ADENINE NUCLEOTIDES AND SELECTED CHEMICAL PARAMETERS AS POTENTIAL MARKERS FOR FERTILITY ASSESSMENT

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Aan Marna, Gysbert en Hannes

Mat. 11:25 Daardie tyd het Jesus gesê: "Ek prys U, Vader, Here van hemel en aarde, dat U hierdie dinge vir slim en geleerde mense verberg het en dit aan eenvoudiges bekend gemaak het."

DECLARATION

I, the undersigned, hereby declare that **"Adenine nucleotides and selected chemical parameters as potential markers for fertility assessment"**, is my own work and has not previously in its entirety, or in part, been submitted at any university for a degree. All the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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ABSTRACT

Diagnostic andrology with its descriptive approach to the diagnosis of defective sperm for the last 40 years has focused mainly on the macro- and microscopic appearance of the ejaculate. More emphasis has recently been placed on the quantification of biochemical components in semen and sperm. In this regard, substances can be measured more accurately and in some cases as multicomponents in the same run. The value of bioenergetics of sperm as a marker for fertility assessment is a controversial topic in the literature. What complicates this issue is the relatively new information on how adenosine triphosphate (ATP), as the major high energy molecule, can be synthesized from other sources which was, until recently, unknown.

This study attempts to investigate the role of adenine nucleotides as potential markers in semen for fertility assessment using the Tygerberg strict criteria. In this study a reverse phase liquid chromatography (RPLC) technique was developed which measures all the nucleotides in a single run. Because the cycle of energy yielding and energy consuming processes operate simultaneously, it is difficult to assess the energy status of spermatozoa by only measuring the ATP concentrations as suggested in the literature. In this study all the adenine nucleotides and their relative ratios were measured. The results did not show significant differences using the Tygerberg strict criteria of morphology evaluation. This is in line with some workers who indicate that there is no value in measuring ATP levels and correlating this with fertilization. This study furthermore indicates that the breakdown products of ATP and the different adenine nucleotide

ratios do not correlate positively with morphology and fertilization in the IVF and GIFT procedures using the Tygerberg strict criteria of morphology.

As an application of the technique preliminary experiments indicated that the duration of incubating semen or spermatozoa is important when measuring nucleotides. An unknown product, that relates to motility, is formed during the incubation period of 18 hours (absent after 40 minutes). It was also shown in this preliminary study that the ATP/ADP ratio is the most sensitive biochemical parameter for changes in motility and that the older concept of the adenylate energy charge (AEC) is not a good indicator of the energy status of spermatozoa.

The experiments with the energy related enzymes Lactate dehydrogenase and Creatine kinase versus the morphological groups, have been proved not to be predictive. The sperm membrane enzyme γ-glutamyl transferase which also correlates negatively with the Tygerberg morphological groups could be more informative if the number of the P pattern group (<4%) were increased. This membrane enzyme could be of more value than previously realized, since it may relate to the lipid peroxidation of sperm which are extremely susceptible to oxidative stress.

The statistical analysis of the fatty acid composition of the sperm membranes for the P (poor <4%) and G (good >14%) pattern spermatozoa correlates negatively with each other using the Tygerberg criteria. However, the number of patients in these groups were only 5 and the results may be different in larger groups.

ABSTRAK

Diagnostiese andrologie met sy beskrywende benadering tot die diagnose van defektiewe sperme het vir die laaste 40 jaar hoofsaaklik gefokus op die mikro- en makroskopiese voorkoms van die ejakulaat. Meer klem word tans geplaas op die kwantifisering van biochemiese komponente in semen en sperm. In hierdie opsig kan substanse meer akkuraat en in sommige gevalle as multikomponente in dieselfde reeks gemeet word. Die bioenergetika van sperme was en is nog steeds 'n teenstrydige onderwerp in die literatuur ten opsigte van die waarde daarvan as merker vir fertiliteitsbeoordeling in die algemeen. Wat die onderwerp bemoeilik is die hele kwessie van onlangse inligting oor hoe adenosien trifosfaat (ATP) as hoof hoë-energiefosfaat-molekuul gesintetiseer kan word vanaf ander bronne wat tot onlangs onbekend was.

In hierdie studie was gepoog om die rol van die adenien-nukleotides te ondersoek as merkers vir fertiliteit deur gebruik te maak van die Tygerberg streng kriteria vir morfologie. Dit is moontlik gemaak deur 'n omgekeerde fase vloeistof chromatografie (RPLC) metode te ontwikkel, wat al die nukleotides in 'n enkele reeks meet. As gevolg van die siklus van energie-vorming en energie-afhanklike prosesse wat gelyktydig plaasvind, is dit moeilik om die energie status van spermatozoa vas te stel deur slegs die ATP-vlakke te meet soos in die literatuur voorgestel. In hierdie studie is al die adenien nukleotiedes en hul verhoudings gemeet.

Met die gebruik van die Tygerberg streng kriteria van morfologiese evaluering was die resultate nie betekenisvol nie. Dit is in ooreenstemming met sommige werkers wat aangedui het dat daar geen waarde in is om ATP-vlakke met bevrugting te korreleer nie. Verder dui hierdie studie ook dat daar geen betekenisvolle waarde is in die meting van die afbraakprodukte van ATP en die verskillende verhoudings ten opsigte van morfologie en bevrugting in die IVB (*in vitro* bevrugting) of GIFO (gameet intrafallopiaanse oordrag) tegnieke soos gebruik met die Tygerberg streng kriteria van morfologie nie.

As toepassing van die tegniek was dit uit voorlopige studies interessant dat die tyd van sperm-semeninkubasie belangrik is wanneer adenien nukleotiedes bepaal word. 'n Onbekende produk wat met spermmotiliteit verband hou het gedurende die inkubasietyd van 18 uur gevorm (afwesig na 40 minute). Verder is ook in hierdie voorlopige studie aangedui dat die ATP/ADP verhouding die mees sensitiewe biochemiese parameter is om veranderinge in motiliteit aan te dui en dat die vroeëre konsep van die adeniel energielading (AEL) nie 'n goeie indikator is van die energie-status van spermatozoa nie.

Die eksperimente met energie verwante laktaat dehidrogenase en kreatien kinase teenoor die morfologiese groepe dui daarop dat geen betekenisvolle verskille voorkom nie. Die spermmembraan γ-glutamiel transferase ensiem wat ook negatief gekorreleer het ten opsigte van die Tygerberg morfologiese groepe, kan moontlik meer van waarde wees indien die aantal pasiënte in die P patroon groep (<4%) verhoog kan word. Hierdie

membraanensiem kan van meer waarde wees as tans die geval is omdat die spermmembraan hoogs sensitief is vir lipied peroksidasie gedurende oksidatiewe stres.

Die statistiese analises van die vetsuur samestelling van die spermmembraan vir die P ("poor" <4%) en die G ("good" >14%) sperm patrone korreleer ook negatief met mekaar indien die Tygerberg kriteria gebruik word. Aangesien die getal pasiënte in hierdie groepe slegs 5 was, mag daar wel verskille in groter groepe voorkom.

CHAPTER I

1 INTRODUCTION

1.1 Review of the literature

In the beginning God made man and blessed him and told him: "Be fruitful and multiply; fill the earth and subdue it." [Holy Bible, 1988]. Indeed, man did respond to that instruction, so much so that the world population in the year 2000 is estimated to be 6 292 million [Populations reference Bureau, INC., 1992]. It is said that the world is overpopulated and that implies serious shortages in supply of basic food and housing which bring suffering and death to many. However, while many couples are blessed with children of their own it is emotionally traumatic for other couples to realise that for some reason or other it is impossible to have children of their own [Menning, 1980; Mahlstedt *et al.*, 1987]. Infertility affects both husband and wife. Estimates of its prevalence are inaccurate but it is estimated that 8 - 15% of couples experience some form of infertility problem. If this is extrapolated in the global context then 50 - 80 million people may suffer from it [Rowe, 1989]. It is only recently that the male factor received attention in the literature.

A large body of research has been performed in the field of *in vitro* fertilisation (IVF) and embryo transfer (ET). Nevertheless, in an article by Odendaal and Kruger [1990] on infertility in perspective, they stated that more research is needed. Male factors contribute to infertility and as yet no major breakthrough has occurred in the treatment of the patient with subfertile semen. Acosta *et al.* [1990], in their book **"Human Spermatozoa in Assisted Reproduction"** state: "We have come a long way since sperm was discovered as one of the main

elements of human reproduction, but we still have a long way to go to unveil all the scientific facts of sperm function." Recently the reproductive literature focuses a lot on the technology of intracytoplasmic sperm injection (ICSI). This technology makes it possible to fertilise an oocyte with one sperm using micromanipulation. This novel technique has been applied successfully to treat male factor infertility, even in patients with severely impaired sperm characteristics [Devroey *et al.*, 1995; Palermo *et al.*, 1996].

Our understanding of the factors which determine the fertilising potential of semen is still poor and there is still no reliable method for screening of the fertilizing ability of sperm in spite of newer methods like the freezing test (FT), hypo-osmotic swelling test (HOS) and migration-sedimentation test (MST) [Paz *et al.*, 1993]. Routine semen analysis is currently used to assess male fertility potential. The analysis includes: (WHO 1986)

1 Physical characteristics such as colour, volume, pH, coagulation, liquefaction and viscosity.

2 Semen biochemistry which include zinc, acid phosphatase, fructose content.

3 Microscopic examination which include agglutination, motility, mixed antiglobulin reaction (MAR) test, supravital staining (SVS), sperm concentration and morphology evaluation.

Of these, the concentration, motility, forward progression and morphology are the more important parameters [Table 1].

TABLE 1: Spermiogram of criteria of normality [WHO 1986]:

Semen parameter	Average values
Volume	2.0 ml or more
pH	7.2 - 7.8
Sperm concentration	20 x 10^6 spermatozoa/ml or more
Total sperm count)	40 x 10^6 spermatozoa or more
Motility	50% or more with forward progression (i.e. categories (a) and (b): Section 2.4.2) or 25% or more with rapid linear progression (i.e. category (a)) within 60 min after collection
Morphology	50% or more with normal morphology
Viability	50% or more live, i.e. excluding dye
White blood cells	Fewer than 1 x 10^6/ml
Zinc (total)	2.4 micromol or more per ejaculate
Citric acid (total)	52µmol (10mg) or more per ejaculate
Fructose (total)	13 micromol or more per ejaculate
MAR test	Fewer than 10% spermatozoa with adherent particles
Immunobead test	Fewer than 10% spermatozoa with adherent beads

Studies have shown that the analysis of standard seminal parameters including the concentration, percentage motility and morphological features of spermatozoa, is inadequate to accurately predict the *in vitro* and *in vivo* fertilising potential of human semen [Chan *et al.*, 1990]. However, several laboratories have made contributions in this field. These include studies on 1) morphology, using strict criteria [Kruger *et al.*, 1986 and 1988]; 2) movement characteristics [Holt *et al.*, 1985, Jeulin *et al.*, 1986, Chan *et al.*, 1989]; 3) penetration of zona-free hamster oocytes [Wolf *et al.*, 1983; Corson *et al*, 1988; Nahhas & Blumenfeld, 1989]; 4) the binding to zona pellucida of human oocytes [Burkman *et al.*, 1988, Liu *et al.*, 1989, Oehninger *et al.*, 1989].

The above illustrates that there is a clear lack of quantitative biochemical parameters to assess sperm fertilising capacity. Further, it has been shown by many investigators that visual semen analysis is technically inaccurate and imprecise [Kjaergaard *et al.*, 1990; Bostofte *et al.*, 1993].

This study is mainly a technique study designed to investigate sperm bioenergetics and certain selected metabolic parameters in semen and its relation to sperm morphology and *in vitro* fertilisation using the Tygerberg strict criteria.

These include:

1 Nucleotides and their role as an energy source.

2 Key metabolic enzymes such as lactate dehydrogenase and creatine kinase as well as the membrane enzyme gamma glutamyl transferase.

3 Composition of lipids.

A review of each of the above will be presented below, as well as a motivation for their inclusion.

2 NUCLEOTIDES

Introduction:

Two systems in cells are responsible for providing energy. The adenine system whereby the reversible enzyme adenylate kinase catalyses the formation or utilization of adenine dinucleotides as follows: $2ADP \rightleftharpoons ATP + AMP$ which will be discussed in the text in more detail. Secondly there is also the phosphagen system. This system provides energy by the dephosphorylation of phosphocreatine to form creatine : ATP + creatine $\rightleftharpoons$ phosphocreatine + ADP - catalysed by the enzyme creatine kinase. When cells in general are in a resting state, that is when the ATP levels are relatively high, the reaction proceeds with the net synthesis of phosphocreatine, whereas at times of high metabolic activity, this is, when ATP levels are low, the equilibrium shifts so as to yield net synthesis of ATP [Voet & Voet, 1995]. This system was not investigated in this study.

2.1 Adenosine triphosphate (ATP) measurements as markers for fertilisation:

ATP is present in significant amounts in semen, both intracellularly in the mitochondria of the spermatozoon, and extracellularly in the seminal plasma [Singer *et al.*, 1983; Halangk *et al.*, 1985].

The energy derived from ATP is used for essential sperm functions such as motility [Calamera *et al.*, 1982], for maintenance of ionic gradients [Borle, 1981], and as a source of intracellular cyclic adenosine 3'5' monophosphate [Menon & Gunaga, 1974].

The literature on ATP as a possible marker for fertilisation, is contradictory. Seminal ATP levels were found to be of no significance to distinguish between fertile and infertile semen samples used for *in vitro* fertilisation of human oocytes [Chan & Wang, 1987; Chan *et al.*, 1987; Mieusset *et al.*, 1989; Chan *et al.*, 1990]. In contrast, Irvine and Aitken [1985] and Comhaire *et al.* [1987] established a correlation between ATP levels and fertilisation potential. In the epididymis, alpha-glucosidase correlated negatively with sperm ATP concentration [Fourie *et al.*, 1993]. Bosman *et al.* [1994] report that ATP levels of sperm did not correlate positively with fertilization after freezing and thawing processes in an IVF programme.

2.2 ATP measurements and its relation to motility:

Various workers measured ATP in spermatozoa and its relationship to motility. Schlegel *et al.* [1985] measured the ATP content of sperm with high motility (>60%) and donors with severe impaired motility (25%) and reported that there was no correlation between ATP content and motility in either group. This was contradictive to reports that there is a correlation between ATP levels and total

motile spermatozoa [Vilar *et al.*, 1980; Ford *et al.*, 1981; Orlando *et al.*, 1982; Ponsette *et al.*, 1986]. Ponsette *et al.* [1986] made a very important contribution to sperm bioenergetics by reporting in the same article that spermatozoa with both high and low motility are capable to synthesise ATP, but that a dysfunction of the phosphorylating particles in the mitochondria are not associated with low sperm motility. In a report by Levin *et al.* [1981] a comparison of semen specimens with normal sperm motility, compared to healthy donors with specimens with significantly lower motility from infertile patients, showed that the initial ATP concentration was similar in all the samples. However those with poor motility had normal concentrations of ATP. Du Toit *et al.* [1993] report that there is a good correlation between the differential motility scoring method and sperm ATP levels.

2.3 The energy charge index:

Only Atkinson appears to have used the energy charge concept, represented by the following equation:

$$AEC = \frac{ATP + \frac{1}{2} ADP}{AMP + ADP + ATP}$$

Akerlöf *et al.* [1987] reported that a relatively high energy charge index (0,8) showed that the spermatozoa tolerated the separation on the percoll gradient well.

Most sperm bioenergetic research is aimed at measuring only the ATP levels in sperm and does not take cognisance of the fact that ATP in sperm is involved in two processes namely, glycolysis (generation) and motility (utilisation). Measurement of the AEC takes care of the whole pool of nucleotides and according to Atkinson *et al.* [1966] and Chulavatnatol *et al.* [1977], provide a

better understanding of the relationship between the dynamics of energy turnover and sperm motility.

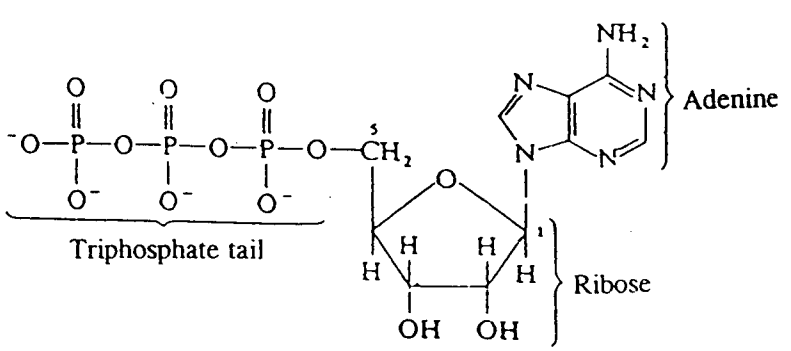
3 REVIEW AND BACKGROUND OF THE CHEMICAL CHARACTERISTICS OF THE NUCLEOTIDE POOL AND NON-NUCLEOTIDES

This review is presented to give a broad view of the nucleotides. Merely reporting on nucleotide values without a background explaining the underlying mechanisms is futile.

3.1 Adenine nucleotide metabolism

3.1.1 Structure and synthesis

Since its discovery in 1930 the role of adenosine triphosphate has been well elucidated in cellular metabolism and later in muscle and microtubular contractions [Dustin, 1980]. The molecule contains a five-membered sugar (ribose), a purine ring system (adenine) and a phosphate "tail" attached to the number 5 carbon hydroxyl group of the ribose ring.



Adenosine triphosphate (ATP)

FIGURE 1: Structure of ATP

It has been firmly established that ATP is the central molecule in the flow of chemical energy in all living cells. Energy released during glucose and fatty acid oxidation is captured and stored as ATP, and when energy is required it is degraded to release energy. The energy is stored in the phosphoanhydride bonds which link the phosphate groups to the ribose. ATP hydrolyses under standard energy conditions into ADP and P_i and releases approximately 30,660 kcal/mol (7,3 kJ/mol) of free energy.

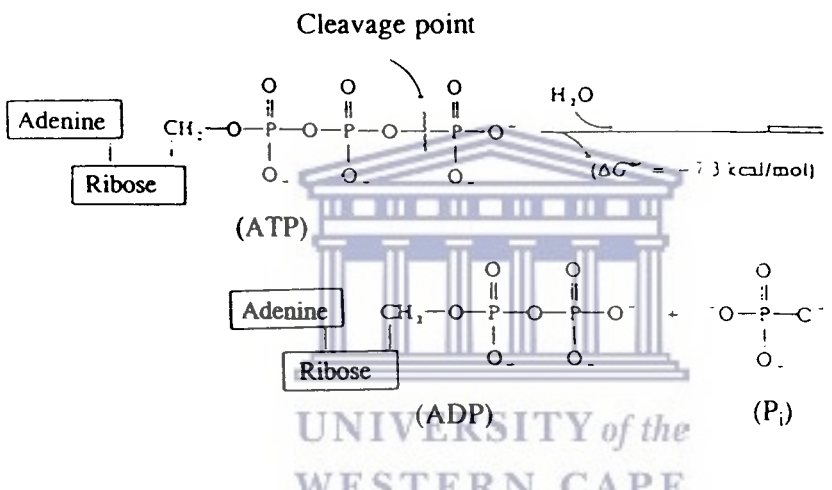


FIGURE 2: Orthophosphate cleavage (Hydrolysis of ATP to yield ADP + P_i)

ATP can undergo a second type of hydrolysis reaction to yield adenosine monophosphate (AMP) and pyrophosphate (PP_i), thereby releasing approximately 7,3 kJ/mol of energy under standard energy conditions according to the reaction [Scheve, 1984].

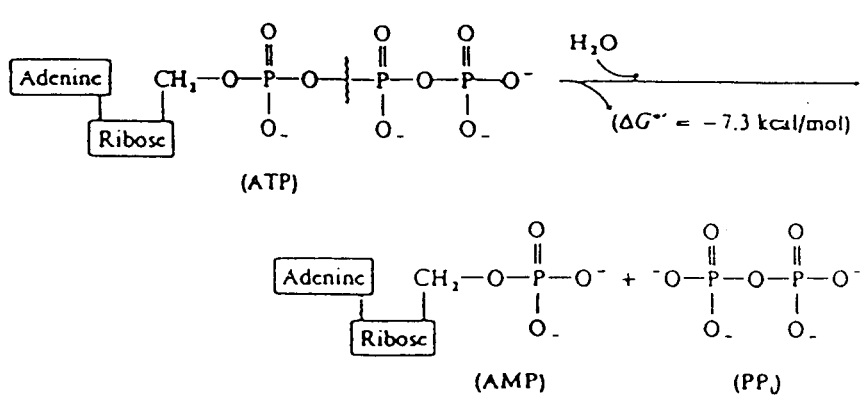


FIGURE 3: Pyrophosphate cleavage (ATP to AMP + PP_i)

This reaction is called pyrophosphate cleavage because it produces inorganic PP_i. The inorganic PP_i can undergo further hydrolysis, as shown in the following equation [Scheve *et al.*, 1984]:

$$PP_i + H_2O \longrightarrow 2P_i \qquad G^o = -8{,}0 \text{ kcal/mol}$$

Metabolic pathways transform glucose, free fatty acids and lactate to acetyl CoA which enters the citrate cycle to produce $NADH_2$. This in turn yields the hydrogen atoms which flow along the cytochrome chain in the mitochondria whereby ADP is converted to ATP by oxidative phosphorylation. The exact mechanism whereby the oxidation of $NADH_2$ is linked to the formation of ATP is still not fully understood. In the presence of oxygen complete oxidation of glucose to CO_2, water and energy, 36 ATP's are produced.

