounced than in the 2 week old rats. Furthermore, while the liver glycogen was at its lowest at 12h00 in 2 week old rats (52,63±1,31 mg/g)(fig 3), it peaked for the 5 week old rats at 46,39±1,23 mg/g (fig 4).

In 20 week old rats no significant differences were recorded with males at 42,42±4,09 mg glycogen/g liver tissue and females at 36,15±1,86 mg glycogen/g liver tissue at 08h00. This observation corresponds with those for 2 week old rats. No significant differences between males and females were recorded at 12h00, 14h00 and 16h00. However, at 18h00 the recorded values for males was 31,07±5,65 mg/g and for females 20,41±0,13 mg/g liver tissue which were significantly different ($p < 0,05$). The values recorded at 20h00 were also significantly different at 26,88±1,88 mg/g for males and 8,46±1,19 mg/g ($p < 0,05$) for females. The liver glycogen content of the 20 week old males was therefore more stable after 12h00 than that of the females (fig 4).

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2. DISCUSSION

It is well known that liver and skeletal muscle glycogen content fluctuates during the course of a 24-hour period (Mc Verry and Kim, 1971; Clark and Conlee, 1979). I have therefore determined the diurnal variation in the glycogen content of liver tissue of suckling (2 week old), sexually immature (5 weeks) and sexually mature (20 weeks) male and female rats.

The diurnal fluctuations displayed sex differences and a tendency for the fluctuations to be more pronounced in adult females (fig 4) especially at 16h00, 18h00 and 20h00. The adult male fluctuation is more pronounced at 10h00. No significant differences were observed between 2 week old males and females. The diurnal fluctuations of the liver glycogen content of suckling rats were also less marked, compared to that of adult rats. This supports the findings of Walker *et al* (1974) who also found that diurnal rhythms of liver glycogen are greatly suppressed or absent in suckling rats but develop rapidly after weaning. The reason for this can be related to diet as suckling rats depend on mother's milk which has a very high lipid content (Walker *et al*, 1974). This may result in free fatty acids being oxidised in preference to glucose (Martin *et al*, 1981; p 254). Suckling rats also feed throughout the day, whereas adult rats are nocturnal feeders (Maritz *et al*, 1985). This could be one of the reasons for the greater fluctuations observed in adult rats.

Conlee *et al* (1976), however, used adult male and female rats of a Wistar strain fed on a normal diet with water freely available. They exposed the animals to 12-h light-dark cycle 5 - 10 days prior to the experimental period, whereas I exposed rats since birth to a 12-h light - dark cycle.

They found that liver glycogen concentration of both sexes rose from a nadir of 29.3± 3.5 m/g at 08h00 to a peak of 53.7± 7.80 mg/g at 12h00. My results show that liver glycogen concentration rose from a nadir of 36,15±1.86 mg glycogen/g liver at 08h00 for females and 42,42 ± 4,09 mg glycogen/g liver for

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CHAPTER 4

THE EFFECT OF *IN VIVO* NICOTINE EXPOSURE ON SUCKLING AND ADULT RAT LIVER GLYCOGEN CONTENT.

The liver plays an important role in maintaining the blood glucose concentration within normal limits by extracting excess glucose from the portal blood after a meal and to store it as glycogen. The glycogen serves as an important source of glucose to maintain blood glucose in between meals and during short term fasting. Interference with glycogenolysis may therefore also affect the liver's ability to maintain blood glucose. In previous studies (Maritz, 1983, 1986) it was shown that nicotine interferes with carbohydrate metabolism in adult as well as fetal and suckling rat lungs.

In this study the effect of nicotine (1 mg/kg/day) exposure *in vivo* on the rat liver glycogen content of 1, 7, 14 and 35 days old postnatal rats, as well as fed and fasted adult (20 weeks old) rats were investigated, to establish whether nicotine is indeed influencing liver glycogen metabolism.

The postnatal rats (n = 26) were exposed to nicotine via the placenta during gestation and via mother's milk during lactation. After weaning on day 21 after birth, they were exposed to nicotine by subcutaneous injection. Fed adult rats that received nicotine subcutaneously daily for three days were sacrificed 1 (n = 22) and 24 (n = 18) hours after the last dose in order to investigate whether the effects of nicotine is short lived or still measurable after a relatively long period of time. Nicotine exposure is associated with adrenalin release (Bizzi *et al.* 1972) which will have a short term effect. Fasted adults (n = 31) were fasted 24 hours before sacrifice.

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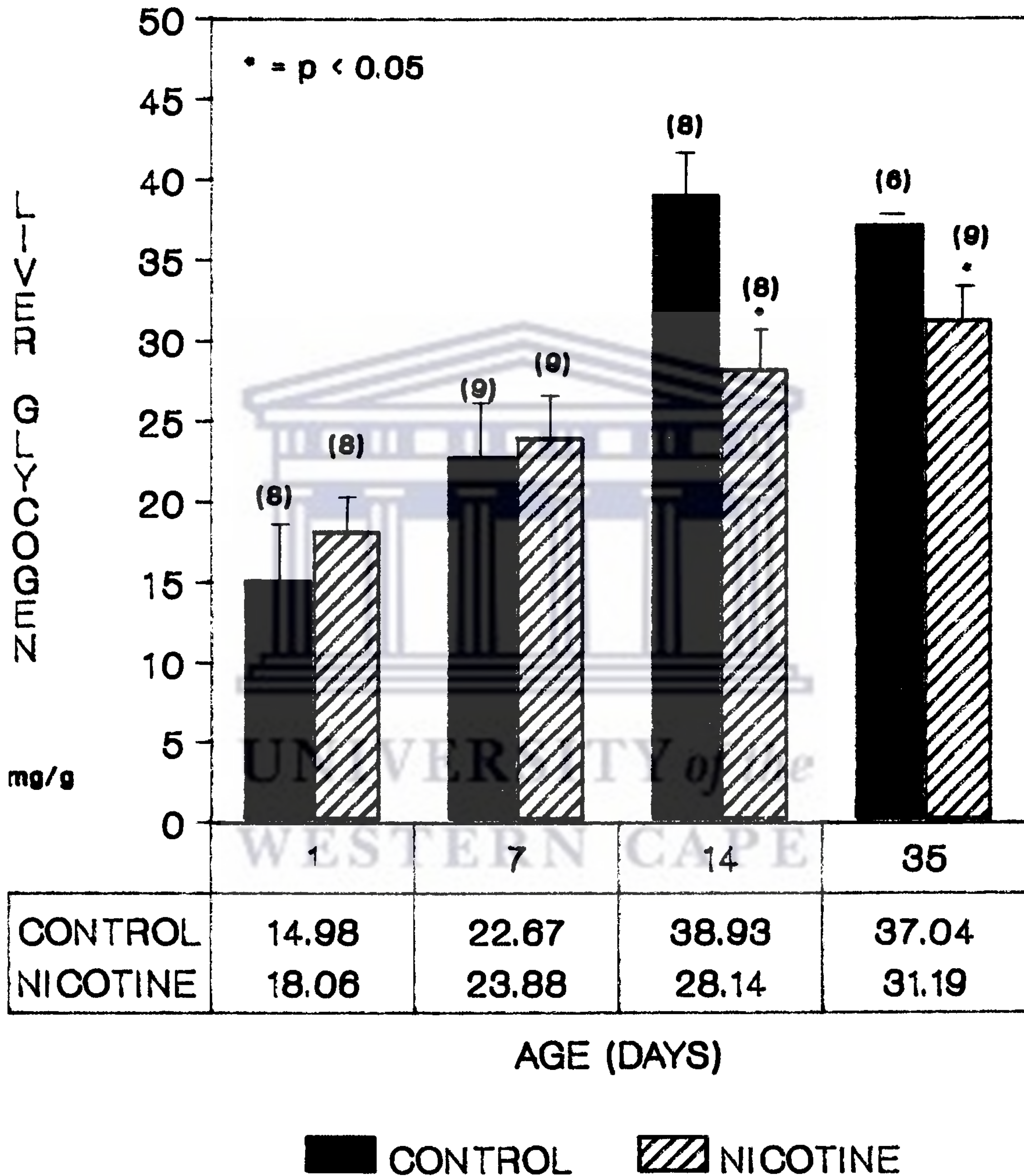
FIG.5 NEONATAL LIVER GLYCOGEN CONTENT: EFFECT OF NICOTINE :1 MG/KG/DAY

Rat ages were 1, 7, 14 and 35 days. Rats were treated maternally and by i.p. injections after weaning
Values : means ± S.E.M. * : value significantly different.