This oxidation reaction presented as $NADH + H^+ \longleftarrow O_2 \rightleftarrows NAD^+ + H_2O$ is coupled to the phosphorylation of ADP:$3ADP + 3P_i \rightleftarrows 3ATP$. Thus 3 ATP molecules are formed for each atom of half-molecule of oxygen taken up (P/O ratio of 3).

In the respiratory chain reducing equivalents derived from $NADH_2$ ($NADH + H^+$), flow along the respiratory transport (electron transmitter) chain in the following manner.

* $NADH + H^+ + \text{Flavoprotein} \rightleftarrows$ reduced flavoprotein + NAD.
* Reduced flavoprotein + coenzyme Q $\rightleftarrows$ reduced coenzyme Q + flavoprotein.
* Reduced coenzyme Q + cytochromes $\rightleftarrows$ reduced cytochromes + coenzyme Q.
* Reduced cytochromes + oxygen $\rightleftarrows$ cytochromes + H_2O.

In the respiratory chain there are three sites, each associated with the production of ATP. Site 1 is between NADH and coenzyme Q; site 2 is between cytochrome b and cytochrome c and site 3 is between cytochrome a and oxygen. Each of these sites yields one ATP and mitochondrial oxidation of NADH produced by the citrate cycle, therefore yields three molecules of ATP per atom of oxygen reduced.

After the production of ATP in the mitochondria it is then transported outwards into the cytosol by the ATP/ADP transport system located in the interchondrial membranes. The free ATP is required for sperm motility while ADP is released. As cytosolic ATP is used, it is replenished by synthesis from ADP in the mitochondria.

The rate at which the citric acid cycle operates, is a major factor controlling the rate of production of ATP by spermatozoa, because this cycle plays an important role in the formation of $NADH_2$. Each turn of the cycle produces twelve molecules of ATP [Conn *et al.*, 1967].

3.1.2 ATP and energy status

Atkinson [1968] examined the general biological significance of the relation between adenine nucleotides and energy production. As less and less of the adenylate pool (ATP + ADP + AMP) exists in the form of ATP and more and more in the form of the breakdown products (ADP and AMP) many enzymes involved in energy production are activated. Atkinson [1968] has proposed a complex (but frequently used) equation to represent the energy status of the cell:

$$E = \frac{2\,ATP + ADP}{AMP + ADP + ATP}$$

$$\text{or} = \frac{[ATP] + 0.5\,[ADP]}{[AMP] + [ADP] + [ATP]}$$

E = energy charge = AEC

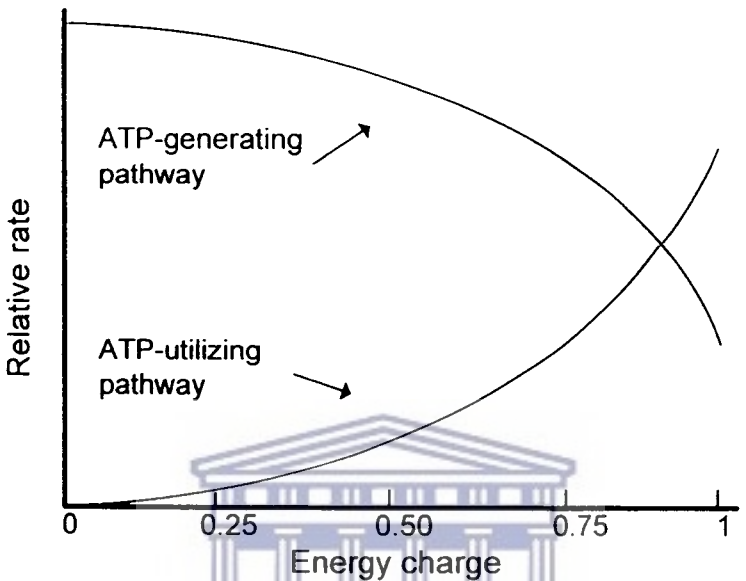


FIGURE 4: Effect of energy charge on the relative rates of a typical ATP-generating (catabolic) pathway and a typical ATP-utilising (anabolic) pathway. [Taken from Stryer 2nd Edition: Biochemistry.]

A fall of ATP stimulates the energy supply systems of glycolysis and more specifically fructolysis in spermatozoa and also the oxidative systems. The detailed mechanisms whereby the decline in high energy phosphate acts on the different pathways vary [see table 2 underneath], yet the principle is the same. It is not the level of ATP itself which regulates the "compensatory" pathways, rather the formation of ATP breakdown products (Adenosine monophosphate, inorganic phosphate, adenosine) which act as regulators. Breakdown of only a small amount of ATP can potentially increase the cellular levels of the real regulators markedly, such as AMP and adenosine. ATP is a negative modulator while ADP and AMP are positive modulators.

TABLE 2: Sites of production of H, CO_2 and ATP

Product	Sites of production	Fate
4 x 2H in total	1. Isocitrate dehydrogenase	Formation of NADH plus H^+ ($NADH_2$) and electron transport to produce 3 ATP.
	2. α-Ketoglutarate dehydrogenase	3 ATP as above
	3. Succinate dehydrogenase	$FADH_2$ formation and electron transport via CoQ to produce 2 ATP
	4. Malate dehydrogenase	3 ATP as above
GTP	1. Succinate dehydrogenase by substrate level phosphorylation	Ultimate formation of 1 ATP
One turn of citrate cycle →	Various dehydrogenase reaction as above →	12 ATP

3.1.3 Control of energy charge

The cell's energy charge is exerted by allosteric regulation of specific enzymes by ATP, ADP and AMP. The major locus for control of glycolysis by means of the ATP/AMP system is the conversion of fructose-6-phosphate to fructose 1,6 diphosphate. The allosteric enzyme phosphofructokinase is strongly inhibited by ATP but stimulated by AMP and ADP. On the other hand, fructose diphosphate phosphatase is stimulated by ATP and inhibited by AMP [Conn *et al.* 1976].

ATP is a negative modulator of liver pyruvate kinase, while glucose-6-phosphate, fructose 1,6-diphosphate and glyceraldehyde-3-phosphate activate it. Therefore, as the ATP/ADP ratio decreases, the flow of carbon from glucose to pyruvate is encouraged but when enough ATP is generated, the flow is again shut.

3.1.4 Adenine Nucleotides as Metabolic Regulators

The central role of the ATP/ADP ratio originate from the stoichiometric participation of the adenine nucleotides in metabolic reactions. A high and constant ATP/ADP ratio reflect a positive energy balance. Furthermore the adenine nucleotide pool consists of ATP, ADP and AMP in a dynamic equilibrium dominated by the enzyme adenylate kinase:

$$2ADP \xrightleftharpoons{\text{Adenylate kinase}} ATP + AMP$$

The equilibrium constant of adenylate kinase is not far from unity, and therefore the energy status of the adenine nucleotide pool is a function of all three nucleotides and not ATP and ADP only. Moreover, the adenine nucleotide pool is small and turns over several times per second. Any imbalance between the rates of ATP synthesis and consumption will therefore cause sharp fluctuations in the cell's energy status. One may therefore expect to find sensitive mechanisms to monitor the state of the adenine nucleotide pool and to ensure an appropriate response. For example, if ATP utilisation suddenly increases, the ATP content of the cell will be decreased. Consequently the rate of ATP generation will increase in an effort to replace the lost ATP.

Such mechanisms take the form of allosteric inhibition as earlier discussed or activation of key enzymes by ATP, ADP, or AMP (figure 6 provide examples). AMP is a particularly effective regulator. The ratio of ATP/AMP or ATP/ADP is therefore important and not necessarily the individual concentrations of the nucleotides. These regulatory effects show a clear relationship to cellular function. Enzymes which are eroded in ATP synthesis tend to be activated when ADP and AMP rises in the pool. Examples of such regulatory enzymes (R for regeneration) include phosphofructokinase, pyruvate

kinase, pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase. Conversely, enzymes involved in ATP consumption exhibit the opposite type of control, being stimulated by ATP but inhibited by ADP or AMP. Cases of regulation (U for use) are phosphoribosylphosphate synthase, aspartate, transcarbamylase, nucleoside diphosphokinase, and the key enzyme in glycogen synthesis, glycogen synthase. At the same time these enzymes are also under feedback control by the end products of their activities.

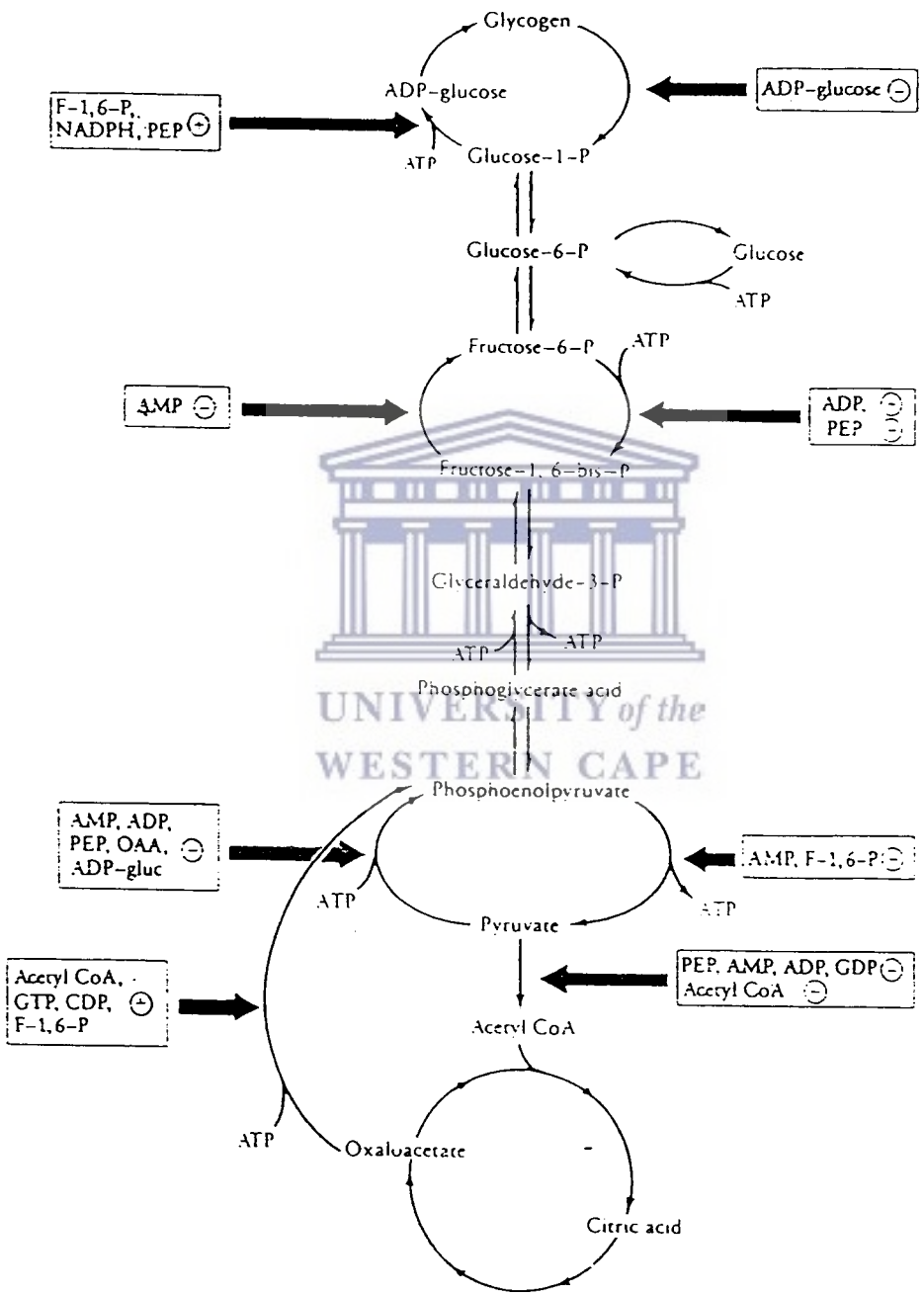


FIGURE 5: Direction and control of sugar metabolism in E. coli. Reactions involved in the utilisation of glycogen and glucose are on the right, those of biosynthesis on the left. Compounds in boxes are modifying ligands, either stimulatory (+) or inhibitory (-). [After Mandelstam & McQuillen, 3rd Ediction, Oxford Scientific, 1982.]

3.1.5 Phosphorylation potential

This term relates changes in high energy phosphates to mitochondrial metabolism, where:

$$\text{Phosphorylation potential} = \frac{[ATP]}{[ADP][P_i]}$$

AMP is omitted because it does not play a direct role in the regulation of mitochondrial oxidative metabolism [Sobell & Bunger, 1981]. A correlation between "phosphorylation potential" and oxygen uptake was described by Giesen & Kammermeich [1980] for the rat heart. As ATP is broken down by increased heart work, oxidative phosphorylation is proportionately stimulated by the formation of the first stages of its breakdown products, ADP and inorganic phosphate. The ATP/ADP ratio is an extremely sensitive measure of both the energy status of the cell and the rate of enzymatic and chemical degradation of the intracellular nucleotides [Opie, 1984].

It is reported by Halangk *et al.* [1985] that lactate concentrations above 1 mmole will sufficiently supply bovine spermatozoa with substrate and the energy turnover is mainly limited by the activity of dynein ATPase rather than by the capacity of mitochondrial oxidative phosphorylation.

3.1.6 Breakdown of ATP

Adenosine diphosphate (ADP)

The best known products of ATP breakdown are ADP and inorganic phosphate, which normally form during the contraction of the microtubules in the sperm. This is illustrated by the following equation:

$MgATP^{2} \rightarrow MgADP^{1} + P_i + 2 + H^{+}$ + free energy.

The exact changes depend on the intracellullar pH. ADP can (i) form from ATP via the creatine kinase reaction; or (ii) be further splitted to form ATP and AMP by the adenylate kinase reaction or (iii) enter the mitochondria under the influence of the adenine nucleotide translocase to stimulate respiration [Opie, 1984].

Adenosine monophosphate (AMP)

Adenylate kinase (AMP kinase or myokinase) allows the breakdown of ADP to proceed to AMP, thereby increasing the amount of AMP:

ATP + AMP → 2ADP. This reaction is reversible and will only proceed towards the formation of AMP when ADP is elevated. Thus under the influence of myosin ATPase:

$2ATP \rightarrow 2ADP + 2P_i$ + 2 (free energy) and under the influence of myokinase to:

2ADP → ATP + AMP and myosin ATPase again catalyses ATP → ADP + P_i + free energy so that the overall reaction is:

$2ATP \rightarrow ADP + AMP + 3P_i$ + 3 (free energy)

It is important however to notice that this equation generates 1,5 times more high energy phosphate than simple ATP hydrolysis [Opie, 1984].

3.1.7 Adenosine, inosine and hypoxanthine

Recently, important advances have been made in the understanding of the actions of adenosine on blood flow, visceral smooth muscle, synaptic transmission, P_2-purinoceptors and kidney (An international conference : Adenosine and ATP receptors, 1990). Adenosine also has effects on the slowing of the sinus node and

conduction through the AV node [Belardinelli *et al.*, 1980], also as an inhibitor of adenylate cyclase [Schrader *et al.*, 1977].

Formation of AMP provides the substrate for the AMP deaminase reaction whereby AMP is broken down to IMP (inosine monophosphate) as shown in the diagram below.

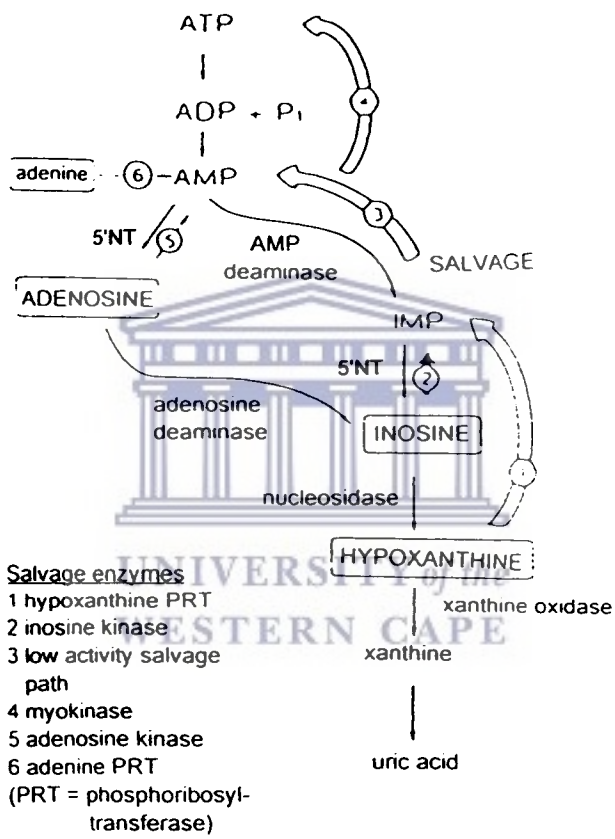


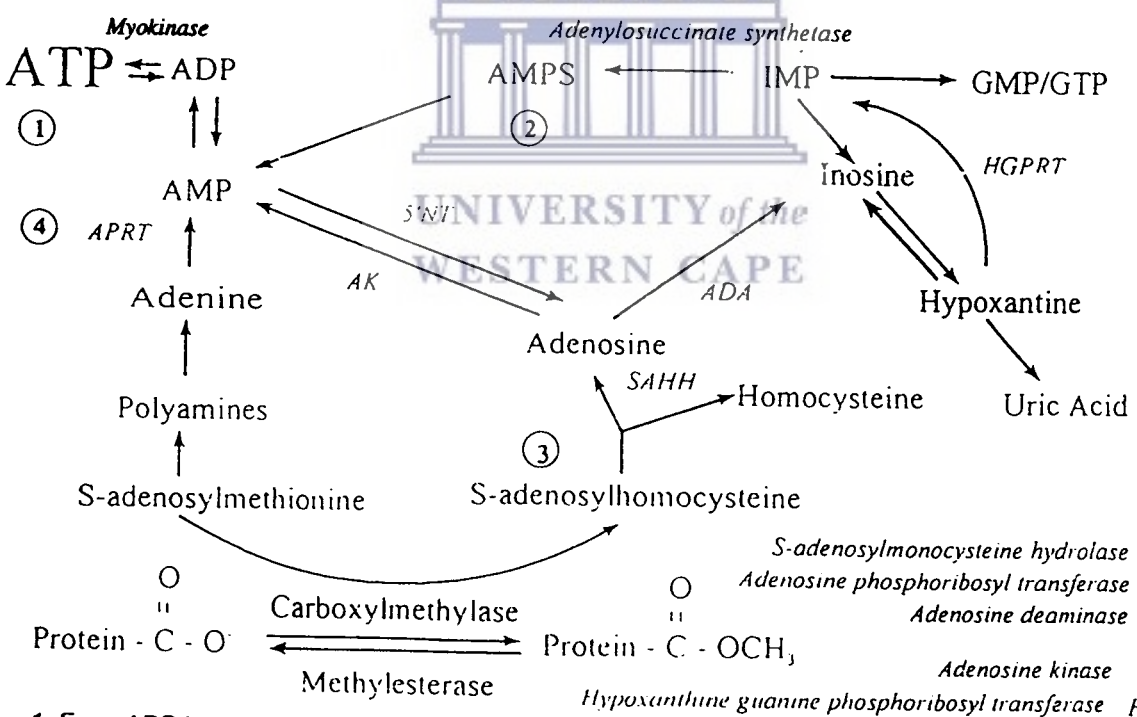
FIGURE 6: Pathway of adenine nucleotide salvage [Taken from: *The heart* by Lionel Opie, 1984]

In a different pathway the metabolism of AMP can be stimulated, since the decline in the "energy status" of the anaerobic cell activates 5'-nucleotidase, the enzyme that converts AMP to adenosine. The extra energy provided by the added breakdown of ATP beyond ADP to AMP is brought about only at the cost of further metabolism of AMP to IMP and adenosine [De Jong, 1979; Itoh *et al.*, 1986; Altschuld *et al.*, 1987]. It might be supposed that the processes breaking down ATP and related compounds normally stop at AMP. This may not be so

for there are deaminase enzymes active in the formation of very small amounts of ammonia (NH_3) in the heart [Opie, 1984]. It is not certain whether this is the case with spermatozoa.