	1	7	14	35
CONTROL	14.98	22.67	38.93	37.04
NICOTINE	18.06	23.88	28.14	31.19

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TABLE 1

EFFECT OF NICOTINE ON THE RATE OF GLYCOGEN DEPOSITION IN THE LIVER OF YOUNG RATS

The effect of maternal nicotine exposure (1 mg/kg/day) on the rate of glycogen deposition in the liver of 1,7 and 14 day old rats.

Control rats received a saline placebo.
Value's given as means ± S.E.

	LIVER GLYCOGEN CONTENT mg/g			RATE OF GLYCOGEN DEPOSITION mg/g/d
Age (days)	1	7	14	
CONTROL	14.98±3.23	22.67±0.59	38.93±0.85	1.78
NICOTINE	18.06±0.87	23.88±1.02	28.14±0.26	0.72
	(+20.56%)	(+5.34%)	(-29.54%)	(-59.55%)

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1. RESULTS

1.1. EFFECT OF NICOTINE TREATMENT ON THE LIVER GLYCOGEN CONTENT OF SUCKLING AND 5 WEEK OLD RATS

From the graph (fig 5) it is clear that the liver glycogen content of the control suckling rats increased as a function of age up to day 14 where it plateaud. It is interesting to note that on day one after birth, the liver glycogen content of nicotine exposed suckling rat pups were higher than that of controls. On day 7 there was no difference in the liver glycogen content but from day 14 onwards the liver glycogen content of the controls (38,94±0,85 mg/g) were significantly higher than that of the nicotine exposed rats (28,14±0,26 mg/g liver tissue)

After calculating the amount of glycogen deposited per day (table 1) it is clear that the rate of glycogen formation of control suckling rats was at 1,78 mg glycogen/g tissue/day, which was 59,55% faster than that of the nicotine exposed rats at 0,72 mg glycogen/g tissue/day. Maternal nicotine exposure had no statistically significant effect on the liver glycogen content of 1 and 7 day old suckling rats. However, in 14 day old rats the liver glycogen content of control rats (38,93 ± 0,85 mg glycogen /g liver) was 29,54% higher compared to that of nicotine exposed rats (28,14 + 0,26 mg glycogen/g liver) (table 1). Similarly the liver glycogen content of 35 day old nicotine exposed rats (37,04 ± 0,22 mg glycogen/g liver) was 15,79% lower compared to control rats (31,19 ± 0,58 mg glycogen/g liver) (fig 5). Although glycogen depositions plataued on day 14 in the control group, glycogen is still synthesized on day 35 in the nicotine exposed animals (fig 5).

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TABLE 2

THE EFFECT OF NICOTINE ON LIVER GLYCOGEN CONTENT OF FED ADULT RATS

The effect of nicotine (1 mg/kg/day) on liver glycogen content after 3 and 30 days administration to adult rats (20 weeks).
Controls received saline placebos. The animals were sacrificed 1 and 24h after final treatment. * Values given as means ±S.E.M. < 0,05: significantly different from control.

	CONTROL	NICOTINE		
TREATMENT TIME (DAYS)		3		30
TIME SACRIFICED AFTER FINAL TREATMENT		1h	24h	24h
Liver Glycogen Content	28,52±1,05 (18)	19,71±0,92 (22) *(-30,89%)	24,97±0,52 (18) *(-12,45%)	26,99±2,47 (7) (-5,36%)

* : $p<0,05$

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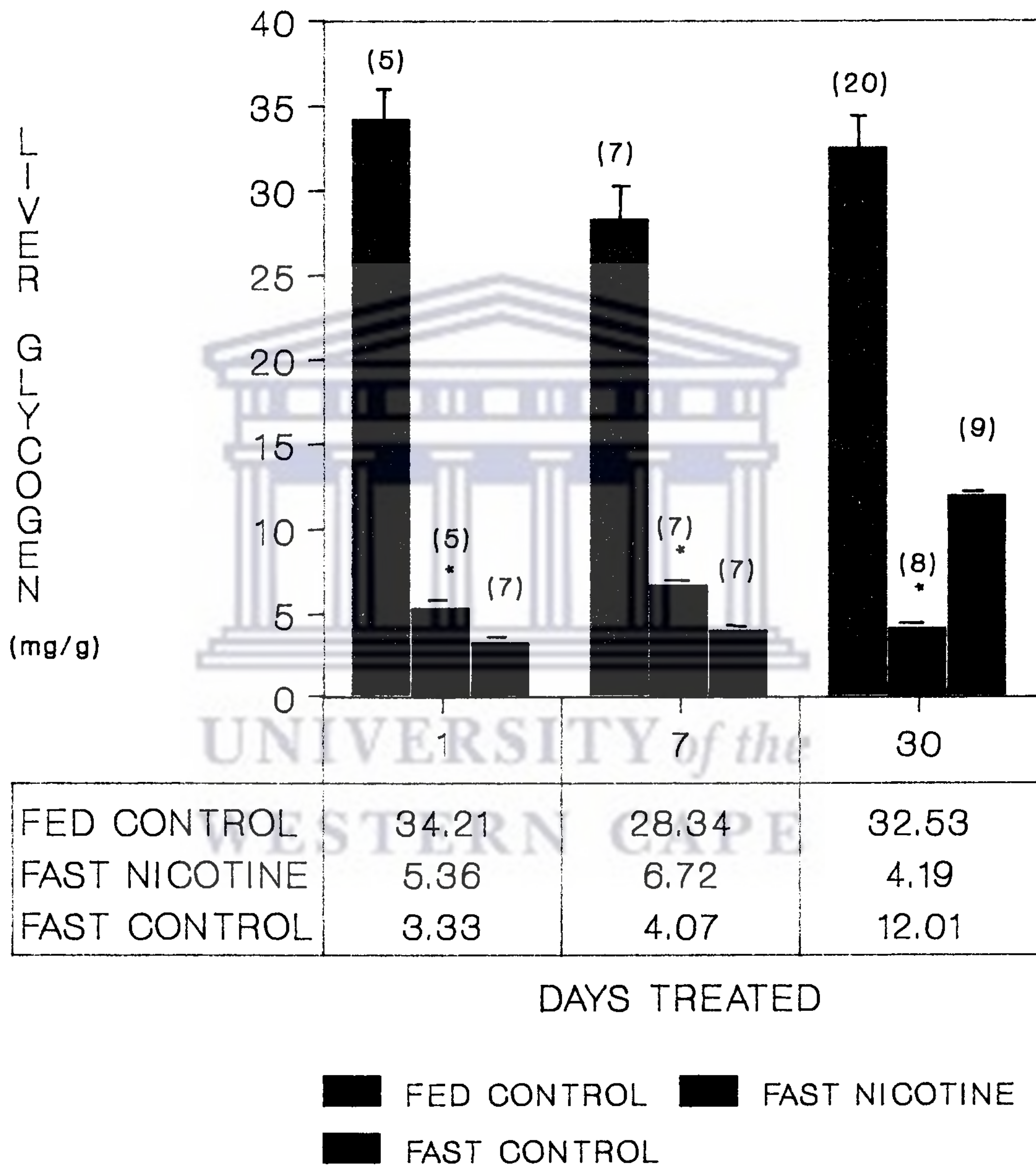

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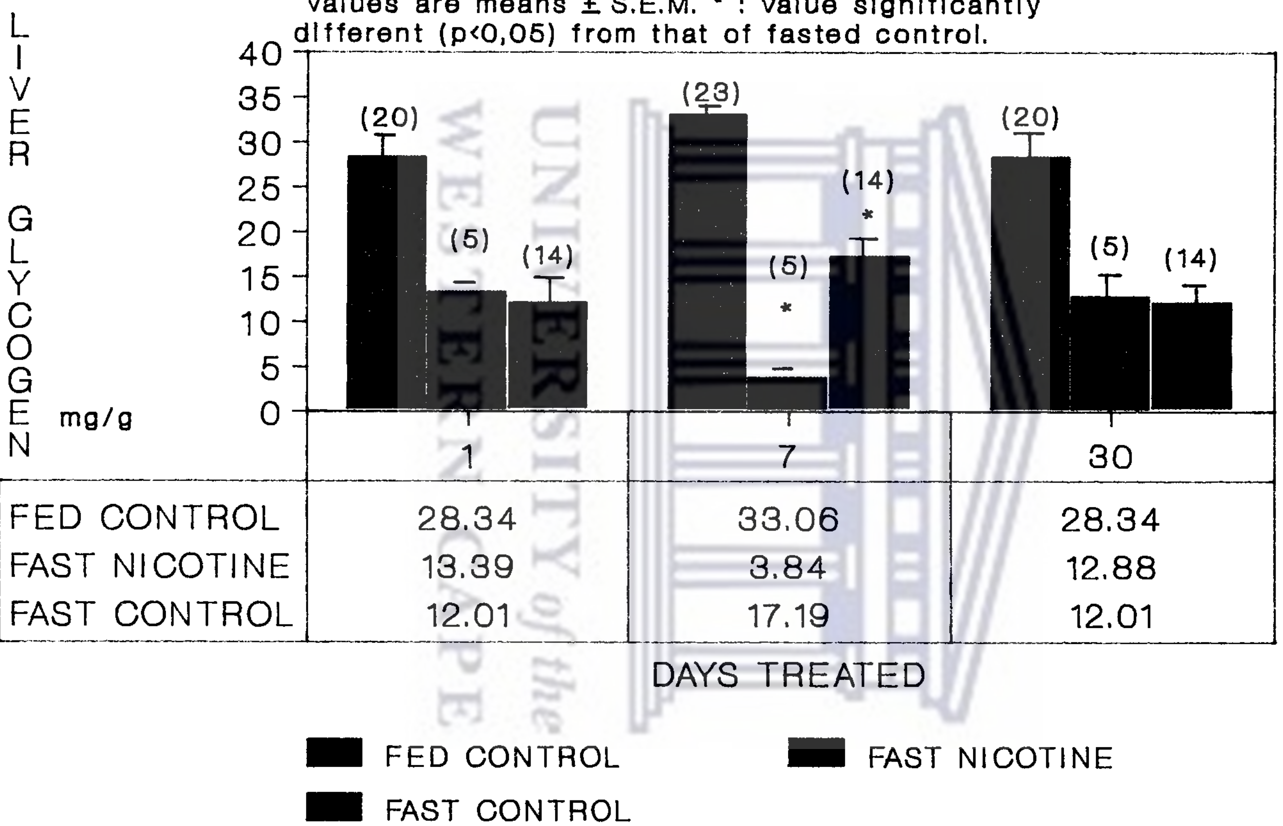

	1	7	30
FED CONTROL	28.34	33.06	28.34
FAST NICOTINE	13.39	3.84	12.88
FAST CONTROL	12.01	17.19	12.01

FIG.7 ADULT FASTED MALE LIVER GLYCOGEN NICOTINE EXPOSURE (1mg/kg/day)
Rats were fasted for 24 h before sacrifice. Values are means ± S.E.M. * : value significantly different ($p<0,05$) from that of fasted control.

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from cessation of transplacental fuel sources to the iniation of suckling(Freinkel, 1980). As a result the glycogen content of the liver decreases to as low as 14,98 ± 3,23 mg/g on day one *post partum* and again increased to 39,40±0,85 mg/g on day fourteen when it plateaud (table 1). Margolis (1983), suggests that a reduced peripheral glucose utilisation in 2- to 10-day old suckling rats and the suppressive effects of a high fat milk diet on pancreatic insulin release probably serves to limit the rate of muscle and liver glycogen accumulation, which explains the slow rate of glycogen accumulation. Therefore, during this period gluconeogenesis using other substrates, including amino acids and glycerol, serves as a source of plasma glucose to maintain euglycemia.

Nicotine (1 mg/kg/day) has no apparent effects on the liver glycogen of 1 and 7 day old rat pups. The rate of glycogen deposition in the glycogen pool of liver tissue of suckling rats was even slower during maternal nicotine exposure than in the control animals. Although it is speculative it could be due to:

i. a suppression of glycogenesis or

ii. an increased rate of utilization of glucose via the HMP pathway to inactivate nicotine.

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Margolis (1983), reported that the amount of glycogen that is synthesized and accumulated increases significantly between day 14 and day 21 after birth. The mechanism for this sudden increase in liver glycogen concentration from day 14 may be related to an ongoing dietary transition from a high fat content found in mother's milk (Margolis, 1983), to a high carbohydrate diet. Furthermore Martin *et al*, (1981; p 536) reported that intestinal epithelial digestive enzymes, such as sucrase and maltase, develops around day 16 after birth, while others reported that hepatic glucokinase develops around days 13-14 after birth (Wakelam and Walter, 1981). With this in mind it is conceivable to speculate

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that nicotine or it's metabolites probably suppress glycogenesis or influence food intake or digestion and absorption of nutrients from the gastro-intestinal tract or development of specific glucokinase isoenzyme patterns or a combination of these factors. Nicotine also increase blood free fatty acids (Bizzi *et al,* 1972) which can also retard glycogen formation (Martin *et al*, 1985; p 268).

Although it is impossible at this stage to pinpoint the exact mechanism of nicotine's action on the glycogen stores, it is clear that the initial breakdown of glycogen during birth is followed by a slower than normal resynthesis of glycogen in the livers of nicotine exposed neonates (table 1).

Fed adult rats exposed to nicotine (i.p.) for 3 days and sacrificed 1 hour after the final dose also show a 30,89 percent decrease whereas adults treated for 3 days and sacrificed 24 hours after the final dose show a significant ($p < 0,05$), 12,45 percent decrease. The initial decrease could be attributed to the effect of adrenaline since nicotine administration resulted in a rapid release of adrenalin (Bizzi *et al,* 1972). However after 24 hours, the adrenalin concentration is expected to be normal. Thus, the lower glycogen content observed at this stage could be due to an increase in glycogenolysis under direct influence of nicotine. This seems to be unlikely since the plasma half-life of nicotine is between 9 and 133 minutes (Feyerabend *et al*, 1985). Furthermore, evidence exists that the liver cannot accumulate nicotine or it's metabolites (Mosier and Jansons, 1972). Another more likely explanation could be a suppression of glycogenesis or food intake and or digestion and absorption thereof. Lower activity of glucokinase seems to be unlikely because this enzyme is already fully developed in the 20 week old rats. However, nicotine could surpress its activity by direct or indirect inhibition. The most likely explanation is the slower synthesis of glycogen under the influence of nicotine (table 1).

It is more likely that the animals treated for 30 days respond the same way as the others. Another posibility is that the adrenal glands become tolerant to

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nicotine (Westfall, 1965) or that the rate of glycogen deposition may be decreased to a larger extent in the older rats (table 1).

Significant decreases of 65% were found in starved females treated for 30 days (figs 6 & 7). This may lead us to conclude that the female liver glycogen stores are more vulnerable to nicotine under stress conditions than the male glycogen stores. This result cannot be explained at this point in time and further investigation is required.

3. SUMMARY

i. Nicotine has no effect on the liver glycogen content of postnates ages 1 and 7 days, as result of the enzymatic activities being much lower in postnates compared with adults.

ii. Nicotine causes a decrease in liver glycogen content of postnates aged 14 days. This may be due to:

a) decreased glycogenesis,
b) decreased digestion and absorption of nutrients,
c) decreased glucokinase activity and
d) decreased food intake.

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iii. Nicotine causes a decrease in the liver glycogen content of postnates aged 5 weeks. This may be due to the stimulation of certain glycogenolytic enzymes or to retarded maturation of liver tissue.

iv. Short term nicotine exposure had a marked effect on the adult liver glycogen content. This may be due to the same factors as set our in ii above.

v. Chronic treatment (30 days) had little effect on the adult glycogen stores. This may be due to the liver adjusting secretion of glycogenolytic enzymes in order to minimize the effect of nicotine. Significant decreases

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of 65% in liver glycogen were found in starved females treated for 30 days. This may lead us to conclude that the female liver glycogen stores are more vulnerable to nicotine under stress conditions than the male liver glycogen stores. The reason for this cannot be explained at this point in time.

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CHAPTER 5

THE EFFECT OF *IN VIVO* NICOTINE EXPOSURE ON THE BLOOD GLUCOSE AND UREA CONCENTRATION OF POSTNATAL, FED AND FASTED ADULT RATS.

Research on the effect of nicotine on carbohydrate metabolism in adult rat lung proved that it interferes with the biochemical pathways subserving energy production in lung (Maritz, 1983). Nicotine suppresses glycogen disappearance from the fetal lung by inhibiting phosphorylase, where the latter is the rate limiting enzyme of glycogenolysis. It also suppresses glycolysis (Maritz, 1986).

One of the functions of the liver is to maintain the blood glucose concentration within normal limits. The liver can fulfill this function through glycogenolysis and gluconeogenesis. If nicotine also suppresses glycogenolysis or gluconeogenesis in the liver, it will result in a drop in the blood glucose concentration.