3.1.8 Synthesis of adenine nucleotides

The nucleotides can be synthesised from four different pathways as indicated below, where AMP serves as the basis for ATP synthesis. Pathway one is the most common and is well described in basic textbooks with the actions of the reversible enzyme myokinase (adenylate kinase, AMP kinase) catalysing the reaction: ATP ⇌ ADP ⇌ AMP



1 From ADP by miokinase.
2 Adenine to AMP by APRT and AMP to ADP.
3 S-adenosyl homocysteine to adenosine by SAHH, adenosine to AMP by AK. AMP to ADP and ATP by myokinase
4 AMPS (Adenylosuccinate to AMP by Adenylosuccinatelyase and then further to ADP and ATP.

FIGURE 7: ATP synthesis by four different pathways (Partially taken from Simmonds *et al.*, 1989)

The second pathway is known as adenylate cycling (purine nucleotide cycling) (refer figure 7). This cycle converts AMP into inosine monophosphate (IMP) and reconverts IMP into AMP via adenylosuccinate (AMPS), producing NH_3 and forming fumarate from aspartate. IMP does not accumulate in cells but is rapidly converted to AMP and GMP. The difference between AMP and IMP is in the replacement of its 6-keto group by an amino group [Van den Berghe *et al.*, 1992].

In the first reaction aspartate's amino group is linked to IMP in a reaction powered by the hydrolysis of GTP to GDP + P_i to yield adenylosuccinate. In the second reaction adenylosuccinate lyase eliminates fumarate from adenylosuccinate to form AMP. GMP can also be synthesised from IMP through the IMP dehydrogenase and GMP synthase enzymes.

It would thus appear that ATP can be synthesised by fructolysis and the tricarbolic acid (TCA) cycle but also by *de novo* synthesis of the adenylate cycle (IMP adenylosuccinate AMP $\rightleftharpoons$ AMP + ATP $\rightleftharpoons$ 2ADP). The latter process is slow and can take several hours [Van den Berghe *et al.*, 1992].

The adenylate cycle was first demonstrated by John Lowenstein who demonstrated it in skeletal muscle which lacks several enzymes that perform anaplerotic reactions in the tricarbolic acid (TCA) cycle. The three enzymes of the purine nucleotide cycle (AMP deaminase, adenylosuccinate synthetase and adenylosuccinate lyase) are several times higher (in μmole) than in other tissues. This cycle is best known to occur in children as an inborn error of purine metabolism, characterised by an accumulation in body fluids of succinyladenosine and succinyl-aminoimidazole carboxamide because of the adenylosuccinase enzyme defect [Jaeken *et al.*, 1988]. In muscle the purine

nucleotide cycle has been shown to function during intense excercise [Van den Berghe *et al.*, 1992]. It is not known if this cycle functions in spermatozoa.

The third pathway of ATP regeneration is by the carboxylmethylation of proteins and phospholipids to form S-adenosyl-L-homocysteine. This product inhibits sperm motility [Goh *et al.*, 1985]. The enzyme adenosylhomocysteine hydrolase converts this product to homocysteine and adenosine. [See figure 7.] Adenosine is then catalysed to AMP by the adenosine kinase enzyme [Simmonds *et al.*, 1989] [See pathway 4 of figure 7].

The fourth pathway of ATP formation is that polyamines synthesized from S-adenosyl-methionine is converted to adenine which in turn is catalysed to form ATP by the adenine phosphoribosyl transferase system [Simmonds *et al.*, 1989].

3.1.9 Adenosine

During the past two decades important discoveries were made to aid in the understanding of the actions of adenosine. Adenosine receptors have been postulated for several effector systems and well-known drugs have been identified as antagonists of adenosine receptors [Proceedings of an international conference on: Adenosine and ATP receptors, 25-26 September 1989]. Two types of adenosine receptors are distinguished which are linked to different components of the adenyl cyclase system. The A_1 receptor interacts with the inhibitory G_i protein and inhibits the cyclase while the A_2 receptor interacts with the stimulatory G_s protein and activates the enzyme.

It was observed that the well known methylxanthines, theophylline and caffeine, in addition to their documented effects as phosphodiesterase inhibitors, are effective antagonists of the adenosine receptor agonists R - PIA and NECA (R-

N^6-phenylisopropyladenosine and 5'-N-ethylcarboxamidoadenosine). It was also observed that the effects of adenosine are independent from cyclic AMP. These important findings were made by Drury & Szent-Gyorgyi [1929] and Sattin & Rall [1970] respectively, on seminal tissue. Initially it was suggested that adenosine itself may have some physiological role since in a variety of systems it had been shown to have potent effects on muscular contractility. Secondly, it was observed that adenosine was an important component of neuronal systems by virtue of its association with adenylate cyclase activity.

Adenosine is a ubiquitous, naturally occurring purinenucleoside that is secreted from a wide variety of cells under conditions of stress, e.g. hypoxia or cell activation under conditions of energy deficit [Mentzer *et al.*, 1975]. Most of the adenosine found extracellularly is generated from the 5'-nucleotidase cleavage of adenosine 5'-monophosphate (AMP). Once released from activated cells, adenosine may either be reabsorbed into neighbouring cells where it is metabolised back to adenine nucleotides or degraded to inosine, hypoxanthine and uric acid, as was reported earlier in the text [Pathway of adenine nucleotide salvage].

3.2 NON-NUCLEOTIDES

Introduction

A few selected enzymes of the glycolytic sequence (Lactate dehydrogenase. LDH) and of energy transfer in the phosphagen system (creatine kinase. CK) and the cell membrane enzyme (gamma glutamyl transferase. γGT) were measured because it was thought that they may be sensitive markers in the spermatozoon metabolism and their concentrations may relate to abnormal sperm morphology. Further that the composition of the spermatozoon membrane may be changed in the abnormal dysfunctional groups and that this could provide valuable information on sperm membrane integrity. In the literature various workers

have used different approaches to study membrane integrity. These include the hypo-osmotic swelling test [Jeyendran et al., 1984 and Check et al., 1995] and the use of fluorescent stains [Harrison et al., 1990]. These approaches were not investigated in this study.

3.2.1 G-proteins

While the G-proteins have not been addressed in this thesis, it is important to review their importance in realtion to ATP briefly.

The action of ligands on receptors by bringing about certain cellular responses is well documented in basic biochemical and physiology textbooks [Ganong, 1989 and Montgomery *et al.*, 1990]. Adenylate cyclase, the enzyme that synthesizes cyclic adenosine 3',5'-monophosphate (cAMP) from ATP, is linked to receptors by guanine nucleotide-binding proteins called the G- and N-proteins.

After a ligand has bound to its specific receptor, the G-protein with GDP (guanosine diphosphate) attached, bind its receptor forming a tertiary complex. This complex in turn bind guanosine triphosphate (GTP), liberating GDP and the specific subunit of the G protein dissociates from the complex.

It is known that cAMP mediates mammalian sperm function and that the epididymal development of the capacity for motility in a number of species has been shown to be associated with an increase in intrasperm levels of cAMP [VijayaRaghavan *et al.*, 1986]. It has further been reported that G-proteins are associated with the plasma membrane outer acrosomal membrane region of acrosome-intact sperm and is involved in the zona pellucida-induced acrosome reaction [Karnik *et al.*, 1991].

3.2.2 Lactate Dehydrogenase (LDH)

LDH measurement is important to demonstrate whether aerobic and anaerobic glycolysis takes place. (Anaerobic glycolysis produces only 2 ATP molecules per molecule of glucose whereas aerobic glycolysis produces 36 ATP's - see page 9 of introduction). LDH is therefore an intricate part of ATP synthesis. This enzyme catalyses the reaction whereby pyruvate is converted to lactate as the end product in the glycolytic sequence and it requires nicotinamide adenine dinucleotide (NAD) as a cofactor. This cofactor acts as an electron/hydrogen acceptor, while the reduced form of the cofactor (NADH) acts as an electron/hydrogen donor. LDH can be separated electrophoretically into five subunits (LDH 1-5).

Each LDH isoenzyme has a different K_m and V_{max} for pyruvate. Tissues that contain large amounts of LDH_1 and LDH_2 will exhibit aerobic glycolysis while tissues with large amounts of LDH_4 and LDH_5 will carry out anaerobic glycolysis. These LDH isoenzymes and LDH_x (C_4) were measured in sperm from normo- and oligospermic men [Casano *et al.*, 1991].

There is a seminal plasma subunit of the LDH system known as LDH C_4 or LDH_X which is specific for germinal epithelium activity and appears first in primary spermatocytes [Orlando C. *et al.*, 1980; Alvareaz & Storey, 1984]. This isoenzyme has also been localised to the post-acrosome region, neck and midpiece of human sperm by immunofluorescence assay. LDH_X mobility differs among species. In man it lies between LDH_3 and LDH_4 [Wang *et al.*, 1990].

3.2.3 Gamma Glutamyl Transferase (γGT)

The possible relationship between membrane integrity and γGT content as well as its relation to lipid peroxidation caused by reactive oxyen species (ROS) served as motivation for the measurement of this membrane enzyme. This enzyme hydrolyses glutathione (GSH a tripeptide) into the products L-glutamate and a dipeptide cysteinyl glycine which break down to cysteine and glycine. This transferase enzyme is found in many tissues with the highest concentration in the renal tubules, liver and pancreas. Its primary occurrence is on the outer surfaces of cell membranes. It is mainly measured as a cholestasis indicating enzyme but is also a screening test for the determination of alcohol consumption [Blick and Liles, 1985].

γ-Glutamyl transferase is involved in the transport of amino acids (Meister cycle) whereby the tripeptide glutathione serves as a donor of a γ-glutamyl group that is transferred to the amino group of the amino acid selected for transport in membranes [Montgomery, 1990].

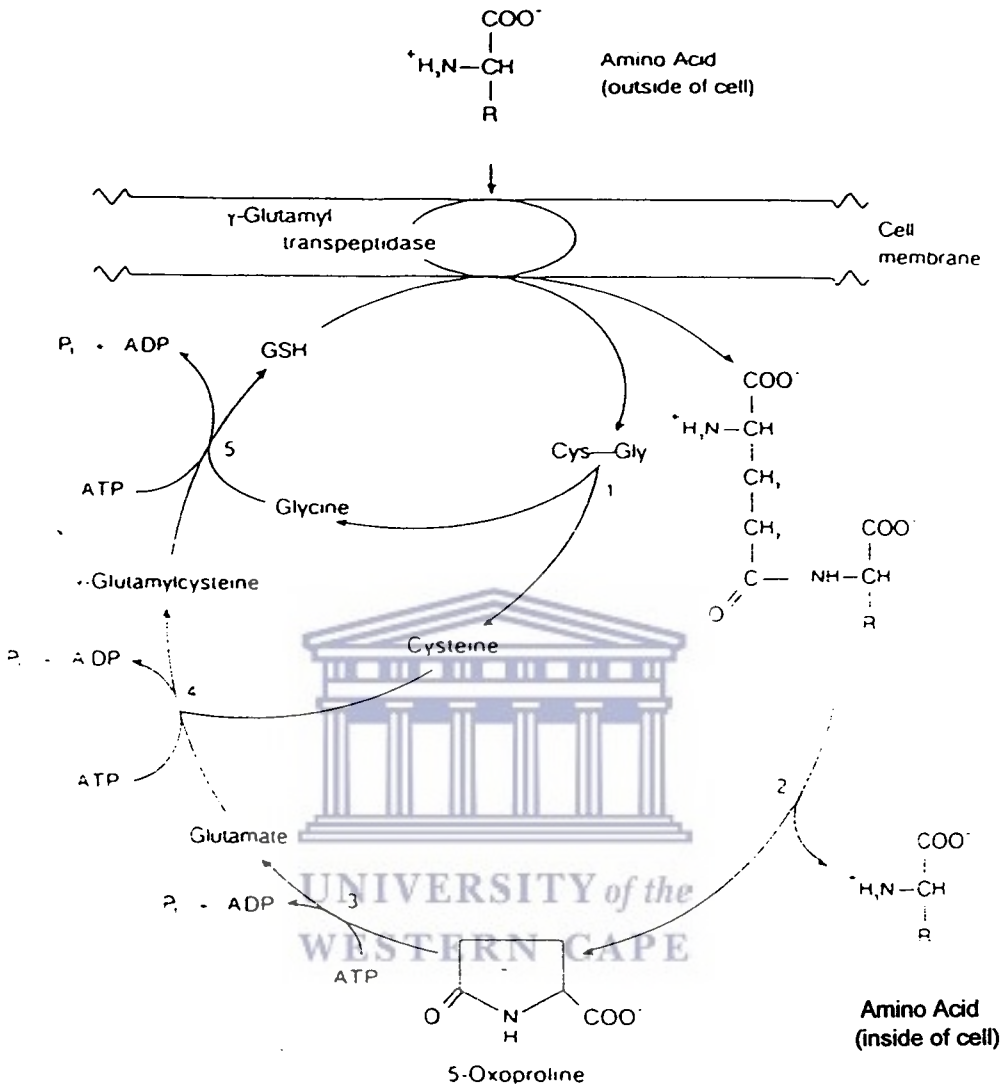


FIGURE 8: The γ-glutamyl cycle. γ-Glutamyl transpeptidase is located in the cell membrane; all other enzymes are cytosolic. These are numbered as 1, peptidase; 2, γ-glutamyl cyclotransferase; 3, 5-oxoprolinase; 4, γ-glutamylcysteine synthase; and 5, GSH (γ-glutamylcysteinylglycine, glutathione) synthase. [Taken from Biochemistry: A case oriented approach by Montgomery, Conway & Spector, 1990]

3.2.4 Creatine-N-phosphotransferase (CPK EC 2.7.3.2)

This enzyme catalyses the phosphorylation of creatine to yield creatine phosphate and as described previously is important in the energy yielding process of the phosphagen system (see page 4 of introduction). The literature is not specific whether spermatozoa use this system to provide energy. Its

measurement is generally used clinically as a marker for muscle damage. A study by Huszar *et al.* [1988] shows that the mean sperm CPK activity is higher in oligozoospermics than in normozoospermics. Recently Huszar *et al.* [1990] also reported that in a group of oligozoospermic subjects from infertile couples who achieved pregnancy after intra-uterine insemination, the mean CPK sperm activity was significantly higher than in a comparable group of oligozoospermic patients from couples who failed to conceive.

3.2.5 Biomembrane Integrity and Composition

As many as 40% of male patients with infertility problems have dysfunctional sperm. There is limited evidence in the literature on the molecular events of sperm motility and its relation to their fertilising potential.

One of the focal points in the current literature is on sperm-oocyte fusion and reactive oxygen species (ROS) generation as criteria for the diagnosis of infertility [Aitken *et al.*, 1987, 1988, 1989, 1991 and Kessopoulou *et al.*, 1992]. The integrity of spermatozoa membranes in the above studies could be related to the peroxidation of the polyunsaturated fatty acid content [Jones *et al.*, 1979] and thus a change in the fluidity of the membrane. As a result of this fluidity change the sperm cannot undergo the acrosome reaction to fertilise the ovum [Ohyashiki *et al.*, 1988].

The lipid composition of the plasma membrane and the various membranes of subcellular organelles have a wide spectrum of lipids which mainly fall within three categories, namely: phosphoglycerides, splingolipids and cholesterol. These compounds are unusual in that the polar and nonpolar portions of the molecules are geographically segregated to give a hydrophylic head and a

hydrophobic tail connected by a belt region of intermediate polarity [Andreoli *et al.*, 1987].

An important feature of the γ-glutamyl cycle is the resynthesis of the glutathione found in the luminal fluid of the epididymis that is utilised in many detoxification reactions and as a defence against electrophylic xenobiotics and intracellular oxidants such as free radicals and reactive oxygen intermediates [Staal *et al.*, 1992]. It is reported by Aitken *et al.* [1987, 1988, 1989 and 1991] that human spermatozoa are capable of generating reactive oxygen species and that this could be related to changes in membrane integrity through lipid peroxidation [Jones *et al.*, 1979].

4 CONTRADICTIONS IN THE LITERATURE AND SUMMARY OF AIMS OF THE PRESENT INVESTIGATION

4.1 Nucleotides

The literature presents conflicting results on the significance of ATP as a marker for fertility assessment. Two schools report contradicting results, namely no correlation between ATP and fertilisation [Chan & Wang, 1987; Chan *et al.*, 1987; Mieusset *et al.*, 1989 and Chan *et al.*, 1990] and those that found a correlation [Irvine & Aitken, 1985 and Comhaire *et al.*, 1987]. A further complication is the type of specimen used for analyses of the nucleotides. Semen which contain seminal plasma and which is high in ATP was used by researchers such as Singer *et al.*, [1983], Comhaire *et al.* [1983]; Irvin & Aitken [1985]; Mieusset *et al.* [1989]; Chan *et al.* [1990] while others like Schlegel *et al.* [1985]; Halangk *et al.* [1985]; White & Aitken [1989] and Vigue *et al.* [1992] used washed spermatozoa. A newly developed Firezyme kit was used by Mahmoud *et al.* [1994] who found a better correlation with sperm parameters than the

fluorescence LKB method. Their work is in line with previous studies that ATP concentration of semen correlates positively with motility [Irvine & Aitken, 1985; Comhaire, 1987], but disagrees with the work of Du Toit *et al.* [1993] showing that lower ATP levels correlated with good motility and good forward progression. A further problem in the literature is that virtually none of the authors took cognisance of the futile cycle of ATP and the processes of energy-yielding (fructolysis) and energy-consuming (motility) reactions that takes place simultaneously. In addition ATP can be synthesised from different biochemical pathways which were not known previously [Goh & Simmonds, 1985, 1989]. Thus ATP measurements alone may therefore not give a true reflection of the energy status of the sperm cell.

4.2 Non-nucleotides

The enzymes reviewed above are important in relation to energy turnover and sperm membrane integrity. Little information is available on the role of these enzymes in fertility assessment and/or their relationship with normal sperm morphology. This applies equally well to the fatty acid component of the cell membrane and its possible relationship to reactive ozygen species (ROS) [Aitken *et al.*, 1987, 1988, 1989 and 1991].

5 AIMS OF THE STUDY

The main aim of this study was to develop a reverse phase liquid chromatography (RPLC) technique for the separation of the adenylate nucleotide pool in human semen and spermatozoa and to apply this technique to the morphological groups using the Tygerberg strict criteria. The motivation for this was to establish a possible relationship between nucleotide content and sperm morphological groups (Tygerberg strict criteria).

Preliminary studies were also performed to measure the enzymes, LDH and its iso-enzymes, CK and the membrane bound γ-glutamyl transferase enzyme for the Tygerberg morphological groups.

The motivation for this is that some laboratories use the Tygerberg strict criteria for sperm morphology evaluation. It was therefore decided to establish whether any correlation exists between the above biochemical parameters and sperm morphology using the Tygerberg strict criteria.

Thirdly, a technique for fatty acid analysis was adapted to see whether one would be able to discriminate between known fertile (G-pattern) and known infertile (P-pattern) patients in a small sample size.

It should however be emphasized that the main aim was to test whether the above could be applied to semen of differential fertility.

CHAPTER II

MATERIALS AND METHODS

1 SPECIMENS

1.1 Specimen collection:

Specimens were collected by masturbation and the respective spermiograms done on the day of donation. Specimens were collected from two groups:

Group 1 specimens

Forty one samples from patients with fertility problems who visited the Andrology Department at Tygerberg Hospital were analysed. These were divided in three groups of different morphological status according to the Tygerberg strict criteria: < 5%, 5-14% and > 14%.

Group 2 specimens

This group of patients (fifty three) were part of the *in vitro* fertilisation program at Tygerberg Hospital. They were selected according to the strict criteria of Kruger *et al.* [1988] and divided in the following morphological groups: smaller than 5%, 5-10%, 11-14% and larger than 14% normal sperm. The following criteria for their selection were also used:

- no donor fertile semen;
- fertilisation of at least 2 ova;
- no "straws";
- no contaminated semen - infection presence of leucocytes;
- women younger than 35 years of age.

2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.1 Specimen Preparation

For HPLC Verification

For semen 100 µl was added to 500 µl 0.5 M Tris, 0,02 M EDTA buffer at pH 7,75 with concentrated sulphuric acid. For seminal plasma 100 µl of the supernatant semen centrifuged specimen at 400 g was used and treated similarly as described above. For the spermatozoa fraction the seminal plasma was removed by centrifugation at 400 g and the pellet washed three times with Hams F10 each time discarding the supernatant. The pellet fraction was also treated with 500 µl 0,5 M Tris, 0,02 M EDTA buffer at pH 7,75 with concentrated sulphuric acid. All three specimens were snap freezed in liquid nitrogen and stored at -80°C until day of analysis.

For nucleotide semen analysis (Group 2 specimens)

Fifty three semen specimens from patients participating in the Tygerberg *in vitro* program and selected as previously described were collected approximately one hour after ejaculation, after which 100 µl of semen was added also to 500 µl 0,5 M Tris, 0,02 M EDTA buffered at pH 7,75 with concentrated sulphuric acid. These specimens were snap freezed with liquid nitrogen and stored at -80°C until day of analysis.