The first part of the experiment was therefore performed in order to determine whether nicotine administered *in vivo* had any influence on the blood glucose and urea levels of rats of different ages, and during fasting. This may serve as an indication whether or not nicotine exposure interferes with the ability of the liver to release glucose into the circulation via glycogenolysis and or gluconeogenesis. Muscle cannot contribute to this due to a lack of glucose-6-phosphatase, whereas the contribution of other tissues are very small due to their low glycogen content compared to the liver (Martin *et al*, 1985; p 260). It is therefore conceivable that significant interference in the ability of the liver to release glucose from its glycogen stores will also influence the blood glucose concentration. Therefore, a decrease in liver glycogen content may result in an increase in the blood glucose concentration. Also, an increase in blood urea would serve as an indication of an increase in gluconeogenesis from amino acids because enhanced gluconeogenesis such as

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TABLE 3

EFFECT OF NICOTINE ON BLOOD GLUCOSE AND UREA CONCENTRATION OF YOUNG RATS

Effect of nicotine on blood glucose and urea concentration of young rats (1 and 5 weeks old). Mothers were treated throughout pregnancy and lactation as described before. Weanlings (5 weeks) were treated by s.c. injection (1 mg/kg/day). Values are given as mean ± SE; $p<0,05$ - significantly different from controls; n values in parenthesis.
Control received saline placebos.

AGE (weeks)		BLOOD GLUCOSE mmol/l	BLOOD UREA mmol/l
1	control	6,19 ± 0,61 (8)	11,33 ± 0,37 (6)
	nicotine	6,18 ± 0,50 (8)	15,09 ± 0,57 (8) *[+33,19%]
5	control	7,16 ± 0,27 (6)	6,69 ± 0,38 (6)
	nicotine	7,21 ± 0,17 (10)	8,27 ± 0,31 (10) *[+23,62%]

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TABLE 4

EFFECT OF NICOTINE ON ADULT RAT BLOOD GLUCOSE AND UREA CONCENTRATION

The effect of nicotine (1 mg/kg/day) on blood glucose and urea concentration after 3 and 30 days administration to adult rats (20 weeks). The animals were sacrificed 1 and 24 hours after final administration.
Values given as means ± S.E. $p<0,05$ - significantly different from control; Controls received saline placebos.

	CONTROL	NICOTINE (1mg/kg/day)		
TREATMENT TIME (days)		3		30
TIME SACRIFICED AFTER LAST DOSE		1h	24h	(24h)
BLOOD GLUCOSE	6,89 ± 0,21 (14)	7,16 ± 0,51 (6) [+3,92%]	7,80 ± 0,30 (10) *[+13,21%]	6,17 ± 0,42 (9) [-10,45%]
BLOOD UREA	7,18 ± 0,14 (12)	9,36± 1,09 (6) *[+30,36%]	9.95 ± 0,48 (15) *[+38,58%]	4,84 ± 0,25 (7) *[-32,59%]

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ii. has the same effect on fed and fasted animals and

iii. has the same effect on suckling rats exposed to nicotine via mother's milk.

1. RESULTS

The blood glucose concentration of 1 week old control suckling rats were 6,19 ± 0,61 mmol/l (n=8) and that of the nicotine exposed suckling rats, 6,18 ± 0,50 mmol/l. Therefore exposure to nicotine via the mother's milk had no effect on the blood glucose levels of these postnates. However, maternal nicotine exposure (s.c.) resulted in a significant increase (33,19%) in the blood urea level of 1 week old pups from 11,33 ± 0,3 to 15,09 ± 0,5 mmol/l, while 5 week old rats showed a 23,62% increase from 6,69 ± 0,35 to 8,27 ± 0,31 mmol urea/l (table 3).

The blood glucose concentration of the 5 week old control group was 7,16 ± 0,27 mmol/l whereas that of the nicotine group was 7,21 ± 0,17 mmol/l ($p > 0,05$). The blood glucose levels of fed adults exposed for 3 days and sacrificed 1 hour after the last dose remained unchanged, between 6.89 ± 0.21 and 7.16 ± 0.51 mmol/l while a significant increase ($p < 0.05$) from 6.89 ± 0.21 to 7.80 ± 0.30 mmol/l was found in those sacrificed 24 hours after the last dose. However, long term treatment over 30 days had no effect on the blood glucose levels (table 4).

Short term treatment (3 days) of adult rats, resulted in an increase in blood urea concentration from 7,18 ± 0,14 to 9,36 ± 1,09 mmol/l (table 4). This increase was observed within lh after exposure to nicotine. Twenty four hours later, the blood urea concentration was still elevated to the same degree. Long term treatment (30 days), on the contrary resulted in a significant ($p < 0,05$) decrease of 32,59% in the blood urea concentration..

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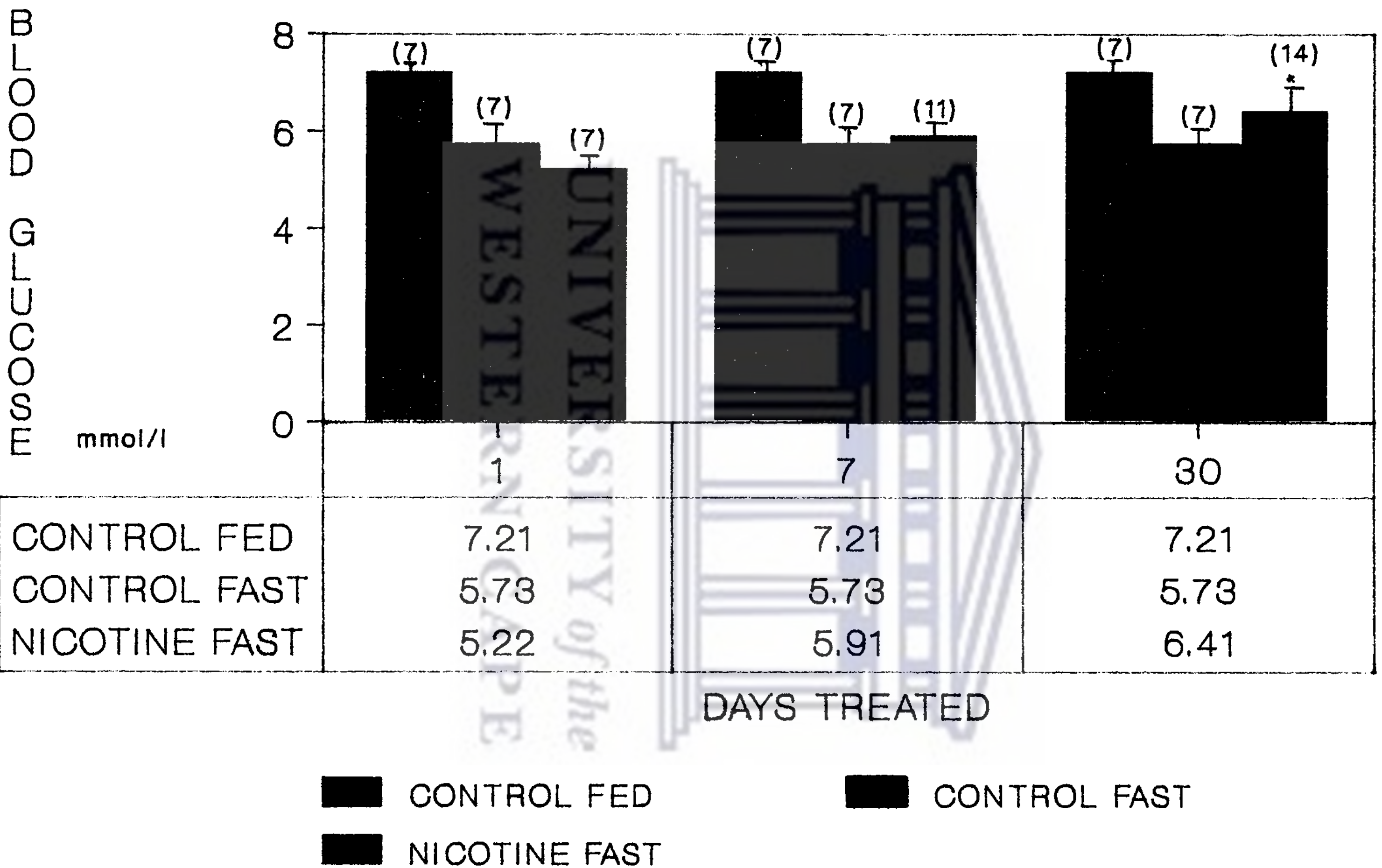
FIG 8. ADULT FASTED RATS: BLOOD GLUCOSE EFFECT OF NICOTINE (1 MG/KG/DAY)

Rats were treated for 1, 7, and 30 days and fasted for 24h before sacrifice. Controls received saline placebos.



	1	7	30
CONTROL FED	7.21	7.21	7.21
CONTROL FAST	5.73	5.73	5.73
NICOTINE FAST	5.22	5.91	6.41

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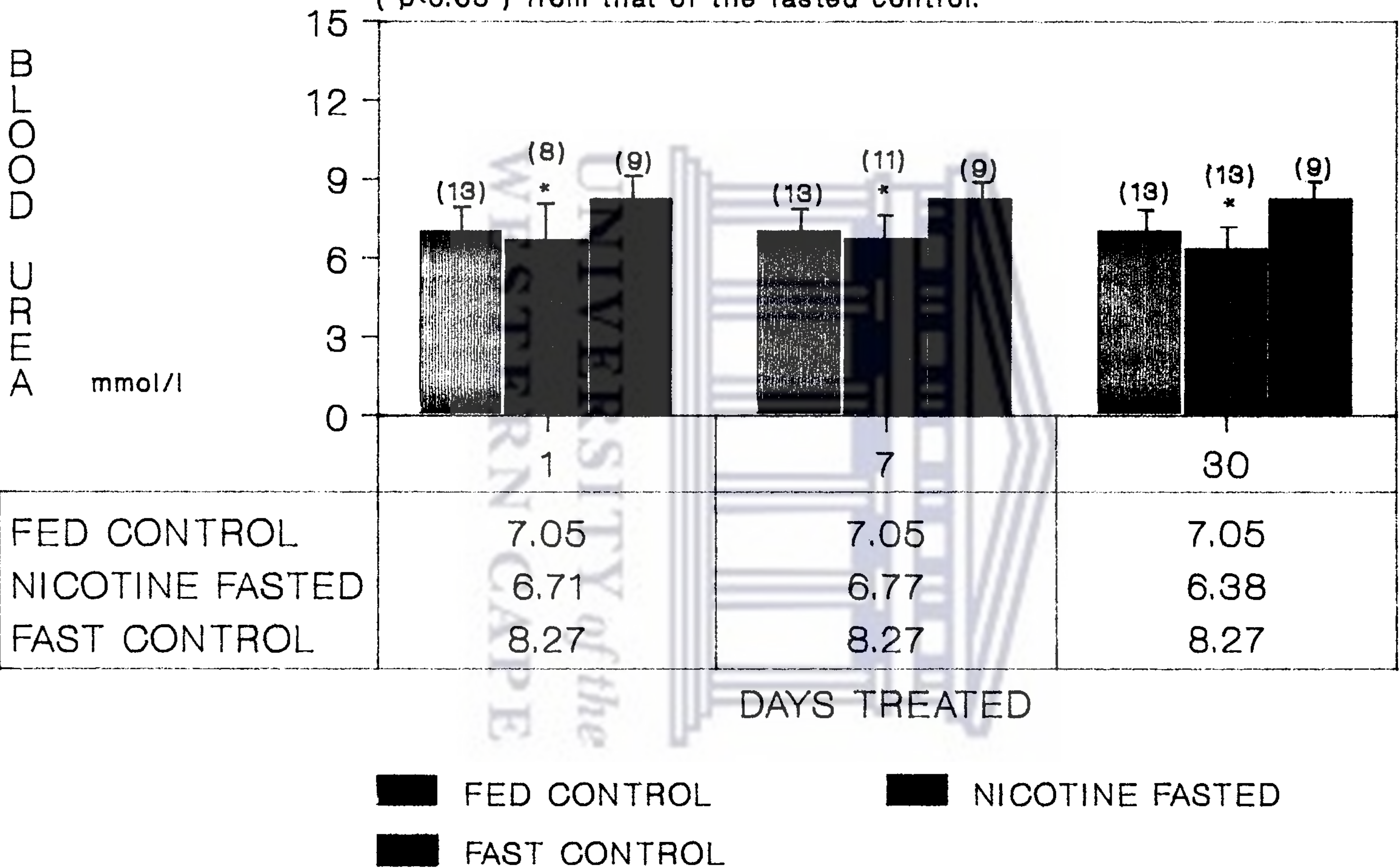
FIG 9. FASTED ADULT RATS:BLD UREA
EFFECT OF NICOTINE (1 mg/kg/day)
Rats were fasted for 24 h before sacrifice
Values are means ± S.E.M. * : values significantly different ($p<0.05$) from that of the fasted control.



	1	7	30
FED CONTROL	7.05	7.05	7.05
NICOTINE FASTED	6.71	6.77	6.38
FAST CONTROL	8.27	8.27	8.27

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Adult rats fasted for 24 hours and exposed to 1 mg nicotine/kg body mass/day for 1 and 7 days previously and sacrificed 24 hours after the final dose showed no significant change in blood glucose levels, which was 5.73 ± 0.11 mmol/l (table 5). Long term treatment (30 days) of males and females resulted in a small but significant increase in the blood glucose concentration from 5.73 ± 0.11 to 6.41 ± 0.14 mmol/l ($p < 0.05$) which is in contrast to the findings for fed adult rats treated for 30 days (table 4) where blood glucose concentration was unchanged. This increase seems to be dependent on the time span of exposure since on day 7 it was higher than on day 1 although not significantly so (table 5). Fig. 8 illustrates this upward trend in the blood glucose level of fasting rats that occur as the time span of nicotine exposure increases from 1 to 30 days.

Fasting for 24h resulted in a 17,3% increase in the blood urea concentration in the fasted control animals. The values for the rats exposed to nicotine for 1 day was 6,71 ± 0,37 mmol/l. On day 7 it was 6,77 ± 0,17 mmol/l and on day 30 it was 6,38 ± 0,21 mmol/l and were all significantly ($p < 0,05$) lower compared to the fasted controls, by 23,25%, 22,16%, and 29,62% respectively (figure 9).

2. DISCUSSION

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The blood glucose concentration at any given time is determined by the balance between the amount of glucose entering the blood stream and the amount leaving it. The principal determinants are gluconeogenesis and glycogenolysis activity of the liver. This function is however not automatic; glucose uptake and glucose discharge are affected by the actions of numerous hormones (Ganong, 1985). Any change in the mechanisms that control glucose homeostasis will have an effect on blood glucose concentration and on those stores involved in it.

In the previous chapter it was shown that maternal nicotine exposure had no effect on the liver glycogen stores of 1 week old rats. As expected maternal nicotine exposure also had no effect on the blood glucose concentration of

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these rats. Contrary to the 1 week old rats, the liver glycogen content of 5 week old rats which were also exposed to nicotine via the placenta and mother's milk, and which received nicotine subcutaneously after weaning (3 weeks after birth) decreased significantly by 15,79% (Fig. 5) However, as for the 1 week old rats, no change in the blood glucose concentration due to nicotine exposure was observed. With the data available it is difficult to explain these findings. The fact that the liver glycogen and blood glucose of the one week old rats were unaffected by maternal nicotine exposure can be attributed to:

i. detoxification thereof by the mother's tissues and

ii. dilution thereof by the mother's body fluids so that the amount received via the mother's milk is too diluted too have an effect.

In the case of the 5 week old animals, nicotine was given directly to the animals (subcutaneously) after weaning. The blood nicotine concentration is therefore higher than in 1 week old rat pups. Thus, the nicotine can give rise to release of catecholamines with a resultant increase in the rate of glycogenolysis and thus a lowering of the liver glycogen stores as observed in this experiment.

One would associate this decrease in liver glycogen with a concomittant increase in blood glucose concentration. A possible reason is that the liver is actively using the glucose which is derived from its own glycogen stores to detoxify the nicotine. This detoxification is largely dependant on the supply of NADPH as a result of increased glucose utilisation via the HMP-path. This assumption is supported by the fact that *in vivo* nicotine exposure increases the activity of certain microsomal enzymes in the lung involved in the detoxification of foreign chemicals (Maritz, 1983).

Apart from the liver glycogen stores, gluconeogenesis is also essential for the maintenance of the normal blood glucose concentration.

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Beaudry *et al* (1977), found that the rate of gluconeogenesis from lactate in perfused livers and isolated hepatocytes of 10 to 15 day old suckling rats was more than twofold that of adult rats. According to them lactate was used as a gluconeogenic precursor in preference to amino acids. My investigation revealed that the blood urea level of 1 week old control neonates (11,33±0,37 mmol/l) (table 3) was significantly higher than that of the control 5 week old (table 3) and adult rats (7,18±0,14 mmol/l) (table 4). If we assume that the increased rate of urea formation by the liver, is an indication of increased glucose synthesis from amino acid precursors, then young suckling rats also utilise more amino acids for gluconeogenesis than adult rats. . This also means that the gluconeogenesis pathway is highly sensitive to the effect of nicotine since only a fraction of the amount given to the mother will reach the neonates via the mother's milk. However the increase in blood urea was not accompanied by a higher blood glucose concentration.