For enzyme (LDH, CK, γGT) analysis (Group 1 specimens)

In forty one semen specimens (group 1 specimens as described previously) the seminal plasmas were collected by centrifugation at 400 g (1500 rpm) while the pellets (spermatozoa fraction) were discarded. These seminal plasma specimens were also snap freezed in liquid nitrogen and stored at -80°C until day of analysis.

"Swim up"

"Swimming up" spermatozoa for one specimen were prepared as follows:

Step 1 To a total semen specimen (±3 ml) was addded 2 ml of Hams F10 medium and centrifuged at 400g (1 500 rpm) for 10 minutes. The supernatant was discarded.

Step 2 Repeat step 1 with another 2 ml of Hams F10. The supernatant was again discarded.

Step 3 The pellet was layered with 1 ml of Hams F10 medium and incubated in a Forma Scientific tissue culture incubator (95% CO_2 5% O_2) in a test tube at 37°C for 1 hour. This specimen was then accepted as the "swim up" specimen.

2.2 18 Hour incubation of semen

A single incubation of semen was done in a Forma Scientific tissue culture incubator at 37°C with gas supplies of 95% CO_2 and 5% O_2. For semen incubation studies specimens were taken at 0 (after liquification), 2 hours, 4 hours, 6 hours, 12 and 18 hours. 100 µl of semen was added to 500 µl of extraction buffer (0,5M Tris, 0,02M Edta buffered at pH 7,75 with concentrated sulphuric acid), snap freezed in liquid nitrogen and then stored at -80°C for batch analysis.

This experiment was done to apply the new HPLC technique and to establish possible changes in nucleotides over time. A motility grading scale used by Tygerberg Andrology was furthermore used to correlate motility and nucleotide content.

2.3 Fertilization groups

These were grouped on the basis of number of oocytes that were fertilized as a percentage of the total number aspirated from the female patients participating in the Tygerberg IVF and Gift programs as 0%, that is where no fertilization occurred, <50 and $>50\%$.

2.4 System I

HPLC was done on a Waters model 440 HPLC equipped with WISP automatic injection and variable wavelength with detection at wavelength 254 nanometers. The stationary phase was a C_{18} reverse phase column (Supelco) with 5 μm packing. The mobile phase consisted of a 0,5 molar potassium dihydrogen orthophosphate buffer containing 2,5% methanol (HPLC grade) and 0,08% trisbutylammonium phosphate (Sigma), adjusted to pH4 using concentrated phosphoric acid. All reagents were analar grade. The flow rate was established at 2 ml per minute [Victor *et al.*, 1987].

Peaks were automatically integrated by the Waters computerised system and compared to standards. Calculation of the unknown (specimens) was done using the equation

$$S = \frac{\text{area of Test}}{\text{area of Standard}} \times \text{concentration of standard.}$$

The settings on the Waters data module system was as follows:

00	00/00/00	Date
01	00:20:28	Time
02	0.30	Chart Speed, cm/min
03	OFF	Plot
04	OFF	Pen 2
05	10	Pen 1 Zero
06	90	Pen 2 Zero
07	OFF	Auto Zero
08	LC	GPC/LC
09	OFF	Calib

20	OFF	Auto parameter
21	15	Peak width
22	25.000	Noise rejection
23	100	Area rejection
24	25.00	Run stop

2.5 System II

Most of the adenine nucleotide separations were done on A Beckman Gold High Pressure Liquid Chromatograph system equipped with a variable wavelength diode array detector, automatic injector with a 20 μl loop and compatible software was used. The wavelength detection was done at 254 nanometers. The sensitivity was set at 0,005 AUFS (absorbance unit full scale) with a 1 cm light path cell in the detector. The rest of the chromatography procedure was similar to the previous description (Waters system).

2.6 Standards

Standards (Sigma) of the nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP) adenosine monophosphate (AMP) and adenosine were adjusted to a concentration of 10 mg/10 ml in H_2O. 10 μl ADP, AMP and adenosine and 20 μl ATP were injected in the column. The final concentrations of these nucleotides were calculated as 210 femtomoles (fmoles) for ATP, 150 fmoles for ADP, 180 fmoles for AMP and 230 fmoles for adenosine. The standards were constituted in HPLC grade water and aliquoted into 500 μl volumes and frozen at -4°C until used.

Concentration of the standards for the Beckman system were: AMP, ADP, adenosine and ATP: 10mg/10ml from which 10 μl of AMP, ADP, adenosine and 30 μl ATP respectively were made up to 4 ml. 20 μl of these diluted standards were injected on to the column. The concentrations of AMP, ADP

and Adenosine were the same as for the Waters system. The ATP concentration was increased for the Beckman chromatography for higher peak areas since the instrument settings were as such not to give integrated ATP peak areas.

3 BUFFERS

3.1 Extraction buffer (Tris-EDTA) WHO

Tris 12,10g

Na_2 EDTA H_2O 0,37g

Sodium azide 0,50g

(All analar grade.)

Dissolve in 900 ml of HPLC grade Milli Q H_2O. Adjust pH to 7.75 with concentrated sulphuric acid. Make up to 1 liter with Milli Q H_2O. Filter with millipore 0,22 μm or 0,45 μm filter.

3.2 Mobile phase for RPHPLC

70g (0,5M) KH_2PO_4 (Merck 4873)

25 ml (2,5%) Methanol (HPLC grade)

0,8g (0,086%) Tetrabutyl ammonium hydrogen sulphate (TBAS) Sigma T1134. pH to 4 with concentrated phosphoric acid. Make up to 2 liters. Filter with 0,45 μm millipore filter.

4 NUCLEOTIDES

4.1 Analysis

The nucleotides were identified by their corresponding retention times of the standards and then measured by the following formula:

$$\frac{\text{Area underneath the peak (AUP) of unknown}}{\text{Area underneath the peak (AUP) of known nucleotide}} \times \text{concentration of known nucleotide}$$

and expressed in nmoles/l. The following ratios were calculated:

ATP/ADP; ATP/AMP; ATP/ADP+AMP and the Atkinsons adenylate energy charge as AEC = $\frac{ATP + \frac{1}{2} ADP}{AMP + ADP + ATP}$

Specimens were batch analysed.

5 ENZYMES

Enzyme analysis on forty one specimens were performed on diluted 1:10 seminal plasma with water and a Hitachi 747 automated spectrophotometer was used.

5.1 Lactate dehydrogenase (LDH E.C.1.1.1.27)

A Boehringer kit (1127977 - R2) was used. Reagents were prepared according to manufacturers instructions. It consisted of buffer, NADH and pyruvate. The test principle is:

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{LDH} \text{lactate} + \text{NAD}.$$

Wavelength setting was 340 nm and results expressed as U/l (international units per liter). The LDH isoenzymes were analysed by electrophoresis using a

Beckman Paragon® 655940 system. After visualisation the electrophoresed isoenzymes were scanned on a Beckman densitometer where intensity of separated component was integrated as percentage absorption of total sub-units.

5.2 Gamma-glutamyl transferase levels (γGT. GGT. E.C.2.3.2.2)

This reagent kit (Boehringer Mannheim code 816345) consists of Tris buffer and a glycylglycine tablet, diluent and substrate: L-γglutamyl-3-carboxy-4-nitroanilide.

L-γ-glutamyl-3-carboxy-4-nitroanilide + glycylclycine $\xrightarrow{\gamma GT}$.

L-γ-glutamyl-glycylgline + 5 amino-2-nitrobenzoate.

The end product (amino-2-nitrobenzoate) is yellow in colour. The rate of formation of the yellow end product is monitored at 415 nanometers on a Hitachi 747 spectrophotometer. The increase in absorbance time ΔA/min is directly proportional to γGT activity.

5.3 Creatinine kinase (CK) (E.C.2.7.3.2.)

A Boehringer kit (1127594) was used. Reagents were prepared according to the manufacturers instructions. It consisted of buffer, enzymes, coenzymes and substrate. The test principle is:

creatine phosphate + ADP $\xrightarrow{CK}$ creatine + ATP glucose + ATP $\xrightarrow{HK}$ glucose-6-P + ADP glucose-6-P + NADP+ $\xrightarrow{G6P\text{-}DH}$ gluconate-6-P + NADPH. Enzyme activity was measured by a change in the absorption spectra of

NADP+ $\longrightarrow$ NADPH which is directly related to the concentration of the enzyme.

6 MEMBRANE COMPOSITION

6.1 Extraction of lipids

Extraction of total lipids was done according to Folch *et al.* [1957]. 500 µl of thawed extraction sperm buffer was added to 4 ml methanol, whirl mixed for 2 minutes and a further 8 ml chloroform was added (chloroform, methanol: specimen 20:1). The samples were then shaken for 15 minutes by a Baird and Tatlock shaker and 2,5 ml of a saline saturated with chloroform methanol was added (v/v= 86:14). After whirl mixing, the specimens were centrifuged in a MSE centrifuge at 500 g for 10 minutes. The infranatant fraction (lower phase) was collected and concentrated with nitrogen gas in a 40°C waterbath. It was then concentrated with 2 ml chloroform methanol saline (v/v/v = 85:14:1) by using nitrogen evaporation in heated block at 20°C. For spotting of TLC plates, 80 µl was used after reconstituted with chloroform-methanol saline and 30 µl was spotted.

6.2 Separation of: Triglycerides, cholesterol and phospholipid classes

For the separation of non-polar lipids the spotted thin layer chromatogram (TLC) plates were run in: Diethyl ether, petroleum ether, acetic acid (30:90:1). The total phospholipids remained at the origin. The plate was dried under nitrogen vapour.

To visualise the chromatograms they were sprayed with B.BOT. [2,5-bis-(5'-tert-butylbenzoxa-zolyl)-[2'] thiophene dissolved in chloroform methanol mixture (1:1, v/v) and viewed under UV light.

The spots containing the cholesterol ester and triglyceride were marked and scraped off. Methylesters of the cholesterol esters and triglycerides were prepared by transmethylation with a methanol-sulphuric acid mixture (95:5, v/v) at 70°C for 2 hours. The methyl esters were extracted with a hexane/water system. The triglycerides were stored under nitrogen until gaschromatographic analysis. The cholesterol esters were separated on TLC (as before) to remove free cholesterol liberated during the transmethylation process. This is an important step because free cholesterol interferes with the gaschromatographic analysis of the methylesters [Folsch, *et al.*, 1957].

6.3 The separation of the polar phospholipids was done with the mobile phase: chloroform, acetone, methanol, acetic acid, distilled water (45:16:15:11:6).

The phosphatidyl choline (PC) and phosphatidylethanolamine (PEA) fractions were scraped off and treated in the same manner as above.

The methylesters were analysed on a Varian 4600 Gas Chromatograph (GC) linked to a Varian Vista 401 chromatography data system which served as a recording integrator. The GC was equipped with two 30m DB-1225 fused silica megabore columns (J+W scientific, catalogue number 125-2232). The GC conditions were:

starting temperature: 165°C

end temperature: 220°C

temperature increments: 3°C/min

injection temperature: 250°C

detector temperature: 250°C

flow rates of gases were: nitrogen: 25 ml/min

hydrogen: 25 ml/min

oxygen: 250 ml/min

The integrator quantified the fatty acid (FA) values in area counts. The GC spectrum of a reference standard was used to identify the FA peaks by comparing the applicable retention times. The individual FA (saturated and unsaturated) were expressed as a percentage of total FA.

CHAPTER III

VERIFICATION OF A HPLC-METHOD FOR DETERMINING THE NUCLEOTIDES IN SEMEN, SPERM AND SEMINAL PLASMA

1 NUCLEOTIDES

The emphasis in this chapter was to establish whether the adenine nucleotides could be clearly separated, measured and quantified in semen, spermatozoa, seminal plasma and the "swim-up" specimen. Furthermore, the technique was then applied in an 18 hour semen incubation experiment.

1.1 RPLC (Reverse phase liquid chromatography)

The literature describes various HPLC techniques for the separation of nucleotides, nucleosides and bases in general but not specifically for semen or spermatozoa analysis [Barrio et al., 1972; Kuttesch *et al.*, 1978; Spielman *et al.*, 1981; Stocchi *et al.*, 1985; Viarengo *et al.*, 1986 and Perrett 1986]. The wavelength detection was done by Kuttesch *et al.* [1978]; Viarengo *et al.* [1986] and Victor *et al.* [1987] at 254 nm. However, Vigue *et al.* [1992] used a wavelength of 256 nm for their analysis of the nucleotide pool. It was therefore important to measure the maximum absorption (λmax) of these nucleotides. Figure 9 gives an indication of the different λmax of AMP, ADP, adenosine and ATP respectively.

To test for the precision of the technique for area under the curve and retention times (amount of time the component spend on the column) 10 consecutive injections (20µl) of the standard sample were made on the C_{18} column fitted with

a C_{18} guard column (Supelco). These injections were run for 25 minutes and the area and retention time of each nucleotide was recorded. These recordings were then used to calculate the mean, standard deviation (SD) and relative standard deviation (RSD: % of SD). These results are presented in table 3.

Further standard nucleotide preparations were prepared in water from stock solutions (10 mg/10 ml for AMP, ADP, adenosine and ATP) and injected. Figures 10 and 11 give the chromatographs of the separated nucleotides and their retention times.

Four different specimens, each from the same donor, were prepared as discussed in Materials and Methods. These semen, spermatozoa, seminal plasma and swim up specimens were then injected without further sample clean up. Figure 12 gives a semen nucleotide separation. The integrator was set not to record the first 5 minutes since many substances elute in this time that are not of interest. AMP, ADP, adenosine an unknown substance and ATP separated with retention times of 7,06 minutes; not integrated, 10,52 minutes; 12,43 minutes and 19,9 minutes respectively. Figure 13 gives the separated nucleotides in the pellet of spermatozoa where seminal plasma was removed and washed (three times) with Hams F10 containing concentrated $NaHCO_3$. Similar separations of the nucleotides with their retention times were obtained. Figure 14 shows the separated nucleotides in seminal plasma. Figure 15 gives the result of a swim up specimen prepared as described under Materials and Methods. As can be seen from the chromatograph, the concentrations were too low for integration. Figure 16 shows the results of a 6 hour incubated semen specimen. The chromatogram indicates a fairly large concentration of an unknown that elutes from the column at a retention time of 13,08 minutes. This unknown is not adenosine and has an absorption spectrum different to the 260 nm of adenosine as

can be depicted from figure 9. The substance absorbs light at 218 nm with a second peak at 278 nm (figure 18).

This substance is not adenosine but close in structure because of its physico chemical properties of interaction with the C_{18} stationary phase. It is absent after liquification (40 minutes) but increases to fairly high concentrations after semen was incubated for 18 hours. The unknown is also present in spermatozoa as seen on the chromatogram displayed in figure 13.

1.2 Application of adenine nucleotides determination during an 18 hour incubation and isolation of unknown nucleotide

The unknown that elutes at 13,08 minutes (see figure 16) was fractionated and hand collected over a period of 24 hours in approximately 2 ml fractions after 20 µl injections. The total volume was ± 250 ml. This total volume was divided in two equal parts of which one was treated with an cation exchanger (Dowex) activated with 0,1M HCL to remove the ion pair (tributylammoniumsulphate) and then with an anion exchanger (Dowex) activated with 0,1M Formic acid to remove the phosphate. The rest was put through a 30 x 160 mm Column packed with approximately 7,5g C_{18} packing material. The extraction was carried out in a three phase step, consisting of conditioning, sample application and elution as indicated in figure 17. The methanol fraction was concentrated by heat and nitrogen vapour to approximately 5 ml. This is currently analysed by mass spray spectrometer analysis. Preliminary analysis indicates that this could be methylthioadenosine.

1.3 Summary of method development results:

It can be seen in figures 10, 11, 12, 13 and 14 that the HPLC technique separated the nucleotides well in seminal plasma, semen and spermatozoa. However, with the swim-up the concentration of spermatozoa in the 100 µl swim-up fraction is too low and need specimen concentration or manipulation to detect the adenine nucleotides as discussed previously. The technique furthermore showed that the nucleotides could be quantified over an 18 hour period of semen incubation and will be addressed in more detail in chapter IV.

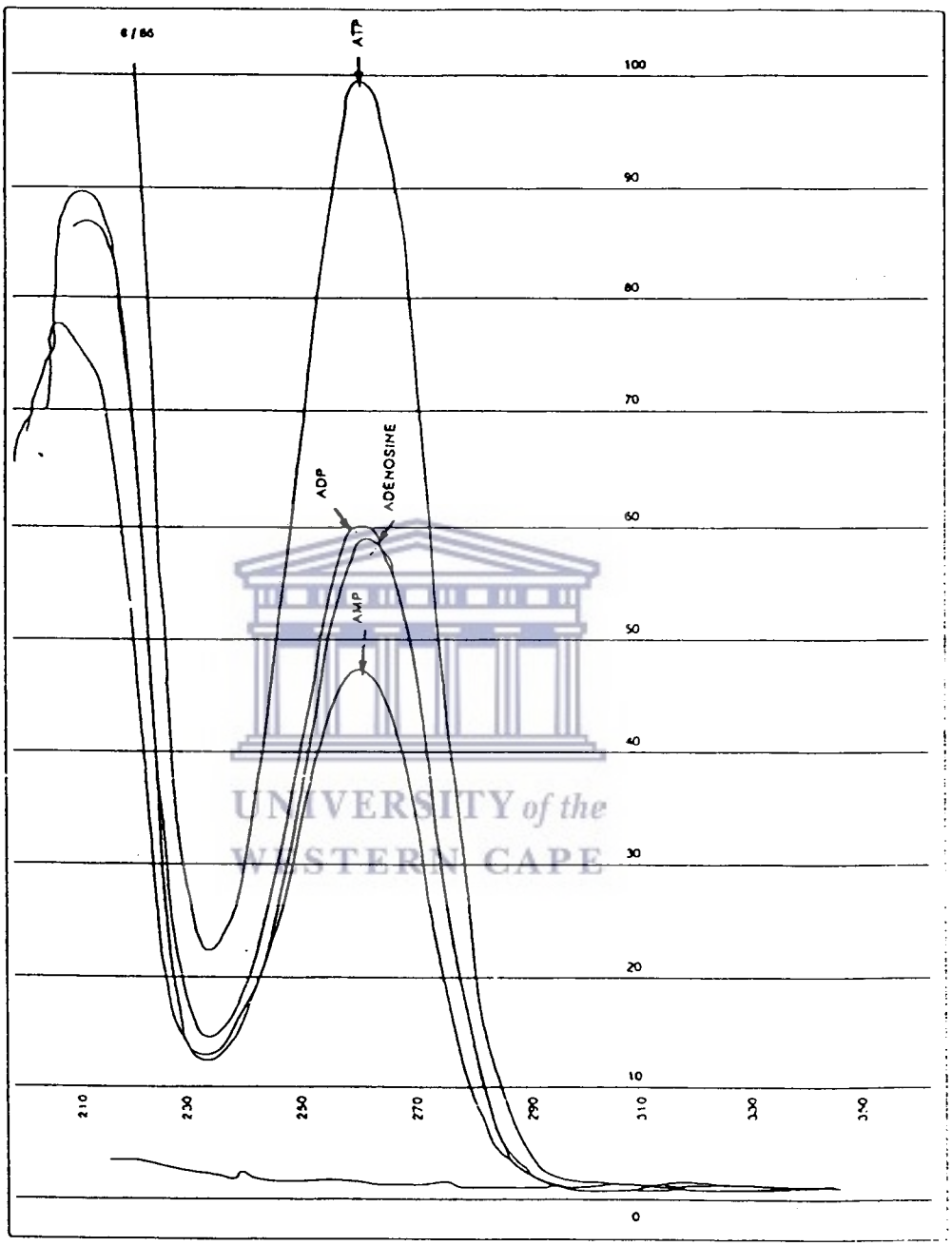


FIGURE 9: Absorption spectra of nucleotides, AMP, ADP, adenosine and ATP on a CARY 19 scanning spectrophotometer. The λmax in water is: 258 nm, 259, 260 and 259 for the nucleotides respectively.

TABLE 3: Mean, standard deviation and relative standard deviation of 10 consecutive injections in water.

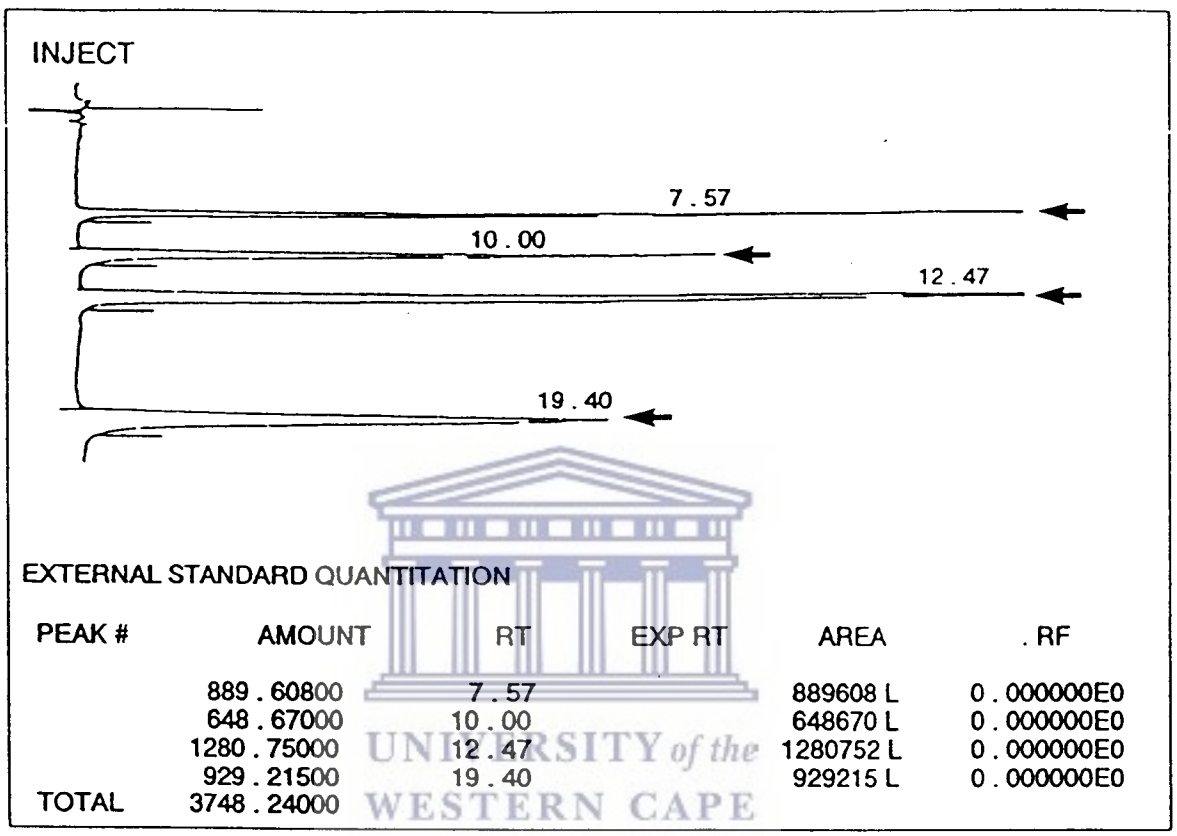
	AMP		ADP		Adenosine		ATP	
Run	Area	Rt	Area	Rt	Area	Rt	Area	Rt
1	900108	7,95	642962	10,32	1276100	13,57	922804	20,14
2	896841	7,87	637437	10,27	1275690	13,37	952313	20,00
3	894554	7,85	637513	10,25	1284004	13,25	931754	19,92
4	892994	7,82	638344	10,20	1282306	13,17	949622	19,90
5	895797	7,82	642347	10,20	1280290	13,15	942061	19,85
6	908797	7,75	657909	10,12	1281145	12,92	978742	19,67
7	891188	7,70	642194	10,10	1278384	12,80	949487	19,60
8	889608	7,57	648670	10,00	1280752	12,47	929215	19,40
9	898743	7,62	649241	10,00	1282579	12,72	951730	19,40
10	898830	7,70	649800	10,20	1282880	12,90	939210	19,50
Mean	896746	7,77	644642	10,17	1280413	13,03	944694	19,74
SD	5162	0,11	6297	0,10	2701	0,31	14958	0,25
RSD	0,58%	1,45%	0,98%	1,02%	0,21%	2,40%	1,58%	1,25%

TABLE 4: Changes in nucleotide concentration that occurs in semen during a 18 hour incubation.

Time in hours	AMP nmoles/l	ADP nmoles/l	Adenosine nmoles/l	ATP nmoles/l	unknown peak area	Total nucleo-tides peak areas	ATP/ ADP	AEC
0	57	40	538	3610	0	4,258	90	0,98
2	38	71	241	3785	0,348	4,336	53	0,98
4	26	64	-	3576	0,722	4,133	56	0,98
6	30	69	-	3625	1,116	4,573	53	0,98
12	53	476	-	4598	2,552	7,238	10	0,94
18	141	601	-	5747	6,345	12,402	10	0,93

TABLE 5: Changes in % motility and forward progression for incubated semen over 18 hours (Tygerberg criteria)

Time in hours	% motility	Forward progression
0	60	3
2	60	3^+
4	50	3
6	20	2^+
12	10	2
18	0	0



EXTERNAL STANDARD QUANTITATION

PEAK #	AMOUNT	RT	EXP RT	AREA	. RF
	889 . 60800	7 . 57		889608 L	0 . 000000E0
	648 . 67000	10 . 00		648670 L	0 . 000000E0
	1280 . 75000	12 . 47		1280752 L	0 . 000000E0
	929 . 21500	19 . 40		929215 L	0 . 000000E0
TOTAL	3748 . 24000				

FIGURE 10: Chromatogram of the standard separated nucleotides in water on a Waters model 440 liquid chromatograph at wavelength 254 nanometers. The retention times were AMP 7,75 minutes, ADP 10, adenosine 12,47 and ATP 19,4.

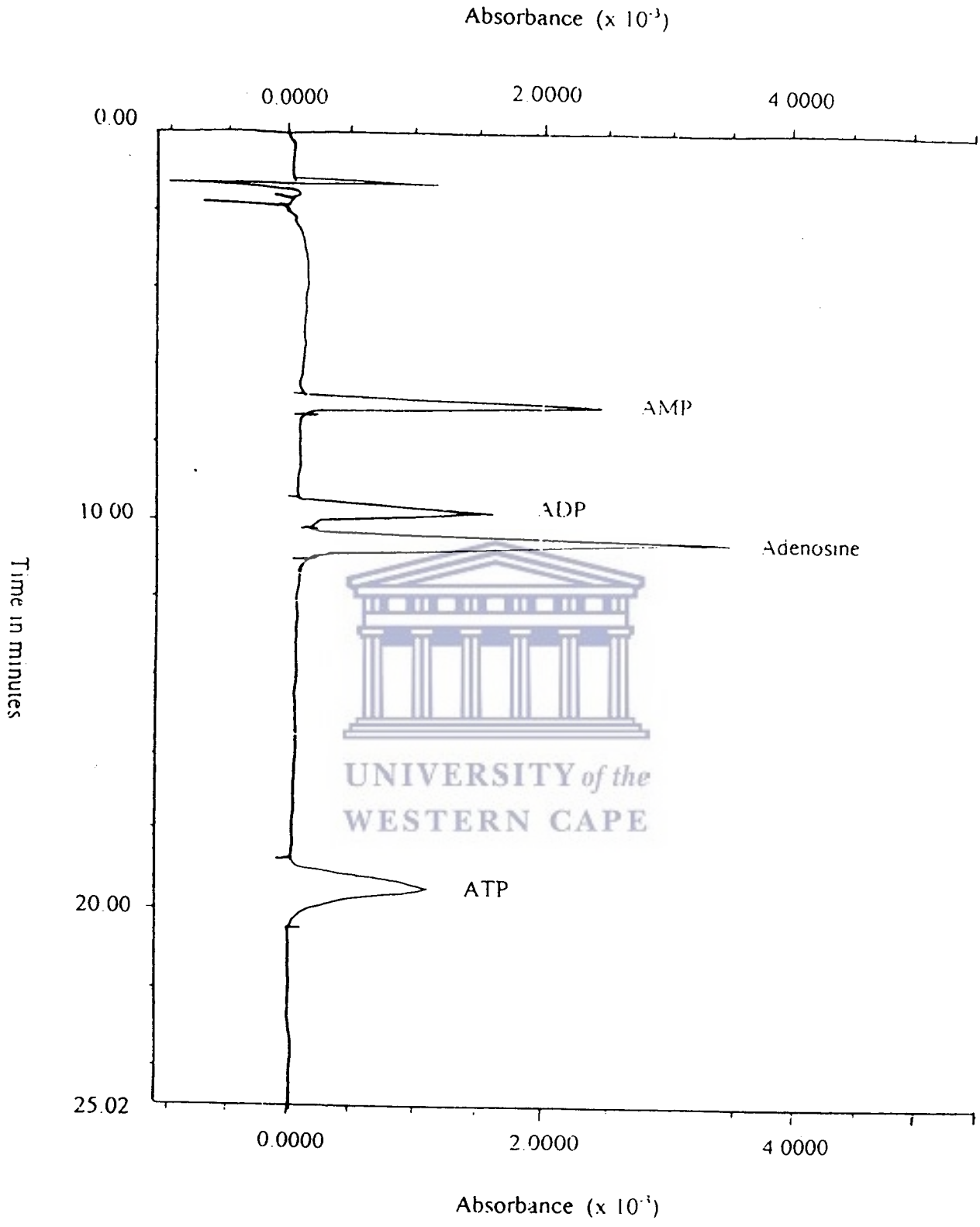


FIGURE 11: Chromatogram of standard separated nucleotides in water on the Beckman System Gold with diode array detection at 254 nonometers. The retention times were: AMP 7,03 minutes, ADP 9,78, adenosine 10,68 and ATP 19,44.

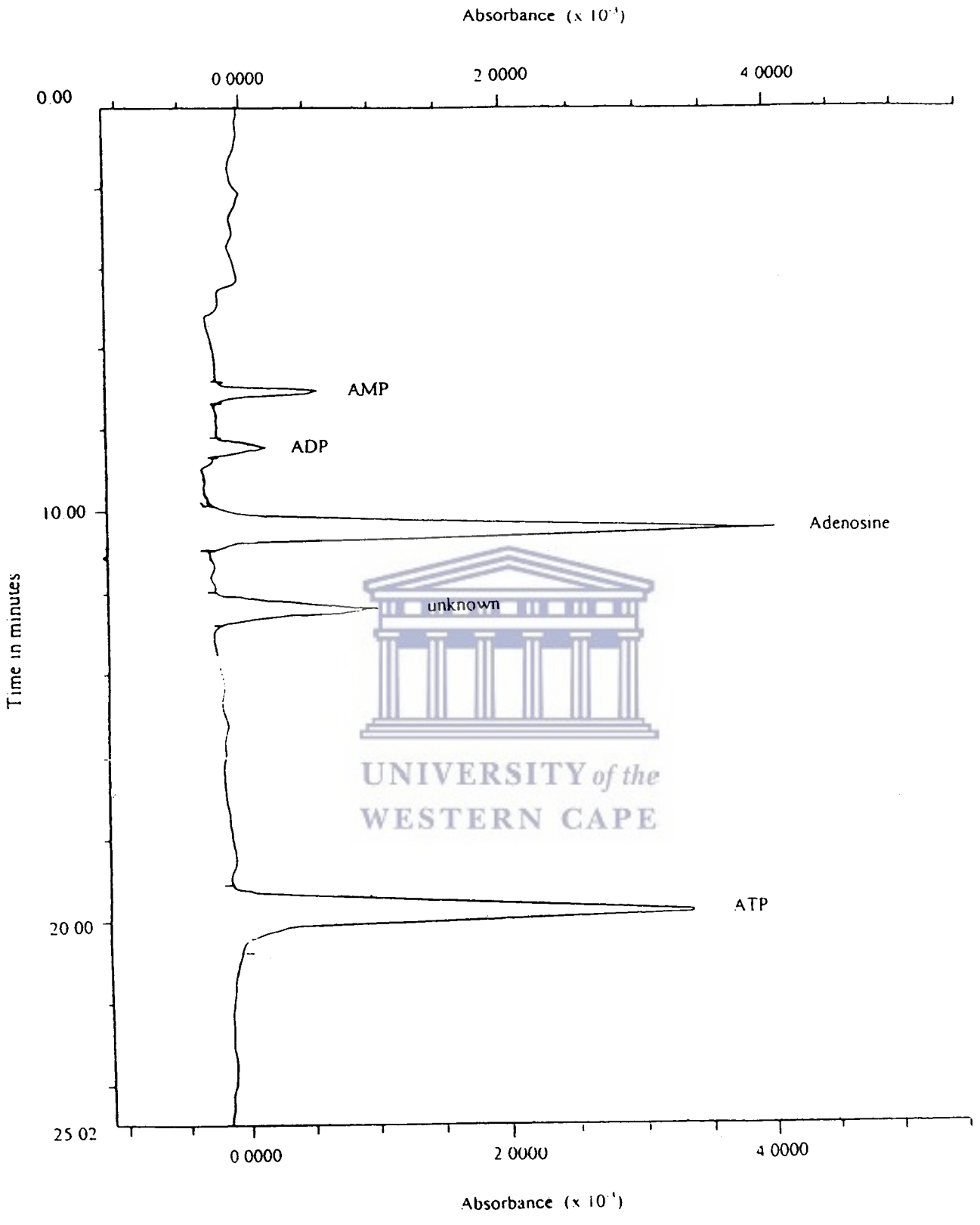


FIGURE 12: Chromatogram of nucleotides in semen (without sample clean-up) Retention times of AMP is 7,06 minutes; Adenosine 10,52; unknown 12,43 and ATP 19.98.

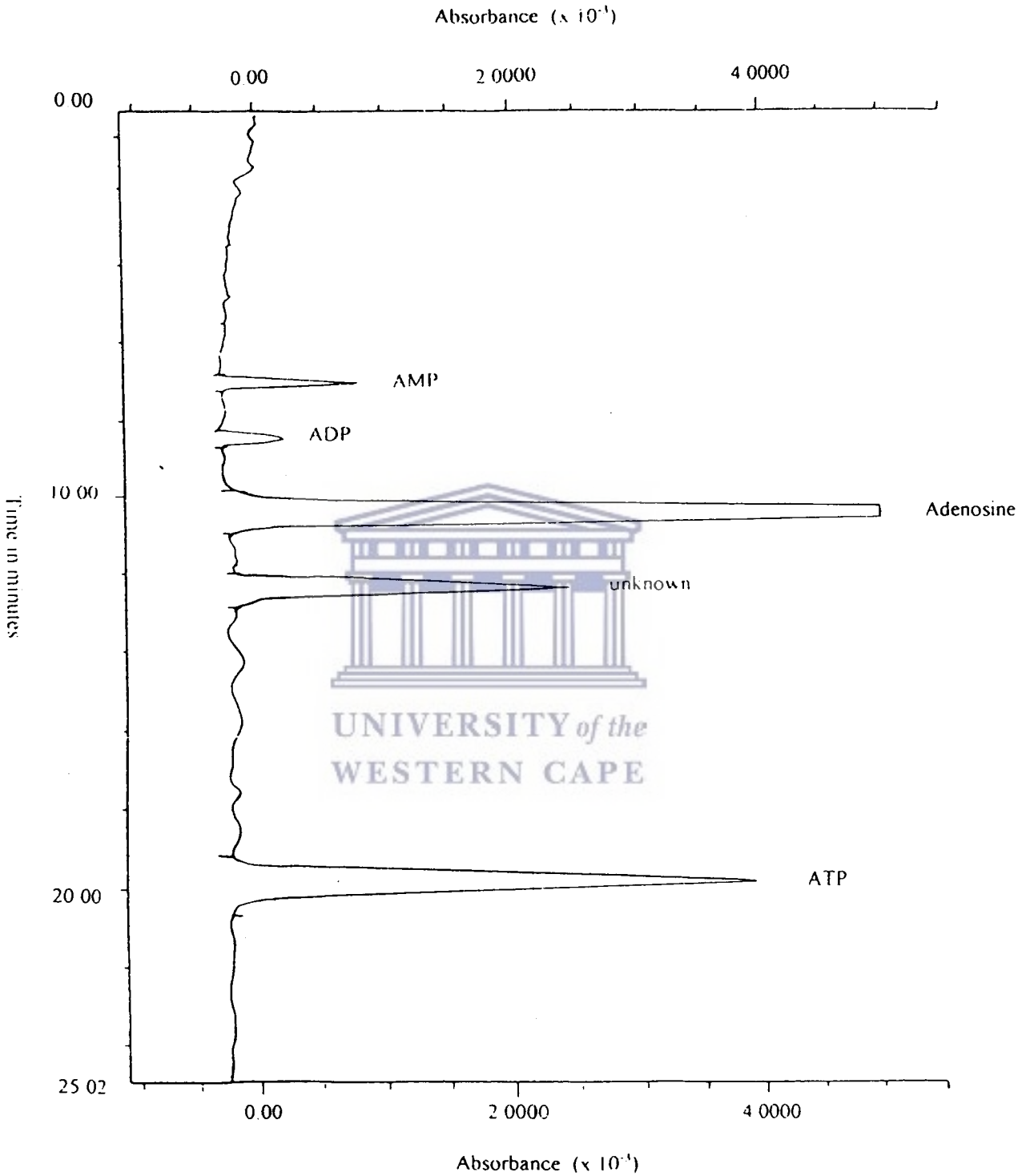


FIGURE 13: Chromatogram of nucleotides in spermatozoa. Retention times of AMP is 7,06 minutes; Adenosine 10,52; unknown 12,41 and ATP 19,95.

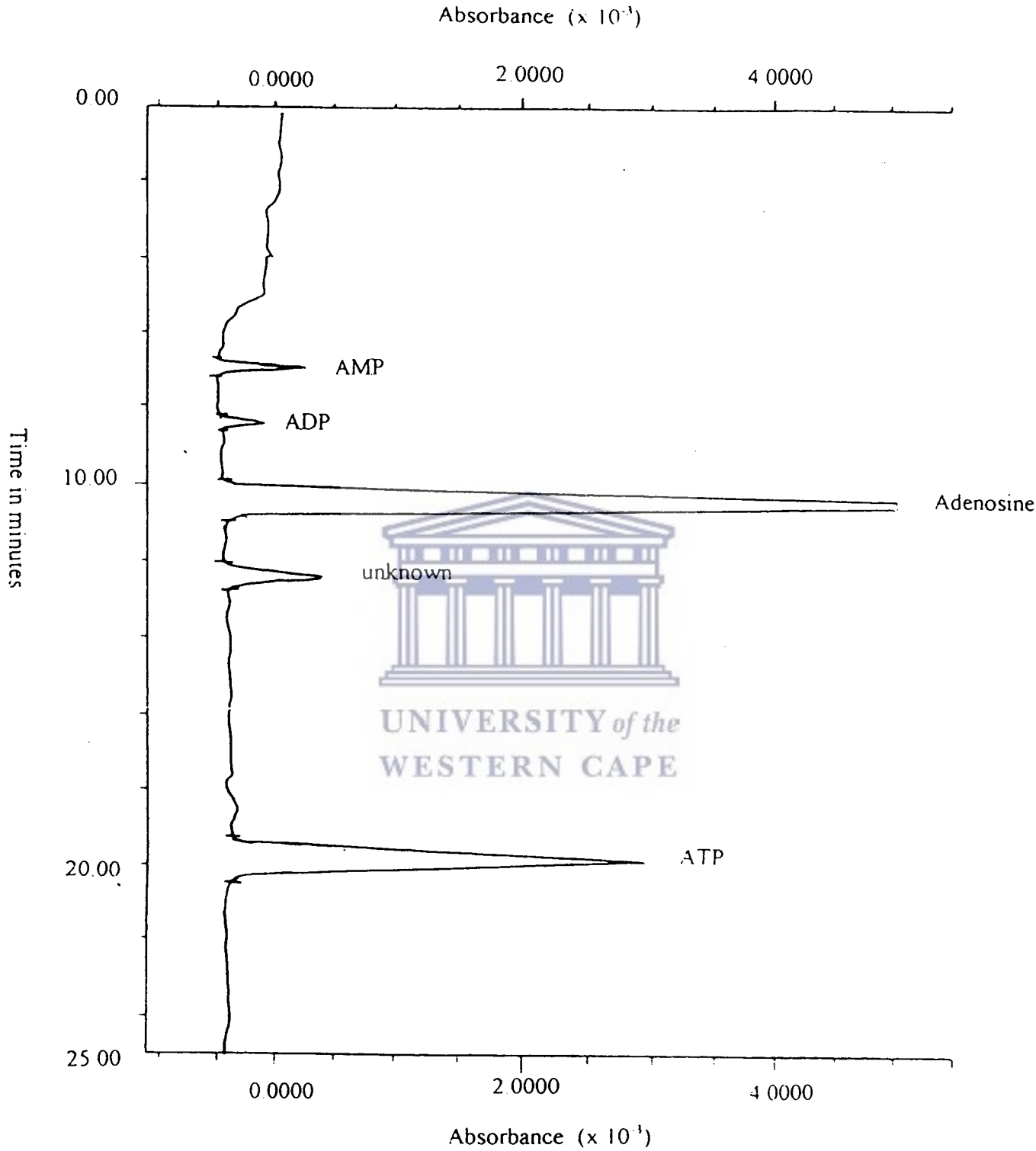


FIGURE 14: Chromatogram of nucleotides in seminal plasma with retention times of AMP at 7,04 minutes; Adenosine 10,46; unknown 12,37 and ATP at 19,77.