In 5 week old rat pups that received nicotine subcutaneously after weaning a 23,62% increase in blood urea concentration occured which is less than the 33,19% increase for 1 week old rats. This is an indication that exposure to nicotine probably enhances gluconeogenesis in both age groups and that suckling rats are more sensitive to the effect of nicotine than after weaning or that gluconeogenesis was maximally stimulated by the low nicotine concentration in mother's milk. However, the possibility of a decreased clearance of urea by the kidney cannot be ruled out although it is unlikely that nicotine had an effect on this function of the kidney (this aspect will be discussed later).

Blood glucose levels of fed adult rats treated for 3 days and sacrificed one hour after the last injection were not significantly affected, however an increase to 7,80 ± 0,30 mmol/l was found in those sacrificed 24 hours after the last dose. The latter case shows the effect of nicotine on the blood glucose levels with the effect of catecholamines released at the time of injection having worn off and when virtually no nicotine is present in the liver. This finding supports

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to that of control animals. The changes (decrease or increase) in liver glycogen content does not seem to have an effect on the blood glucose concentration of our experimental animals. This implies that the glucose set free after glycogen degradation, is used via another pathway. Further work needs to be done to find out exactly what happens to the glucose released, because it appears as if a substantial part of the glucose would either be completely oxidized or used in other pathways e.g. to detoxify nicotine. It is also possible that some of the glucose released from the stores of the liver is incorporated into the glycogen stores of the lung and heart because nicotine exposure resulted in an increase in these carbohydrate stores (Maritz, 1988).

In contrast to the above, fasted males and females treated daily for 30 days showed a significant increase (+12%) in their blood glucose levels from 5,73 ± 0,11 to 6,41 ± 0,14 mmol/l. Animals treated for 1 and 7 days under the same experimental conditions showed no change in blood glucose levels which remained at 5,73 ± 0,11 mmol/l. Short as well as long term nicotine treatment resulted in significant decreases of 18,86% and 22,85% respectively in blood urea levels of the same groups of rats. This implies that another substrate is used for gluconeogensis during fasting instead of amino acids (see chapter 7).

3. SUMMARY

The above results clearly show the following:

i. Maternal nicotine treatment had no effects on the blood glucose levels of 1 week old suckling rats. The 5 week old postnates who received nicotine intraperitoneally upon weaning (day 21), also showed no change in their blood glucose concentration. The same treatment resulted in marked increases in the blood urea level of postnates of the same age groups. This may be the result of a stimulation of gluconeogenesis by nicotine utilising an amino acid precursor.

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ii. Fed adult rats treated for 3 days and sacrificed one hour later showed no change in blood glucose while fed adults treated for the same period of time but sacrificed 24 hours later showed a significant increase (+ 13,21%) in blood glucose concentration.

However, treatment for 30 days had no effect on the blood glucose 24h after the last dose was administered (i.p.). Marked increases in blood urea levels of up to 38,58% were recorded in the same groups of animals treated over 3 days, pointing to a stimulation of gluconeogenesis utilising an amino acid precursor. No changes in blood urea levels were recorded in animals treated for 30 days. This may probably be due to adaptation of the liver to nicotine. I am not sure at this stage whether the effect of nicotine is a direct effect or via catecholamines or other hormones.

iii. Fasted males and females treated once daily for 30 days showed a significant increase (+ 12%) in their blood glucose levels, but a 22,85% decrease in blood urea levels 24h after the last exposure. This may be as result of nicotine causing a shift in substrate preference for gluconeogenesis. Fasted males and females treated once and for 7 days showed decreases of 18,86% and 18,14% respectively in blood urea levels 24h after the last exposure, but no changes in blood glucose levels were recorded for the same groups. This implies that another substrate is used for gluconeogenesis during fasting instead of amino acids.

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TABLE 6

THE EFFECT OF NICOTINE ON IN VITRO UREA AND GLUCOSE RELEASE IN ADULT RAT LIVER TISSUE

The effect of nicotine (1 mg/kg/day) on the *in vitro* urea and glucose release of adult Wistar Rat liver tissue slices. Nicotine was administered to rats for 30 days and the animals were fasted for 24 hours before sacrifice and after the last dose of nicotine. Nicotine was not included in the incubation medium.
The KRP medium was used for this experiment (pH 7.4; Temp 37,5%)
The controls received saline placebos.
$p<0,05$ - significantly different from control.
n: number of experiments. In each experiment 3 animals were used.

TREATMENT	n	GLUCOSE RELEASE µmol/g/hr	UREA PRODUCTION µmol/g/hr
CONTROL: fed (male & female)	6	47.0 ± 1.80	12.0 ± 0.19
CONTROL : fasted (male & female)	8	21.0 ± 1.90 (-55.32%) ($p<0,05$)	17.0 ± 0.60 (+41.67%) ($p<0.05$)
NICOTINE: Males (1 mg/kg/day) for 30 days 24hr fast)	5	19.0 ± 1.40 ($p>0.05$)	20.0 ± 0.80 (+17.65%) ($p<0.05$)

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rate of glucose release after a 24 hour fasting period. Fasting resulted in an increase in urea release of 41,67% from 12,0 ± 0,19 μmol urea/g wet tissue/hour for liver tissue of non-fasting animals compared to 17,0 ± 0,60 μmol urea/g wet tissue/hour by liver tisue of rats fasted for 24 hours. Exposure to nicotine for 30 days resulted in a further 17,65% increase in urea production to 20,0 ± 0,80 μmol urea/g wet tissue/hour ($p < 0,05$).

2. DISCUSSION

Glucose is produced via two pathways namely gluconeogenesis, in which lactate, pyruvate, glycerol, and amino acids act as precursor molecules, and glycogenolysis (Hultman, 1976). My results were consistent with the present view that glucose turnover is practically halved during starvation (see Table 6). From the data available it is also clear that *in vivo* exposure of the rats to nicotine had no detrimental effect on the ability of the liver to release glucose into the surrounding incubation medium *in vitro*. This implies that the dephosphorylation of glucose-6-phosphate to yield free glucose as well as the process of release of the free glucose into the surrounding incubation medium was not affected by the *in vivo* exposure to nicotine. However, the rate of urea production is increased which shows that nicotine exposure indeed increase urea production by the liver. This means that the increase in blood urea concentration of the nicotine exposed animals can be ascribed to the stimulatory effect of nicotine on amino acid deamination in the liver and conversion thereof into glucose.

In chapter 5 it was shown that as the time of nicotine exposure increases, the decrease in blood glucose concentration due to fasting becomes less pronounced (table 5). It is therefore conceivable to speculate that prolonged nicotine exposure in some way or another either increases the ability of the liver of the fasting animal to synthesize glucose from amino acids or that the body tissue utilizes less glucose or both.

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It is known that the metabolic control processes during fasting result in a switch of the body tissues away from glucose to other substrates such as fatty acids for energy production in order to conserve glucose for nervous tissue, erythrocytes and other cells dependant on glucose for energy. It has also been proved that nicotine supresses glycolysis in lung tissue (Maritz, 1983). Nicotine also increase blood free fatty acids (Williams and Kanagasabai, 1983) which can replace glucose as substrate and at the same time inhibit glycolysis. Although nicotine exposure will result in a lower rate of glucose utilisation via the glycolytic pathway, it is unlikely that it plays a significant role because nicotine also stimulates glucose turnover via the HMP-path (Maritz, 1983). This latter stimulation of the HMP path overrides its inhibitory effect of glycolysis so that the total glucose utilisation is sharply increased despite inhibition of glycolysis (Maritz, 1983).

It seems to be more likely that prolonged nicotine exposure increases the rate of glycogen synthesis by the liver of fasting rats. This is supported by the fact that nicotine have no detrimental effect on the release of free glucose but do result in an increased rate of urea release *in vivo* and *in vitro*. Another finding that support this is that prolonged nicotine exposure results in a trend for blood glucose to become less and less affected by fasting (table 5).

The results therefore imply that prolonged nicotine exposure reduces the impact of fasting on the blood glucose concentration by gradually increasing the ability of the liver to synthesize glucose from amino acid precursors. The fact that the rate of glucose release was not increased to the same rate rate as urea release into the medium can be ascribed to the utilisation of the glucose by the liver. This also explains why the blood glucose concentration is not affected by exposure of the animals to nicotine as observed in the previous chapter.

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CHAPTER 7

THE EFFECT OF NICOTINE ON *IN VITRO* OXYGEN CONSUMPTION, GLYCOGEN UTILISATION AND LACTATE PRODUCTION OF RAT LIVER TISSUE SLICES IN A KRP-MEDIUM.