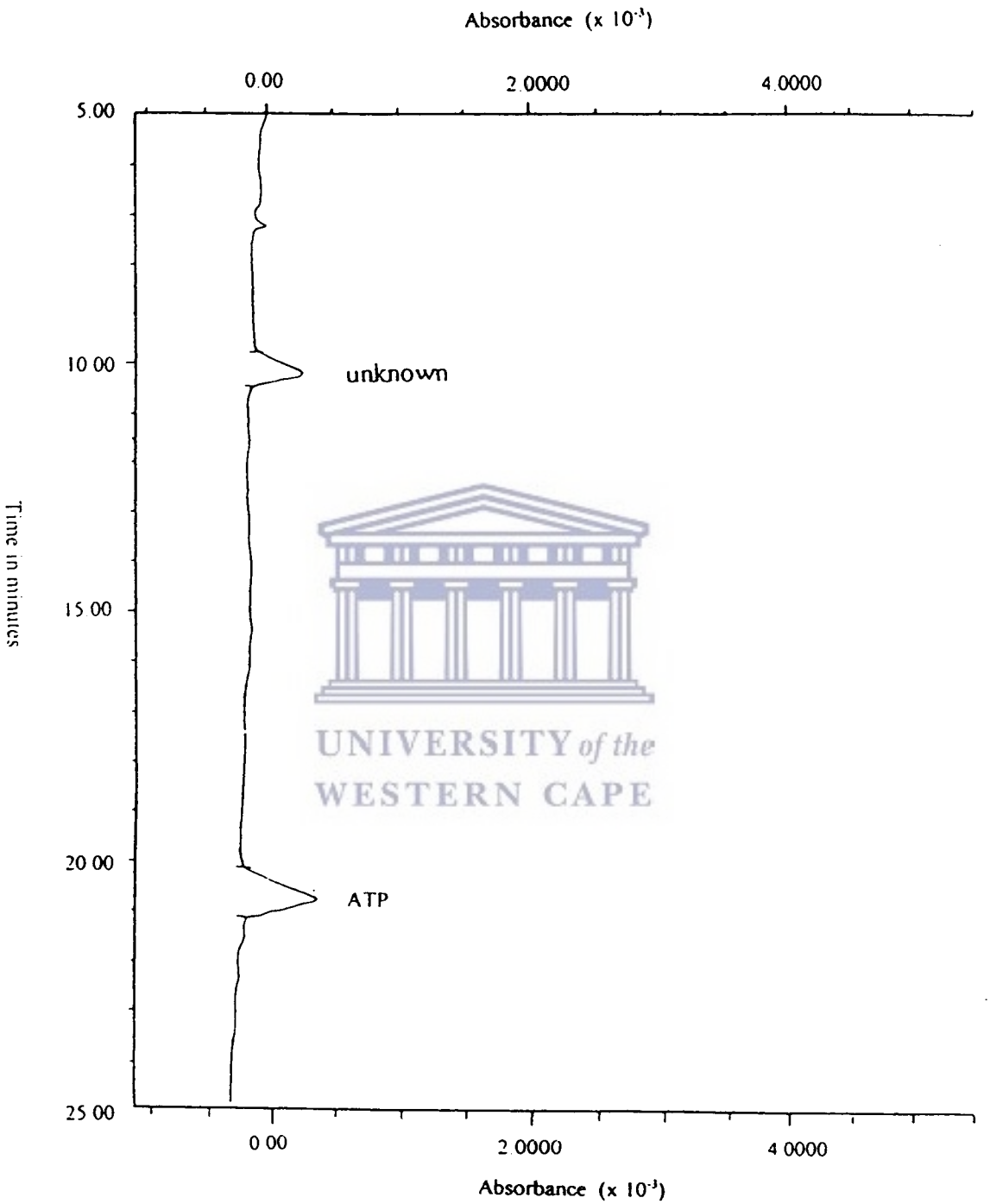


FIGURE 15: Chromatogram of ATP in a swim up specimen (100µl swim-up and 500µl extraction buffer). ATP gives a retention time of 20,08 minutes.

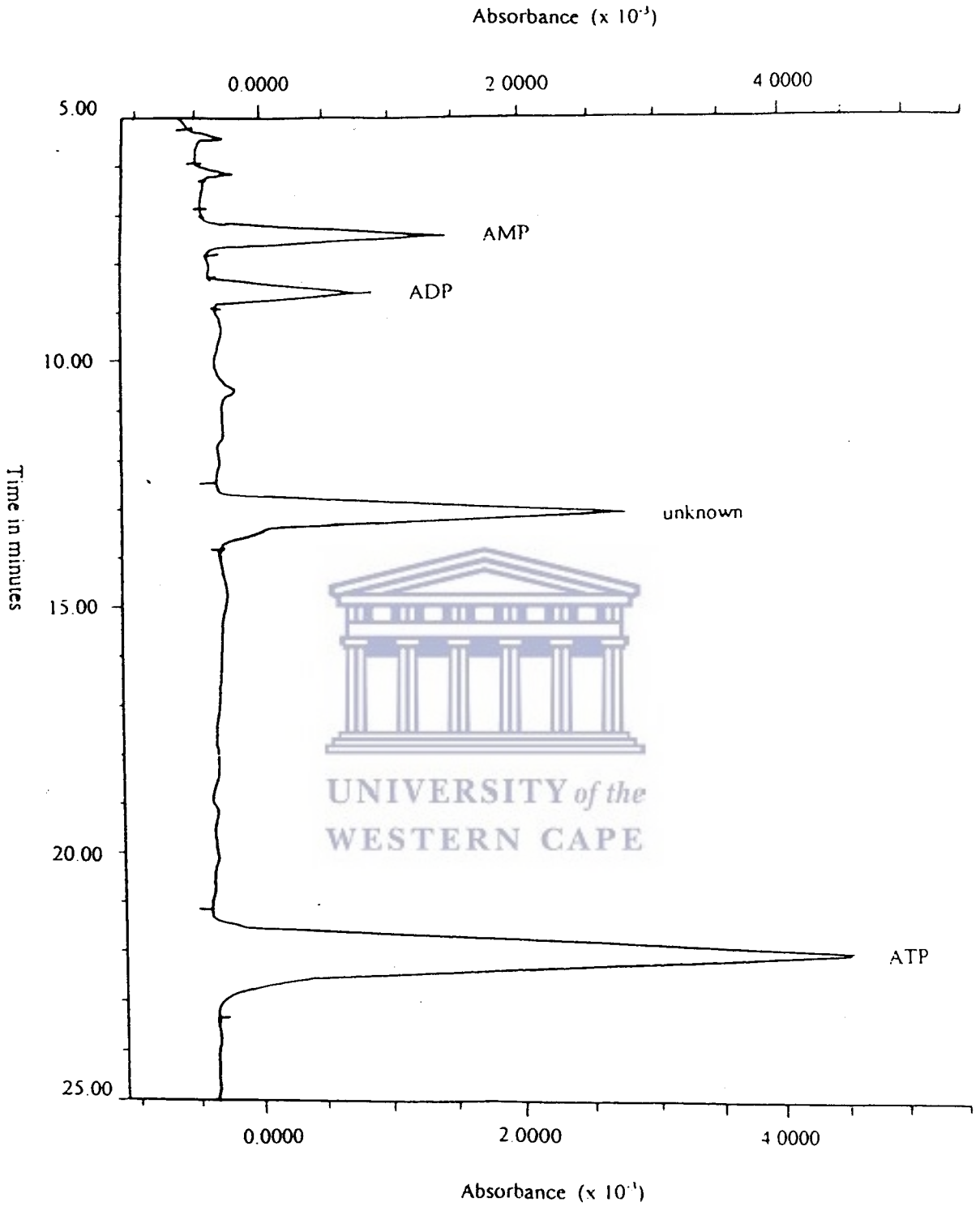


FIGURE 16: Chromatogram indicating the separation of an unknown at 13.08 minutes after 6 hours of incubated semen (absent after ejaculation).

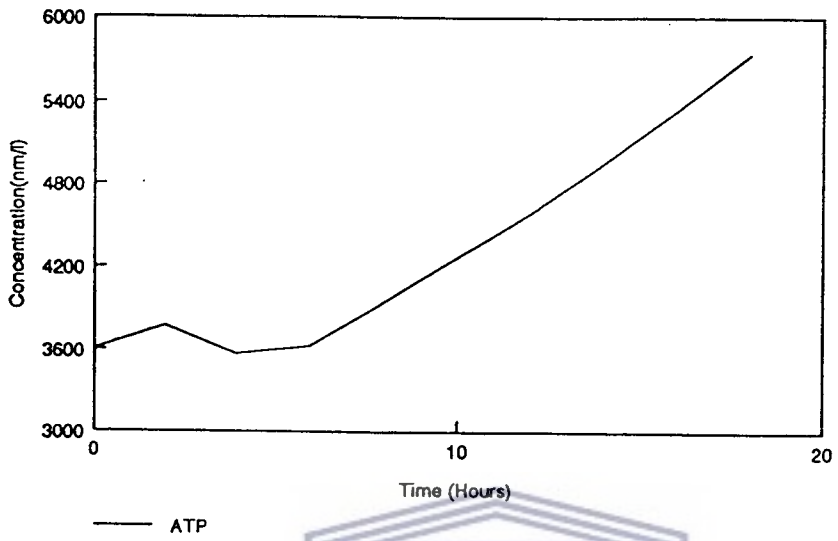


FIGURE 17A: Changes in the concentration of ATP during incubation.

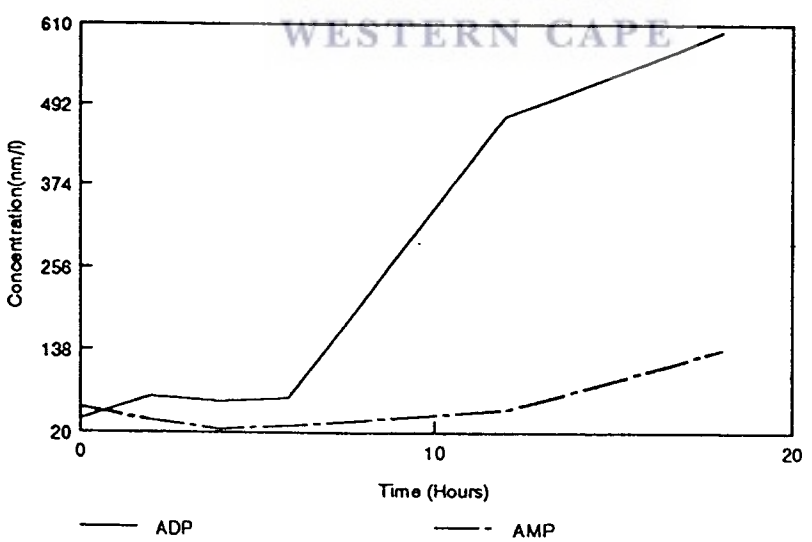


FIGURE 17B: Changes in the concentration of ADP and AMP during incubation.

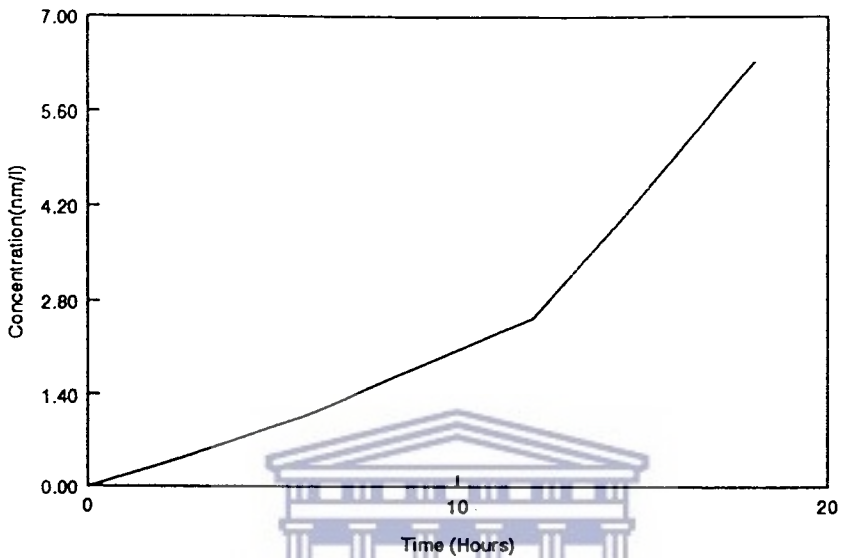


FIGURE 17C: Changes in the concentration of the unknown nucleotide during incubation.

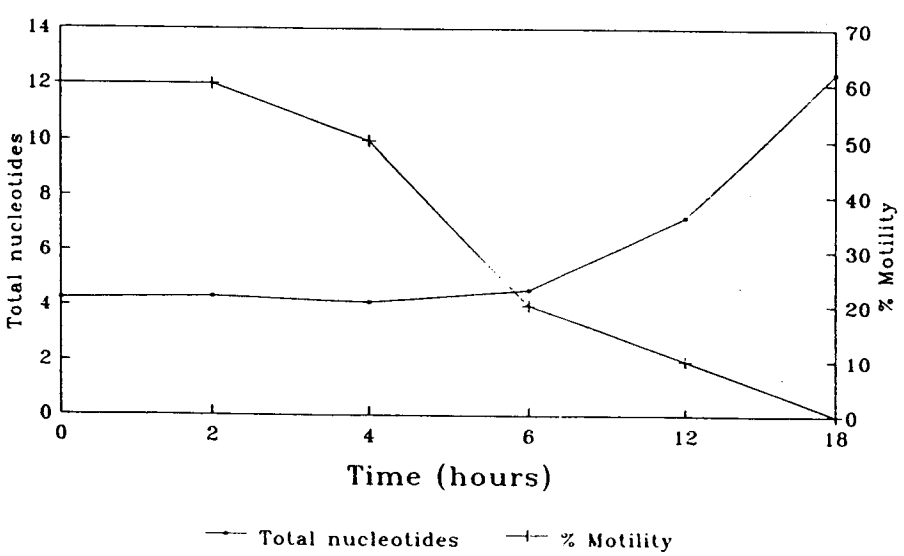
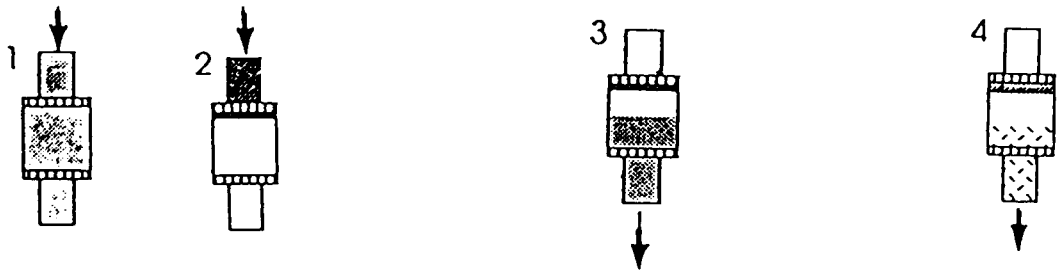


FIGURE 17D: Changes in the concentration of adenine nucleotides and motility during incubation (Tygerberg motility rating).



CONDITIONING

Steps 1 and 2
Methanol followed
by water

SAMPLE APPLICATION

Step 3
Apply sample and
wash with water

ELUTION

Step 4
Elute component
with methanol

FIGURE 18: Steps in using the C_{18} sep-pak for isolation of unknown.

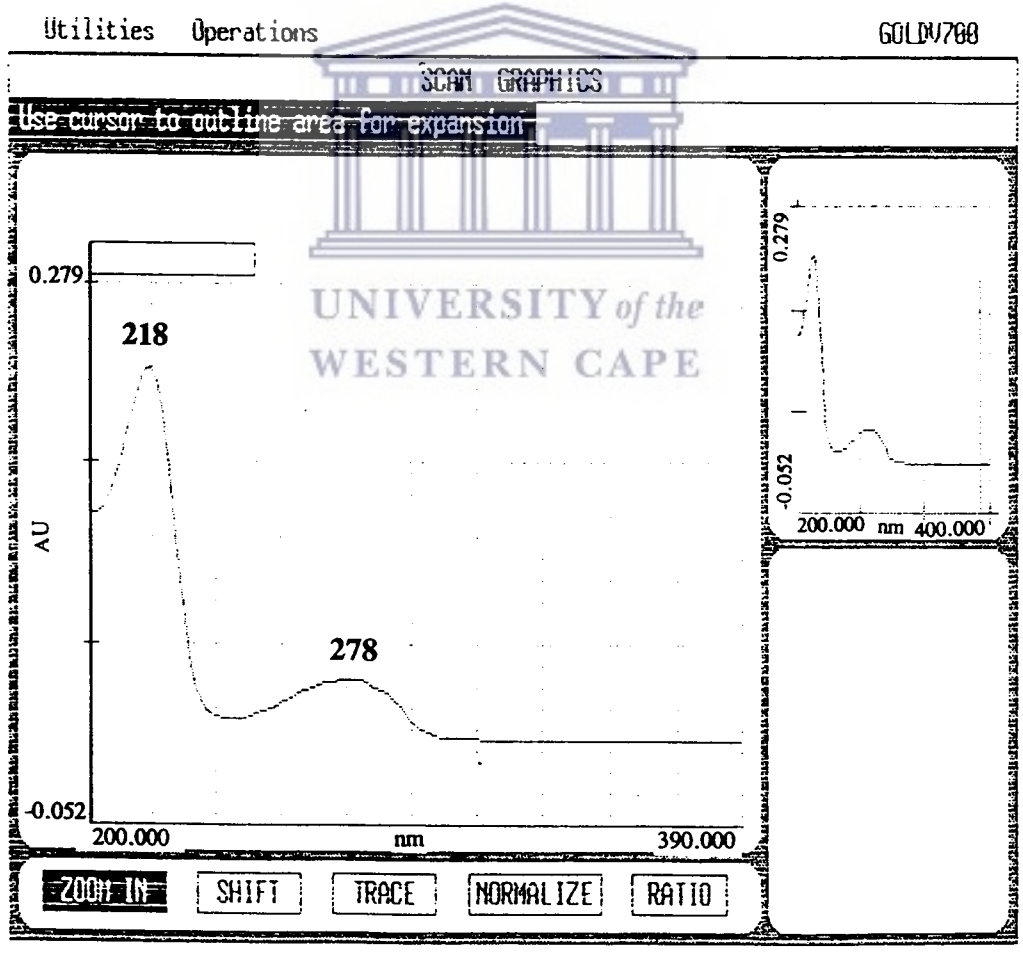


FIGURE 19: Diode array absorption spectra of 13 minute unknown in mobile phase (phosphate buffer pH4) indicating a maximum absorption of 218nm and 278nm.

CHAPTER IV

RESULTS

1 NUCLEOTIDE CONTENT

1.1 Nucleotide changes in incubated semen with changes in motility parameters

The results in table 4 describe the changes in nucleotide content in nmoles/l of incubated semen over an 18 hour period. The AMP, ADP and ATP content as well as the total nucleotide pool increased. The concentration of adenosine, however, decreased rapidly and disappeared after 2 hours of incubation. The ATP/ADP ratio, as an indicator of the phosphorylation potential, decreased from 90 at 0 hours to 10 after 12 hours with the highest change occurring from hour 6. The AEC showed no changes during the 18 hour incubation period. The synthesis of the unknown substance doubled between 12 and 18 hours (figure 13) and a small change occurred from 0 - 6 hours. In the last 6 hours the total nucleotide peak area increased threefold. Table 5 shows the visual motility scores for the incubated semen over the same incubation times as for the nucleotide determination. It is important to note that ATP concentration is high when motility ceased. Figures 17A-D depict a summary of the general trends of nucleotides during the incubation period of 18 hours as described above. It is interesting to note that the ATP and ADP content showed very little change during the first 6 hours of incubation (figures 17A and 17B). However, after 6 hours the ADP and ATP content increased markedly. AMP on the other hand initially showed a gradual decrease during the first 4 hours of incubation from 57 nmol/l at 0 hours to 26 nmol/l at 4 hours, and then it gradually increased from 26 nmol/l at 4 hours to 141 nmol/l after 18 hours (figure 17B).

1.2 Semen nucleotides and relationship to the morphological groups <4%, 5-14% and >14% (Tygerberg strict criteria)

Table 6 shows the calculated values for the nucleotides in nmoles/l and their ratios for the different morphological groups. These calculated values for the 5-9%, 10-14% and >14% strict criteria morphological groups were not significantly different from each other of any for the nucleotides ($p > 0,05$).

1.3 Adenine nucleotides in semen with the fertilization groups 0, <50% and >50%

Table 7 gives the results of the nucleotide content of semen versus fertilization outcome. The data show that the content of the various adenine nucleotides was not related to the fertilization outcome.

2 Enzymes

Table 8 illustrates the activity of three enzymes in the seminal plasma, namely Lactate dehydrogenase (LDH), Creatine kinase (CK) and Gamma glutamyl transferase (γGT), where the latter enzyme is involved in transporting amino acids over cell membranes. The table gives the number of patients in the different morphological groups of 0-4%, 5-14% and >14%. The activity of the enzymes were the same for the different groups ($p > 0,05$).

Figure 20 gives a typical electrophoretic separated and densitometric scanned pattern of the traditional 5 bands of LDH as well as the LDHX or LDH_4 in seminal plasma. Table 9 gives the iso-LDH in U/l of the different fractions (1-5) for the three morphological groups: 0-4%, 5-14% and >14%. Figure 21 is a

representation for the isoenzyme pattern in plasma of a healthy adult. Table 10 gives the summary statistics of the LDH isoenzyme ratios for the different morphological groups: <4%, 5-14% and >14%. Table 11A-D give the fatty acid fraction results for the <4% and >14%.

No significant differences could be established for isoenzymes of groups 1 and 2. However, groups 1 and 3 differed significantly for LDH3 (p = 0,03). For isoenzyme 4 (LDHX included) significant differences were also calculated (p = 0,03) between groups 1 and 3. Table 10 gives the iso-LDH isoenzyme ratios for the different morphological groups.

3 Total fatty acid sperm composition of the P(poor <4% morphology) and G (good >14% morphology) groups.

Tables 11 gives the phosphatidyl choline, phosphatidyl ethanolamine, triglyceride and cholesterol ester fractions in the p (<4%) and G (>14%) morphology groups. C16 and C18 are saturated fatty acids containing no double bonds while C18:1, C18:2, C20:3, C20:4 and C22:6 are examples of unsaturated fatty acids containing 1, 2, 3, 4 and 6 double bonds individually. All of these different fatty acids indicate no significant differences for the two groups.

TABLE 6: Nucleotides in nmoles/l and their ratios of the different morphological groups <5%, 5-9%, 10-14% and >14%.

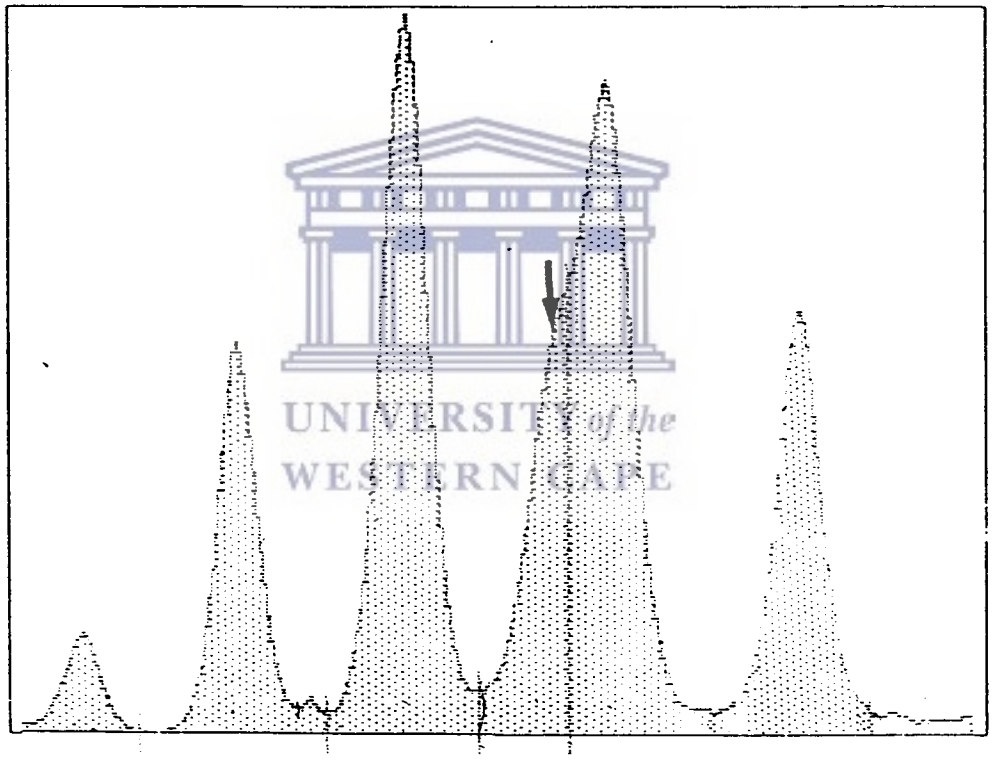
Nucleotides nmoles/l	% Normal morphology			
	<5% n = 6 $\bar{x}$ ± SEM	5 - 9% n = 14 $\bar{x}$ ± SEM	10 - 14% n = 13 $\bar{x}$ ± SEM	>14% n = 20 $\bar{x}$ ± SEM
ATP	2342,7 (453,73)	2516,45 (224,76)	2569,38 (231,09)	2519,31 (198,48)
ADP	644,79 (185,02)	256,63 (77,66)	242,56 (67,62)	465,64 (130,09)
AMP	815 (352)	691,69 (145,71)	649,15 (121,39)	679,5 (95,81)
ATP/ADP	3,63 (2,45)	9,81 (2,89)	10,59 (3,42)	5,41 (1,53)
ATP/AMP	2,87 (1,29)	3,64 (1,54)	3,96 (1,90)	3,71 (2,07)
ATP/ADP + AMP	1,60 (0,84)	2,65 (1,01)	2,88 (1,22)	2,20 (0,88)
AEC = (ATP + ½ ADP) (AMP + ADP + AMP	0,70 (0,04)	0,76 (0,01)	0,78 (0,02)	0,75 (0,02)
Total nucleotides	3802,49 (990)	3527,14 (448,13)	3461,09 (420,01)	3664,45 (424,38)

TABLE 7: Semen adenine nucleotides for the fertilization rates: 0%, <50% and >50%.

Nucleotides nmole/l $\bar{x}$ ± SEM	% fertilization of ova		
	0% GIFT = 10 n = 10 $\bar{x}$ ± SEM	<50% GIFT 9 IVF 2 n = 11 $\bar{x}$ ± SEM	>50% GIFT 16 IVF 17 n = 33 $\bar{x}$ ± SEM
ATP	2722,34 (290,3)	2298,6 (269,8)	2491,06 (179,97)
ADP	357,28 (129,51)	274,48 (118,78)	376,28 (85,69)
AMP	826,05 (214,95)	433,15 (84,72)	801,13 (104,77)
ATP/ADP	7,61 (2,24)	8,37 (2,27)	9,02 (2,10)
ATP/AMP	3,3 (1,35)	5,3 (3,18)	3,11 (1,72)
ATP/ADP + AMP	2,30 (0,84)	3,25 (1,33)	2,31 (0,27)
AEC = (ATP + ½ADP) (AMP + ADP + AMP)	0,74 (0,03)	0,81 (0,02)	0,94 (0,02)
Total nucleotides	3905,67 (390,16)	2964,01 (330,81)	3578,51 (245,89)

TABLE 8: Enzyme levels of total LDH, γGT and total CK levels of the morphological groups <4%, 5-14% and >14%.

Patients	Total LDH U/l $\bar{x} \pm$ SEM	γGT U/l $\bar{x} \pm$ SEM	Total CK U/l $\bar{x} \pm$ SEM
Group I 0-4% n = 12	2305,65 (293,10)	6418,82 (117,97)	537,916 (93,79)
Group II 5-14% n = 18	1692,13 (167,45)	4944,35 (394,87)	559,79 (61,52)
Group III >14% n = 11	2990 (434,147)	5849,45 (895,07)	593,64 (93,4)



Fraction	Rel%	IU/L
1	3.1	65
2	13.3	278
3	28.1	587
4	12.5	261
5	25.8	539
6	17.1	357

Total IU/L 2090 LDH1/LDH2:0.23

FIGURE 20: This figure gives a typical seminal plasma isoenzyme LDH separation. Note (arrow) the additional C4 (LDHx) fraction which is seminal plasma specific.

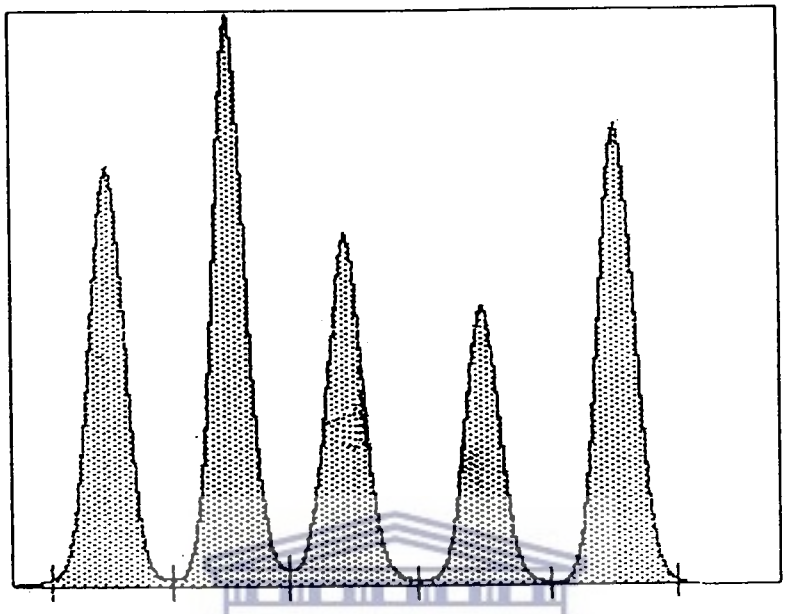


FIGURE 21: This figure depicts a typical normal serum LDH separation indicating the five isoenzymes.

TABLE 9: LDH isoenzyme 1, 2, 3 4, 5 of the morphological groups <4%, 5-14% and >14%. (LDHx has been calculated with the 4 isoenzyme).

	Iso-LDH				
PATIENTS	1 $\bar{x} \pm$ SEM	2 $\bar{x} \pm$ SEM	3 $\bar{x} \pm$ SEM	4 $\bar{x} \pm$ SEM	5 $\bar{x} \pm$ SEM
Group I 0-4% n = 12	264,8 (58,7)	545,67 ((87,2)	617,08 (154,63)	811 (106,56)	300,67 (82,16)
Group II 5-14% n = 17	195,11 (29,47)	458,82 (43,45)	605,59 (54,32)	545,06 (115,64)	143,88 (19,42)
Group III >14% n = 11	240,64 (58,6)	639,09 (99,4)	1129,18 (46,9)	750,46 (218,3)	218,36 (62,7)

TABLE 10: LDH isoenzyme ratios of the morphological groups <4%, 5-14% and >14%. (LDHX has been calculated with the 4 isoenzymes).

ISO-LDH ratios										
Patients	1:2 $\bar{x}$ ±SEM	1:3 $\bar{x}$ ±SEM	1:4 $\bar{x}$ ±SEM	1:5 $\bar{x}$ ±SEM	2:3 $\bar{x}$ ±SEM	2:4 $\bar{x}$ ±SEM	2:5 $\bar{x}$ ±SEM	3:4 $\bar{x}$ ±SEM	3:5 $\bar{x}$ ±SEM	4:5 $\bar{x}$ ±SEM
Group I <14% n = 12	0,458 (0,45)	0,515 (0,11)	0,375 (0,08)	1,98 (0,68)	1,045 (0,16)	0,744 (0,11)	3,76 (1,06)	0,82 (0,61)	4,15 (0,74)	4,14 (0,15)
Group II 5-14% n = 17	0,455 (0,07)	0,429 (0,13)	0,447 (0,08)	1,722 (0,32)	0,813 (0,09)	1,1 (0,17)	4,1 (0,61)	1,4 (0,20)	5,19 (0,70)	4,2 (0,47)
Group III >14% n = 11	0,35 (0,05)	0,22 (0,06)	0,45 (0,13)	2,63 (1,14)	0,591 (0,08)	1,1 (0,168)	4,1 (0,61)	2,17 (0,42)	5,19 (0,70)	5,52 (1,05)

TABLE 11: % Total fatty acids of the phosphatidal choline, phosphatidyletanolamine, triglyceride and cholesterol ester fractions for the P and G pattern morphological groups (n=5).

A) Phosphatidyl choline % fatty acid fractions

	C16	C18	C18:1	C18:2	C20:3	C20:4	C22:6
<4% P-pattern $\bar{x}$ ± SEM	26,73 (1,04)	22,52 (1,54)	14,71 (2,25)	7,10 (0,8)	8,42 (0,85)	4,38 (0,8)	15,62 (1,6)
>14% G-pattern $\bar{x}$ ± SEM	27,22 (1,33)	23,73 (0,45)	13,76 (1,54)	6,78 (0,37)	7,51 (0,53)	3,51 (0,31)	16,93 (1,99)

B) Phosphatidyletanolamine (PEA) % fatty acid fractions

	C16	C18	C18:1	C18:2	C20:3	C20:4	C22:6
<4% P-pattern $\bar{x}$ ± SEM	25,75 (1,2)	11,55 (0,53)	13,93 (3,21)	4,01 (2,92)	3,79 (0,46)	7,44 (0,48)	32,93 (3,91)
>14% G-pattern $\bar{x}$ ± SEM	22,56 (1,05)	11,85 (0,54)	12,19 (1,41)	3,98 (0,46)	3,58 (0,54)	7,73 (0,90)	37,38 (2,77)

C) Triglyceride (TAG) % fatty acid fractions

	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:6
<4% P-pattern $\bar{x}$ ± SEM	34,40 (1,81)	2,48 (0,72)	8,78 (0,30)	17,76 (0,60)	15,82 (0,93)	3,69 (0,82)	2,93 (0,66)	13,18 (1,08)
>14% G-pattern $\bar{x}$ ± SEM	38,41 (1,67)	1,92 (0,34)	8,37 (0,35)	17,87 (0,66)	10,80 (0,85)	3,34 (0,82)	1,64 (0,48)	16,82 (2,69)

D) Cholesterol ester (CE) % fatty acid fractions

	C16:0	C16:1	C18:0	C18:1	C18:2	C20:3	C20:4	C22:6
<4% P-pattern $\bar{x} \pm$ SEM	70,69 (4,43)	0,53 (0,22)	4,61 (1,35)	10,9 (2,05)	10,0 (0,49)	0,83 (-)	1,49 (0,52)	0,95 (0,58)
>14% G-pattern $\bar{x} \pm$ SEM	65,55 (3,44)	0,91 (0,58)	5,39 (0,16)	13,79 (1,32)	8,62 (0,88)	2,02 (0,36)	2,27 (0,46)	1,47 (0,54)

CHAPTER V

DISCUSSION

Development and verification of HPLC-method for determining the nucleotides in semen and sperm.

1 NUCLEOTIDES

Previous methods for ATP-assessment in human semen and spermatozoa were based on bioluminescence techniques [Comhaire *et al.*, 1992; du Toit, 1988]. This technique is based on the principle that ATP, when reacting with luciferin-luciferase, results in the production of light, the intensity of which is proportional to the concentration of ATP. This technique, although very sensitive, is problematic as far as the following is concerned: 1) cannot measure ATP, ADP and AMP simultaneously, 2) speed of analysis is slow, 3) needs an extraction procedure, 4) is tedious. An advantage of this technology is that it can be fully automated to process a large quantity of specimens even in the absence of the analyst, with results calculated in absolute units.

The above analytical problems are overcome when using HPLC as all the adenine nucleotides can be determined with good precision, accuracy, sensitivity and reproducibility in one run of 20 minutes. The λmax (maximum absorption) in water is 258 nm, 259, 260 and 259 for AMP, ADP, adenosine and ATP respectively. Various workers quoted in the literature use different wavelengths for detection of nucleotides. Viarengo *et al.* [1986] used 260 nm, Vigue *et al.* [1992] 256 nm, Stocchi *et al.* [1985] and Victor *et al.* [1987] both used wavelengths of 254 nm. In this study the wavelength was set at 254 nm with a mobile phase consisting of an isocratic ion-pair containing 52 mM KH_2PO_4, 0,08% tetrabutylammonium hydrogen sulphate and 0,8% methanol at pH4. Previous

methods for HPLC analysis for high energy phosphates (HEP) used either of the above two assays or a gradient technique to determine both creatine compounds and adenine nucleotides. Sellevold *et al.* [1986] describe a method where detection for both creatine and adenine measurements were done at 206 nm in a mobile phase consisting of 215 nanomoles KH_2PO_4, tetrabutylammonium hydrogen sulphate (2,3 mM) and acetonitrile 3,5%, at pH 6,25.

The C18 column tolerated the injections of standards well as seen in the results of table 3. In this table the relative standard deviations (RSD) gives an indication of the reproducibility of the test results. Slight variations occurred in the retention times (Rt) with ADP as the lowest (1,02%) and AMP the highest (1.45%). The area of the nucleotides was consistent with adenosine being the best (0.21%) and ATP the worst at 1,58%.

In both chromatographic systems (Waters and Beckman) good separations of nucleotides were achieved in water (refer to figures 10 and 11). In the Beckman system (figure 11) the ADP and adenosine separated close to each other, but could be integrated. The time for complete separation of the four compounds was 20 minutes. In the work of Vigue *et al.* [1992] the separation almost took twice the time and poor resolution of peaks occurred. In their HPLC system the AMP eluted with other substances which makes its quantification difficult. They used acetonitrile instead of methanol in their mobile phase.

Figures 12 to 15 are the chromatograms of semen, spermatozoa, seminal plasma and swim-up specimens respectively. Apart from the swim-up, good separations occurred with no interferences where the nucleotides eluted.

No sample cleanup were done and the guard C18 column tolerated the injections well. However, a certain amount of absorption did occur within the first 5

minutes and the integrator was set not to record these. The first peak of interest, AMP, eluted at 7 minutes.

The discussion will further focus on the results of the seminal plasma, semen, sperm and the different methodology:

In the seminal plasma the ATP content appears to be higher than in semen of the same sample volume as seen in figure 14. This is in line with the work of Singer *et al.* [1983] who reported that the ATP content of seminal plasma could be as high as 35%. It appears that fairly large quantities of ATP is transported from the mitochondria to the cytoplasma and even outside the sperm outer acrosomal membrane. The literature is not specific on the physiological role of this high ATP concentrations in the seminal plasma. Kupitz [1993] suggested a role for extracellular ATP as having receptors on oocytes and that ATP-induced increase in sodium permeability mediates the initial sperm egg signal in the fertilization process. As the sperm most probably swim out of the seminal plasma, the presence of this ATP is paradoxal. It may be that the ATP is derived from sperm or sperm-seminal plasma that reached the fallopian tubes by uterine contractions or cilia movement or that the oviductal fluid contains high concentrations of ATP. This stresses the importance of the role of adenine nucleotides in other functions than energy metabolism [Proceedings on extracellular ATP: Annals of New York Academy of Sciences, 603, 1990].

It is common knowledge that polymorphonuclear leucocytes that derive from infections of the prostate or seminal vesicals could also contribute to the seminal nucleotide pool.

Semen was used in this study (tables 4, 6 and 7) for quantifications of the nucleotide pool in samples of differential morphology. According to the

literature [Comhaire *et al.*, 1985; Ivine and Aitken, 1985; Ponsette *et al.*, 1986; Calamera *et al.*, 1986; Mieusset, *et al.*, 1989; Chan *et al.*, 1990] measurement of only ATP was done by utilizing bioluminescence technology. High pressure liquid chromatography (HPLC) used in this study made the quantification of the nucleotide pool easier in the sense that no analytical procedures were necessary for the measurements apart from extraction. ATP, ADP and AMP were all determined in one single run under the same experimental (automated) conditions (figures 12 - 14). In the bioluminescence techniques the AMP and ADP had first to be enzyme converted before analysis. Pyruvate kinase were used for conversion of ADP to ATP and pyruvate kinase and adenylate kinase for the conversion of ADP and ATP [Du Toit, 1994].

The results in table 4 and figure 17D indicate that ATP concentrations increase while motility decreases which indicate a total synthesis process and clearly show that when ATP content is high, motility has stopped. From this preliminary observation it is concluded that ATP, ADP and total nucleotide levels in semen are inversely related to motility. This is in agreement with the work of Du Toit *et al.* [1993] who indicated that lower ATP levels are associated with good motility. The ATP/ADP ratio (table 4) seems to be a more valuable parameter in motility assessment. Here a higher ratio of ATP/ADP is related to good motility. This observation needs further investigation and could become an important method of assessing the motility score than the current subjective microscopic evaluation.

The calculated Adenyl Energy Charge (AEC) in the incubated semen experiment (table 4) does not seem to be a valuable parameter in evaluating changes in nucleotide content. This index hardly changed over the 18 hour incubation period (0,98 - 0,93). Further it was observed that an unknown product that

related to the adenine nucleotides (absorb at same wavelength) is formed versus time (absent at time 0 - after liquification). This compound was fairly high in concentration where sperm were immotile but with a low ATP/ADP ratio (table 4). Although this product was partially isolated (figures 18 and 19) it could not be fully identified. This substance would not be measured with the firefly luciferase methodology and could even interfere with measurements of nucleotides (not specific for the luceferase enzyme).