In the previous experiments, it was shown that the rate of glycogen deposition in the liver of postnatal nicotine exposed rats were slower than in the controls. Nicotine also reduces the liver glycogen content of fed adult rats. Some of these aspects were discussed in an earlier section. It is however possible that low concentrations of nicotine enhances mitochondrial energy metabolism and thus glucose and glycogen utilisation via the Krebs cycle in liver tissue. If this is so, then it will:

i. also increase the oxygen consumption of liver tissue *in vitro,*

ii. contribute to the slower rate of glycogen formation.

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Consequently, in these experiments the direct effect of nicotine on the oxygen consumption, lactate production and glycogen breakdown of liver tissue were investigated. This production excludes the effect of all external factors such as hormones and changing substrate concentrations which may interfere with the direct effect of nicotine on liver tissue. The experiments were divided into two groups:

i. exposure to nicotine *in vivo* whereafter the tissue was incubated in a nicotine-free KRP-medium and

ii. incubation of liver tissue slices in a KRP-medium containing nicotine (18nM).

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TABLE 7

EFFECT OF IN VIVO NICOTINE EXPOSURE ON IN VITRO OXYGEN UTILISATION OF RAT LIVER TISSUE SLICES IN YOUNG RATS

Effect of maternal nicotine treatment (1 mg/kg/day i.p.), on the *in vitro* oxygen utilisation of neonatal (1-5 weeks) rat liver tissue slices. Mothers were treated throughout pregnancy and lactation and neonates treated by subcutaneous injection after weaning on day 21 after birth. Rats were sacrificed 24h after the final dose. Values given as means ± SE, n values given in parenthesis, $p<0,05$ - significantly different from controls.
Control received saline placebos.

SAMPLE	OXYGEN UTILISATION ($\mu l O_2$/mg ± SE)		
	1hr	2hr	3hr
Control	1,26 ± 0,04 (6)	2,95 ± 0,06 (6)	3,44 ± 0,07 (6)
Nicotine 1 week old	1,48 ± 0,06 (12) $p<0,05$	2,56 ± 0,09 (11) $p>0,05$	3,63 ± 0,08 (12) $p>0,05$
Control	1,26 ± 0,07 (7)	2,40 ± 0,17 (6)	3,38 ± 0,18 (7)
Nicotine 5 week old	1,32 ± 0,04 (8) $p>0,05$	2,51 ± 0,07 (7) $p>0,05$	3,38 ± 0,16 (12) $p>0,05$

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In these experiments no exogenous substrates were used. The liver slices were totally dependent on endogenous substrates such as glycogen. As in the previous experiments young sexually immature (1-5 weeks old) and adult (20 weeks old) rats were used.

1. RESULTS

1.1. Young Rats (1 and 5 weeks old)

Oxygen utilisation *in vitro* was determined hourly over a three hour period and after a 15 minute equilibration period. After one hour the oxygen utilisation of liver tissue slices of 7 day old control pups was 1.26 ± 0.24 μl O_2 /mg/hr and for nicotine exposed pups it was 1.48 ± 0.06 μl O_2/mg/hr ($p < 0,0,5$). After 120 min and 180 min the nicotine had no influence on the oxygen utilization. Nicotine did not influence the *in vitro* oxygen utilisation of liver tissue slices of 5 week old pups (table 7). Glycogen utilisation of 1 and 5 week old rats was surpressed by 12,73 and 15,63% respectively. This inhibition of glycogen utilisation corresponds with the inhibition of 17,16% in lactate production (table 8).

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1.2. ADULTS (20 WEEKS OLD)

However, in adult rat liver slices of untreated rats incubated in KRP medium containing 18nM nicotine, a significant increase ($p < 0,05$) from 1,15±0,07 μlO_2/g/hr in controls to 1,22±0,06 μl O_2/g/hr was recorded.A definite decrease in lactate production from 22,81±1,32 to 12,93±3,43 μmol/g/h and an inhibition in glycogen utilisation from 39,7±1,60 to 22,74±2,16 μmol glucose/g/h was also recorded (Table 9).

Glycogen utilisation (table 10) in rats treated for 3 days, and slaughtered 1h after the final dose, was not significantly different from the controls whereas the lactate production for this group was surpressed by 36,48 percent. In rats treated for 3 days and slaughtered 24 hours after the final

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TABLE 8

THE EFFECT OF NICOTINE IN VIVO ON IN VITRO LACTATE PRODUCTION AND GLYCOGEN UTILISATION BY RAT LIVER TISSUE SLICES

Effect of nicotine (1mg/kg/day) on the *in vitro* lactate production and glycogen utilisation of liver tissue slices of suckling (1 week) and young (5 weeks) Wistar rats. Rats received nicotine via the placenta and mothers milk and subcutaneously after weaning on day 21 after birth. Rats were killed 24 hours after the final dose was administered. Values: mean ± SEM. $p<0,05$ - significantly different from control.

TREATMENT	LACTATE PRODUCTION µmol/g/h	GLYCOGEN UTILISATION µmol glucose/g/h	
AGE	1 WEEK	1 WEEK	5 WEEKS
Control	22.09 ± 1.85 (6)	34.97 ± 2.34 (6)	64.60 ± 1.33 (6)
Nicotine	18.30 ± 1.52 (12) $p<0.05$ (-17.16%)	30.52 ± 1.33 (8) $p<0.05$ (-12.73%)	54.50 ± 1.95 (9) $p<0.05$ (-15.63%)

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TABLE 9

THE EFFECT OF IN VITRO NICOTINE ON IN VITRO LACTATE PRODUCTION, OXYGEN AND GLYCOGEN UTILISATION OF ADULT RAT LIVER TISSUE SLICES

The effect of 18nM nicotine on *in vitro* lactate production, oxygen and glycogen utilisation of adult (20 weeks) rat liver tissue slices. The KRP medium (pH 7.4) was used as incubation medium at 37°C and nicotine was added to the medium before incubation started.

SAMPLE	LACTATE PRODUCTION µmol/g/hr	OXYGEN UTILISATION µl/g/hr	GLYCOGEN UTILISATION µmol gluc/g/hr
CONTROL	22.81 ± 1.32 (14)	1.15 ± 0.07 (7)	39.70 ± 1.60 (11)
NICOTINE	12.93 ± 3.42 (6) $p<0.05$ -43,31%	1.22 ± 0.06 (6) $p<0.05$ +6,09%	22.74 ± 2.16 (6) $p<0.05$ -42,72%

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TABLE 10

THE EFFECT OF NICOTINE ON IN VITRO GLYCOGEN UTILISATION AND LACTATE PRODUCTION IN ADULT RAT LIVER TISSUE SLICES

Effect of nicotine (1 mg/kg/day for 3-30 days i.p.) *in vivo* on *in vitro* glycogen utilisation and lactate production of adult Wistar rat liver tissue slices (20 weeks old). Rats treated for 3 days were sacrificed 1 and 24h after the final dose whereas those treated for 30 days were sacrificed 24h after final dose. Values given as mean ±SE, n values given in parenthesis, and $p<0,05$ taken as significantly different from the controls.
Controls received saline placebos.

SAMPLE	LACTATE PRODUCTION µmol/g/hr ± SE	GLYCOGEN UTILISATION µmol glucose/g/hr ± SE
Control	22,53 ± 1,33 (9)	27,80 ± 2,56 (13)
Nicotine (3 days, 1h)	14,31 ± 1,94 (6) p<0,001 (-36,48%)	30,75 ± 1,09 (6) p>0,05 (+10,61%)
Nicotine (3 days, 24h)	17,73 ± 1,35 (9) p<0,05 (-21,30%)	37,14 ± 4,11 (9) p<0,001 (+33,60%)
Nicotine (30 days, 24h)	30,09 ± 2,06 (7) p<0,001 (+33,56%)	53,87 ± 6,06 (6) p<0,001 (+93,78%)

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dose the glycogen utilisation was significantly increased by 33,60 percent whereas the same group showed a 21,30 percent decrease in lactate production. Rats treated for 30 days and slaughtered 24 hours after the final dose showed and 33,56 percent increase in lactate production with a concomitant 93,78 percent increase in glycogen utilisation (table 10).

2. DISCUSSION

The data in Table 7 demonstrate that the *in vitro* oxygen utilisation of rat liver slices was linear for the three hour incubation period. This implies that the incubation techniques were adequate by not having an effect on the *in vitro* energy metabolism.

There are two organellae in the liver cell that can use oxygen from the incubation medium namely the mitochondria and microsomes. The mitochondria use oxygen to capture energy in the form of ATP while the microsomes use it for detoxification of foreign chemicals. The mitochondria consume far more oxygen than the microsomes and a stimulation or inhibition of mitochondrial function is reflected in its ability to utilise the oxygen that is available in the surrounding body fluids or incubation medium. From the above-mentioned results it is clear that the *in vivo* exposure of liver tissue to nicotine had no effect on the *in vitro* oxygen consumption of liver tissue slices. It is known that *in vivo* nicotine exposure increases lung microsomal enzyme activity (Maritz, 1983) and it is therefore also possible that it will increase liver microsomal enzyme activity. It is however unlikely that an increase in liver microsomal enzyme activity will mask the effect of nicotine on liver mitochondrial oxygen consumption. It is therefore clear that prolonged nicotine exposure *in vivo* and even *in vitro* exposure had no detrimental effect on the function of the mitochondria as energy generators. This is in contrast to the findings of Maritz (1983), who demonstrated that nicotine (1-10nM) inhibits the *in vitro* oxygen consumption of rat lung tissue slices. However, my

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data corresponds with that of Weiss *et al* (1972) and Rosenkrantz and Sprague (1969) who found that nicotine had no effect on the oxygen consumption of brain and liver tissue slices respectively.

Despite the fact that nicotine had no effect on the in vitro oxygen consumption of liver tissue, it supresses the *in vitro* glycogen utilisation and lactate production of animals exposed to nicotine during pregnancy, lactation and for short periods of time. This inhibition of lactate production implies that nicotine supressed the glycolytic pathway. This is in agreement with the findings of Maritz (1983) who demonstrated that nicotine inhibits glycolysis in lung tissue. The slower breakdown of glycogen one hour after nicotine administration (table 10), can be ascribed to the inhibition of the glycolytic pathway before the HMP-path is activated and thus the slower entry of glucose-6-phosphate into this pathway and consequently its conversion to lactate.

Nicotine induces the synthesis of microsomal enzymes (Maritz, 1983). The resultant higher microsomal enzyme concentration in the presence of nicotine results in a faster utilisation NADPH for the detoxification of nicotine. The resultant change in the NADPH/NADP ratio results in an increased glucose-6-p dehydrogenase activity. This enzyme is the rate limiting enzyme of the HMP-path. An increase in the glucose-6-p dehydrogenase activity will result in more glucose entering the pathway to supply the NADPH which is required by the microsomal enzymes for detoxification of nicotine. This explains the increase in glycogen breakdown 24 hours after the last nicotine administration despite the lower glycolytic activity. This finding is the same as for lung tissue exposed to nicotine (Maritz, 1983). The prolonged exposure probably result in an optimal increase in microsomal enzyme activity and is reflected in the 93,78% increase in glycogen breakdown.

Exposing the animals for 30 consecutive days to nicotine resulted in a 33,56% increase in lactate production compared to the controls and this is contrary to

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the inhibition of 21,30% for liver tissue of animals exposed for 3 days and sacrificed 24 hours after the last administration. This increase in lactate production is difficult to explain. However, an increased lactate production is associated with an increased glycolytic activity during anaerobic glucose oxidation such as during strenous exercise. This is accompanied by an increased glucose utilisation via the glycolytic pathway. In this experiment nicotine had no adverse effect on the oxygen consumption of liver tissue slices. This implies that nicotine exposure had no effect on the ability of the mitochondria to extract oxygen from the surrounding tissue and incubation fluids. Therefore oxidation is aerobic and not anaerobic. With this background information in mind it is conceivable to speculate that the huge increase of 93,78% in glycogen breakdown due to the proposed increase in HMP-path activity, will result in the formation of large quantities of 3-phospho-glyceraldehyde. The latter substrate enters the glycolytic pathway below the major allosteric enzymes. This may result in a substrate driven increase in lactate production. In this instance therefore, the higher rate of lactate formation is not an indication of an increased glycolytic activity.

It is known that lactate serves as an important substrate for glucose synthesis in liver (Newgard *et al*, 1983). Although no data is presented in this experiment to proof this, it is possible that the liver tissue utilises lactate and 3-phospho-glyceraldehyde for gluconeogenesis and less amino acids. This may result in the lower blood urea concentration which is observed when the rats were exposed to nicotine for 30 days (chapter 5, table 4).

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CONCLUSION

The effect of nicotine was investigated on the *in vitro* oxygen utilisation and liver carbohydrate metabolism. The concentration of nicotine used was approximately similar to that found in the blood of smokers . From the investigation we can draw the following conclusions:

i. At two weeks, a diurnal rhythm for liver glycogen content already exists while no other differences occur at any point between males and females. However in 5 week old rats, differences between males and females were observed at 08h00 and the diurnal rhythm was more pronounced than that of two week old rats. Significant differences in hte diurnal rhythms of 20 week old males compared to females were also recorded at 16h00, 18h00 and 20h00.

ii. Maternal nicotine exposure (1 mg/kg/day) has no apparent effects on the liver glycogen of 1 and 7 day old pups. The amount of glycogen deposited in the glycogen pool of liver tissue of suckling rats was slower during maternal nicotine exposure and this may be due to a suppression of glycogenesis or an increased rate of utilisation of glucose via the HMP pathway to inactivate nicotine.

   Nicotine exposure (1 mg/kg/day) resulted in a lower liver glycogen content of 2, 5 and 20 week old rats. This may be due to decreased glucokinase activity or decreased food intake. The same treatment suppressed the *in vitro* glycogen utilisation of 1 and 5 week old rats. The fact that the *in vitro* results were different to the *in vivo* results was because all physiological control mechanisms were removed during the experiment and the direct effect of nicotine on this aspect of liver carbohydrate metabolism could therefore be observed.

   Fed adults treated over a short period (7 days) however showed an increase in glycogen utilisation and in this instance the *in vitro* work

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support the *in vivo* findings. Chronic treatment (1 mg/kg for 30 days) had no effect on the liver glycogen stores probably due to adaptation to nicotine.

iii. Maternal nicotine exposure (1 mg/kg/day) had no effect on the blood glucose levels of 1 week old rats. The same held true for 5 week old rats who received nicotine intraperitoneally upon weaning (day 18). However, marked increases in the blood urea levels of postnates of the same age groups were recorded. This may be the result of a stimulation of gluconeogenesis by nicotine utilising an amino acid precursor or decreased urea clearance by the kidney.

Short term nicotine exposure (1 mg/kg for 3 days) resulted in a 13,2% increase in the blood glucose concentration of adult rats sacrificed 24h after the final dose was administered. A concomittant increase in blood urea levels of up to 36% was also recorded, which implies a stimulation of gluconeogenesis by nicotine for the same reasons set out in the previous point.

Nicotine exposure (1mg/kg/for 7 and 30 days) resulted in a 55,32% decrease in the rate of *in vitro* glucose release by liver tissue slices. In adult rats treated over the same period of time and fasted for 24h the nicotine exposure had no effect on the *in vitro* glucose release but an expected increase (17,65%) was recorded in urea production. This implies that the dephosphorylation of glucose-6-phosphate to yield free glucose as well as the release of the free glucose into the surrounding incubation medium was not effected by the *in vivo* exposure to nicotine. The increase in the rate of *in vivo* and *in vitro* production points to the stimulatory effect of nicotine on the amino acid deamination in the liver and the conversion thereof into glucose and thus we can speculate that nicotine directly influences gluconeogenesis.

Prolonged exposure to nicotine (1 mg/kg for 30 days) however resulted in an increased rate of lactate production and a significant decrease in

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blood urea concentration. This is an indication of metabolic adaptation of the liver due to the presence of nicotine.

iv. Nicotine exposure (1 mg/kg/day)had no effect on the rate of *in vitro* oxygen utilisation of 1, 5 and 20 week old rats except for 1 week old pups where a significant increase of 15% was found during the first hour of incubation.

Nicotine (18 nM) administered *in vitro* resulted in an increase of 6% in oxygen utilisation but inhibited lactic acid production and glycogen utilisation both by 43%. We can therefore assume that nicotine enhances glucose turnover via the HMP shunt and suppresses glucose oxidation via the glycolytic pathway as indicated by the lower lactate production.

Long term nicotine treatment (1 mg/kg for 30 days) resulted in an increase of 25,12% in *in vitro* lactate production accompanied by a increase in liver glycogen turnover and this in turn pointed to a stimulation of glycogenolysis and glycolysis. These results cannot be explained at this stage of the investigation and warrants further investigation.

v. In summary the data of this study demonstrate that nicotine does not affect the ability of the liver to maintain the blood glucose concentration within the normal range. It is impossible at this stage to pinpoint the exact mechanism of nicotine's action on the glycogen stores, but it is clear that nicotine in some way or the other influences the maturational aspect of some liver enzymes and this warrants further investigation.

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