Sperm which were washed free from seminal plasma, were used by Levin *et al.*, 1981; Schlegel *et al.*, 1985; Halangk *et al.*, 1985; Du Toit, 1988, 1994 and Du Toit *et al.*, 1993. A drawback of the current study was that the adenine nucleotides were not measured in sperm only. From the literature it is not clear whether the contradictions discussed under the Introduction in the published data on the role of ATP in motility and fertilization could be contributed to whether semen, seminal plasma or sperm specimens were used.

The standard measured technique for ATP analysis used in the literature is described by the WHO laboratory manual for the examination of human semen and semen cervical mucus interaction [1986, page 52-54]. This procedure followed in the literature is based on chemoluminescence and is one of the safest, simplest and most sensitive methods according to Acosta *et al.* [1987]. In this technology the creation of an excited state is through an enzyme-catalysed (chemical) reaction where the decay from the excited state to the ground state is accompanied by emission of light which is termperature-independent, long lasting and measurable by a special photometer (ATP luminometer - for reaction of firefly luceferase see reaction under appendix, par. 7.8.). The disadvantage of this technique is mainly that it cannot be automated. Further, that other

nucleotides (ADP and AMP) can only be measured indirectly which could be problematic with enzymes used and that differences in batches of enzymes may occur. The cost per assay is also fairly expensive.

High pressure liquid chromatography (HPLC) has the advantage of being a fully automated procedure. It measures all the adenine nucleotides simultaneously including adenosine which make the calculation of the ATP/ADP ratio easier. The cost per assay is less than the cheminuminesce procedure and a further advantage of HPLC technology is also that it can be used for other quantifications and separation applications. The bioluminesce technique do not have the technology to measure the phosphagen system (creatine/creatine phosphate - see Introduction) and adenine nucleotides simultaneously. Although not investigated in this study, HPLC has the capacity to measure both high energy phosphate systems at two wavelengths in the same run under the same experimental conditions.

Swim-up sperm samples (see 2.1 specimen preparation under Materials and Methods, page 31) are standard procedures in *in vitro* technology and seem to compensate for sperm with poor morphology and motility. This type of specimen was used by Akerlöf *et al.* [1987] and Vigue *et al.* [1992] for ATP measurements. In this study the same experimental high pressure liquid chromatography (HPLC) conditions as for the analysis of seminal plasma, semen and sperm were used. The swim-up results (figure 15) indicate integrated peaks for the unknown and ATP but not for Adenosine, AMP and ADP. This sensitivity problem was disappointing in view of the fact that most *in vitro* work for assisted reproduction is done on the swim-up fraction. However, this could be overcome by manipulating the technique by: 1) Concentrating larger volumes

of sample on a Sep-pack C18® cartridge whereby the nucleotides can be removed by methanol extraction, 2) coupling the primary amino group to fluorezenic reagents [Imai *et al.*, 1984], 3) injection of larger volumes onto the column. It was not the scope of this study to develop a HPLC technique for swim-up adenine nucleotide analysis.

The unknown substance is not adenosine because the retention time and the absorption spectra are different, as seen in figure 18. This substance is absent after liquification (40 minutes) but increases over time and has not previously been described in the literature.

1.1 Nucleotide levels in incubated semen.

Table 4 shows average values of nucleotides in semen incubated over a time period of 18 hours. From the table it is clear that nucleotide synthesis occurred which is reflected in the increase in the total nucleotide pool. The fact that the ATP content remained constant during the first 6 hours of incubation implies that the rate of synthesis was balanced by the rate of ATP degradation during sperm movement. The increase in the concentration of all the other nucleotides after 6 hours of incubation is however difficult to explain. This observation demonstrates that ATP can be synthesized from other sources which was until recently not known in the literature. This indicates that apart from the more common pathway of resynthesis of ATP from AMP by the adenylate kinases it is also possible that ATP can be synthesized by a *de novo* path from: 1) adenylosuccinate, 2) S-adenosylhomocysteine and 3) polyamines [Goh *et al.*, 1985; Jacken *et al.* 1988; Simmonds *et al.*, 1989; Van den Berghe *et al.*, 1992]. Apart from this *de novo* synthesis as discussed above, ATP is also continuously involved in futile cycling so that the interpretation of ATP levels is far more complex than previously discussed in the literature. ATP levels were high when

sperm were completely immotile which suggests that ATP levels inversely relate to motility. It is also possible that most sperm were dead after 12 hours of incubation because the motility was low at 6 and 12 hours and no motility was detectable at 18 hours (Immotile sperm may not be dead - they may have good functional mitochondria). However, this may not apply to ATP levels in sperm only and may be one of the reasons for the conflicting results in the literature concerning the value of ATP levels as markers for fertility status [Levin *et al.*, 1981; Singer *et al.*, 1983; Schlegel *et al.*, 1985; Ponsette *et al.*, 1986; Gottlieb *et al.*, 1987; Comhaire *et al.*, 1987 and Chan *et al.*, 1989].

Maritz *et al.* [1992] indicated that the ATP/ADP ratio control phosphofructokinase activity and thus glycolysis in lung tissue. This ratio is a more sensitive indicator of control of glycolysis than the AEC. The AEC (adenylate energy charge) which takes care of all the nucleotides is not a good indicator of the energy status of whole semen but is a good indicator of cell viability and whether cells are involved in synthesis reactions. The value of this index hardly changed over time (0 = 0,98; after 18 hours = 0,93) [Atkinson *et al.*, 1967; Viarengo *et al.*, 1986]. The fact that the AEC remained virtually constant for the entire 18 hour incubation period is not necessarily an indication of high sperm viability because synthesis in seminal plasma may proceed even after sperm death. Preliminary results in this study indicate that the ATP/ADP ratio is fairly sensitive to changes in motility (see table 4 and figure 17D) and may therefore become more important as a measureable motility index in future studies. Du Toit [1993] did not use the ATP/ADP as a motility parameter, but measured only ATP levels. He reports that low levels of ATP correlate with good motility and forward progression which is contradictive to the results of Mahmoud *et al.* [1994] reporting the opposite.

It is reported in the literature that the formation of S-adenosylhomocysteine from protein carboxylmethylation or other methyl transfer reactions may inhibit sperm motility [Goh *et al.*,1985].

The presence of an unknown substance after a period of incubation is the first record of a substance other than the conventional adenine nucleotides. It appears that the presence of this substance is inversely related to motility. If the unknown substance (13,83 minutes on semen chromatograph, figure 16), which could be methylthioadenosine can be fully identified by electron spray mass spectrometry, it could be a breakthrough in controlling motility and thus would provide a basis for contraception work.

1.2 Semen nucleotides and morphological groups

Kruger *et al.* [1986] describe a spermatozoon with normal morphology as a head with a smooth oval configuration with a well-defined acrosome involving about 40% to 70% of the sperm head, as well as the absence of a neck, midpiece or tail defects; no evidence of cytoplasmic droplet of more than half the size of the sperm head. Kruger *et al.* [1986] also describe the percentage normal sperm morphologic features in human *in vitro* fertilization (IVF) program as well as the predictive value of abnormal morphology [Kruger *et al.*, 1988]. They referred to the patients with $<4\%$ normal forms as the P pattern, poor prognosis, and those with $>14\%$ as the G pattern, good prognosis. While these criteria are not accepted by WHO it is used by a few laboratories and referred to as the Tygerberg strict criteria.

In the literature no work is reported on the relationship between different morphological groups and the adenine nucleotide content of the various groups. The only group reporting on the ATP/ADP ratios was Grishchenko *et al.* [1989]

who found a significant decrease in the ratio of oligozoospermic versus normozoospermic males. According to them a decrease in ATP levels and an increase in AMP levels was found in asthenozoospermic patients when compared to fertile patients who also had a high percentage normal sperm morphology according to WHO criteria.

1.3 Semen nucleotides and fertilization of groups where 0, <50% and >50% of ova were fertilized.

Table 7 shows the student t test statistics (not significant) for the effect of the nucleotide concentration and ratios on fertilization of aspirated ova where no fertilization, less than 50% and more than 50% fertilization occurred. These results are in accordance with the literature indicating that there is no correlation between ATP and fertilization in IVF or GIFT programs [Mieusset *et al.* 1989; Gwatkin *et al.* 1990; WHO Task Force on the Prevention and Management of Infertility, 1992].

The unknown "nucleotide" which was detected during the incubation experiment did not show any correlation with the morphological groups. However, no experiments were done in this study to test the effect of the unknown on fertilization rates.

2 ENZYMES

2.1 Lactate dehydrogenase (LDH), creatinine kinase (CK), γglutamyl transferase (γGT)

In this study no statistically significant differences were found between any of the enzymes (LDH, γGT, CK) and the different sperm morphological groups.

The literature does not report on any correlation between changes in the isoenzyme pattern and other characteristics of metabolic enzymes and sperm morphological groups. Total LDH was previously measured by Casano *et al.* [1991] in spermatozoa and was significantly higher in oligozoospermic men than normozoospermics. The data summarised in table 8 shows that the activity of LDH, γGT and CK of the morphological groups were the same. Creatinine phosphokinase (CPK, CK) was previously measured by Huszar *et al.* [1988, 1990] in oligozoospermics and normozoospermics and was highly significantly inversely correlated in men with sperm concentrations greater than 30 million sperm/ml. Vigue [1992] investigated the relationship between ATP, ATP/ADP and creatine kinase levels in normozoospermic, oligozoospermic and asthenozoospermic swim-up fractions and found no difference in adenine nucleotides and CK concentrations in these groups. However, Orlando [1994] measured CK, LDH, LDHx and ATP content in washed spermatozoa from oligozoospermic and normozoospermic and reported significantly higher levels in the oligozoospermic group and were related inversely to sperm concentration.

γGT, a membrane enzyme was thought to be of value in measuring cell damage. Determination of reactive oxygen species (ROS) which is involved in peroxidation of fatty acids seem to correlate with differential fertility and this was studied extensively in the literature [Kessopoulou *et al.* 1992; Aitken *et al.* 1987, 1988, 1991, 1994]. In this study all the enzymes were measured in seminal plasma and it would appear from the literature that more informative results could be obtained by measuring their levels in spermatozoa.

2.1.1 LDH isoenzymes and ratios

The results in tables 9 and 10 indicate no significant differences between morphological groups and LDH isoenzymes and their ratios. The relationship between spermatozoa with poor morphological score and fertilization is poorly understood (Lin *et al.*, 1992]. Although the Tygerberg strict criteria is making a major contribution in the evaluation of the infertile donor, there are also shortcomings. Mortimer [1981] reports that light microscopic staining of the acrosome does not permit accurate determination of the state of the acrosome. Furthermore, it may not be the apparent abnormal acrosomal morphology that is prejudicial to fertilization *in vivo* [Jeulin *et al.*, 1986].

3 FATTY ACID COMPOSITION

The results of fatty acid composition in table 11 of the P pattern (<4% morphology) and G pattern (>14% morphology) show no significant differences between the two groups. Methyl group transfer involving phospholipids gives rise to the formation of S-adenosyl-L-homocysteine which is known to inhibit sperm motility [Goh & Hoskins, 1985]. Calamera *et al.* [1989] report a significant decrease of ATP between control and peroxidation groups which was observed in asthenozoospermics at 120 minutes of incubation. De Lamirande *et al.* [1992] indicate that reactive oxygen species (ROS) inhibit sperm motility after several hours of incubation. Human spermatozoa are extremely susceptible to oxidative stress [Jones *et al.*, 1979; Alvarez *et al.*, 1987 and Aitken *et al.*, 1987] because of the abundance of unsaturated fatty acids in the sperm plasma membrane. This composition gives the structure fluidity it needs to engage in membrane fusion, for example the acrosome reaction involved in fertilization. It is the presence of double bonds between adjacent carbon atoms in such sperm-

membrane molecules that favours attack by oxygen-free radicals and the initiation of lipid peroxidation.

4 CONCLUSION

This study makes a contribution towards describing a fully automated technique for measuring the adenine nucleotides in semen, seminal plasma and spermatozoa. When this technique was applied to semen of differential fertility using the Tygerberg strict criteria, no correlation was apparent between the adenine nucleotides and sperm morphology. This may suggest that there is no relationship of strict criteria with fertility in a Gift programme and/or that nucleotide content may not relate to fertility status at all. However, preliminary results show an important inverse relationship between sperm motility and semen nucleotide content.

Selected enzymes (CK, LDH and γGT) do not correlate with the Tygerberg morphological criteria. A technique for the analysis of fatty acids was adapted for sperm membrane analysis which could become more important for assaying membrane composition with ROS (Reactive Oxygen Specie) damage. This also did not correlate with the Tygerberg strict morphological criteria. It must be stressed that the data was limited and that no clear conclusions could be made from the available data. This study furthermore highlights that care should be taken in measuring ATP levels only because of the processes of generation and utilization. ATP can also be synthesized from other sources which was until recently not known.

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APPENDIX

7.1 Tygerberg criteria for motility assessment.

Grading of forward progression.

0	no movement
1	no forward movement
1+	few sperm move now and then
2	undirected slow movement
2+	movement slow but directly forward
3-	movement fast but undirected
3	movement fast directed forward
3+	very fast forwardly directed motility
4	extremely fast (wave-like) movement directed forward

7.2 Spermatozoa terminologies (WHO 1986)

Oligozoospermic	< 10 million/ml
Normozoospermic	normal ejaculate and sperms
Asthenozoospermic	bad motility
Teratozoospermic	too many abnormal forms
Azoospermic	no sperm in ejaculate
Hipospermic	small volume < 1,5 ml
Hiperspermic	> 6,0 ml
Necrozoospermic	all dead sperm
Polizoospermic	> 250 million/ml
Microstrongylozoospermic	spermheads smaller than normal
Macrostrongylozoospermic	spermheads larger than normal

7.3 Morphology: Tygerberg strict criteria [Kruger *et al.*, 1986, 1987]

Normal spermatozoa were described as follows:

Head with smooth oval configuration with well-defined acrosome involving 40 percent to 70% of sperm head.

No defects to neck, midpiece and tail.

No cytoplasmic droplets of more than half the size of the spermhead should be present.

That borderline forms are counted as abnormal.

7.4 P and G Patterns [Kruger *et al.*, 1988]

P Pattern (Poor prognosis pattern)

Mean normal morphological of 1,8% and mean slightly amorphous forms of 18% with a morphology index $< 30\%$.

G Pattern (Good prognosis pattern)

Mean normal morphology of 7,7% and mean slightly amorphous forms of 34,3% with morphology index $> 30\%$.

7.5 LIST OF TABLES

7.6 LIST OF FIGURES

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FIGURE 9: Absorption spectra of nucleotides, AMP, ADP, adenosine and ATP on a CARY 19 scanning spectrophotometer. The λmax in water is: 258 nm, 259, 260 and 259 for the nucleotides respectively.

FIGURE 10: Chromatogram of the standard separated nucleotides in water on a Waters model 440 liquid chromatograph at wavelength 254 nanometers. The retention times were AMP 7,75 minutes, ADP 10, adenosine 12,47 and ATP 19,4.

FIGURE 11: Chromatogram of standard separated nucleotides in water on the Beckman System Gold with diode array detection at 254 nanometers. The retention times were: AMP 7,03 minutes, ADP 9,78, adenosine 10,68 and ATP 19,44.

FIGURE 12: Chromatogram of nucleotides in semen (without sample cleanup) Retention times of AMP is 7,06 minutes; Adenosine 10,52; unknown 12,43 and ATP 19.98.

FIGURE 13: Chromatogram of nucleotides in spermatozoa. Retention times of AMP is 7,06 minutes; Adenosine 10,52; unknown 12,41 and ATP 19,95.

FIGURE 14: Chromatogram of nucleotides in seminal plasma with retention times of AMP at 7,04 minutes; Adenosine 10,46; unknown 12,37 and ATP at 19,77.

FIGURE 15: Chromatogram of ATP in a swim up specimen (100µl swim-up and 500µl extraction buffer). ATP gives a retention time of 20,08 minutes.

FIGURE 16: Chromatogram indicating the separation of an unknown at 13.08 minutes after 6 hours of incubated semen (absent after ejaculation).

FIGURE 17A: Changes in the concentration of ATP during incubation.

FIGURE 17B: Changes in the concentration of ADP and AMP during incubation.

FIGURE 17C: Changes in the concentration of the unknown nucleotide during incubation.

FIGURE 17D: Changes in the concentration of adenine nucleotides and motility during incubation.

FIGURE 18: Steps in using the C_{18} sep-pak for concentration of unknown after removal of phospates.

FIGURE 19: Diode array absorption spectra of 13 minute unknown in mobile phase (phosphate buffer pH4) indicating a maximum absorption of 218nm and 278nm.

FIGURE 20: This figure gives a typical seminal plasma isoenzyme LDH separation. Note the additional C4 (LDHx) fraction which is seminal plasma specific.

FIGURE 21: This figure depicts a typical normal serum LDH separation indicating the five isoenzymes.

7.7 BUFFERS

7.7.1 Extraction buffer for semen, seminal plasma and swim-up

TRIS 12,10g

Na_2 EDTA H_2O 0,37g

Sodium azide 0,50g

(all analar grade)

Dissolve in 900 cm^3 of HPLC grade Milli Q H_2O. Adjust pH to 7,75 with sulphuric acid. Make up to 1 liter with Milli Q H_2O. Filter with millipore 0,22 µm or 0,45 µm filter.

7.7.2 Mobile phase for RPHPLC

70g (0,5M) KH_2PO_4 (Merck 4873)

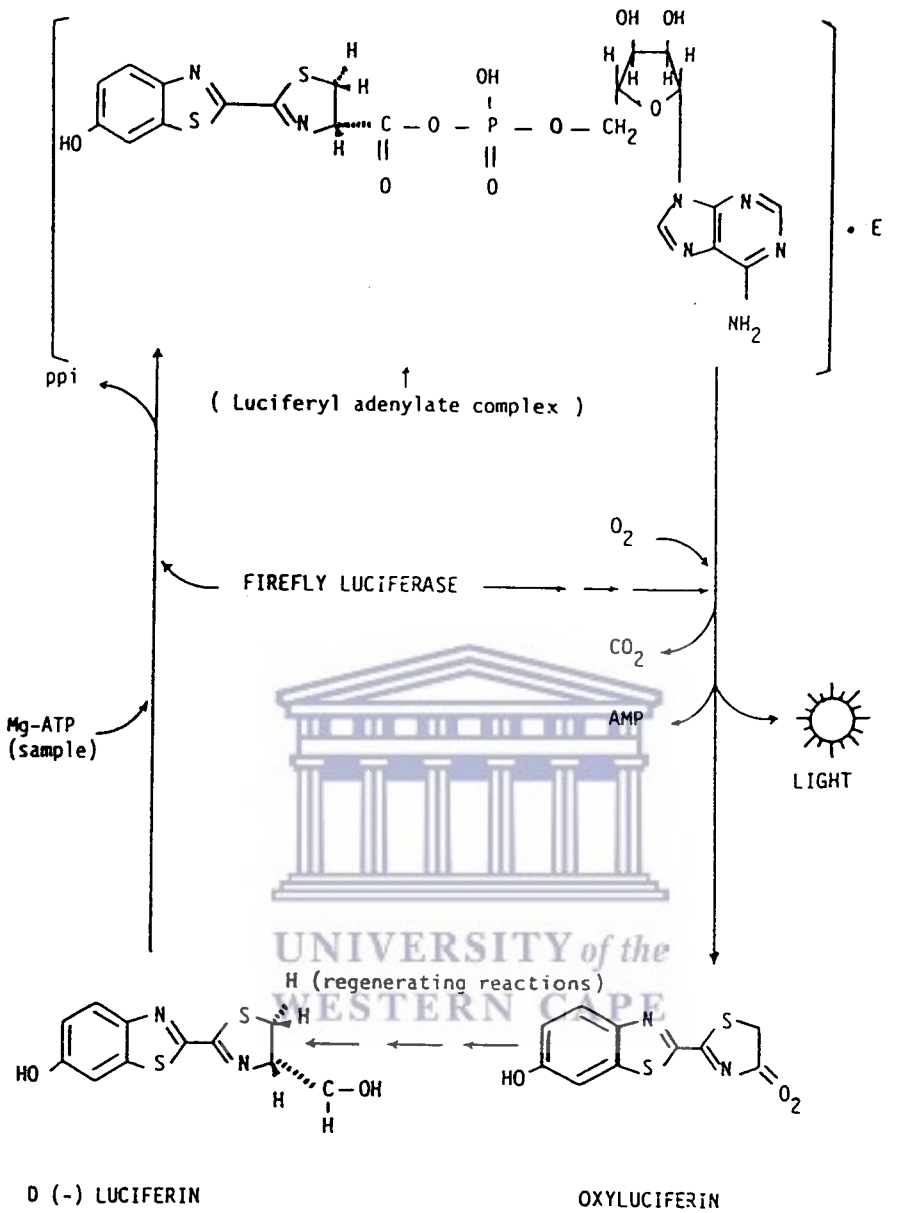
25cm^3 (2,5%) Methanol (HPLC grade)

0,8g (0,086%) Tetrabutyl ammonium hydrogen sulphate (TBAS) Sigma T1134.

Adjust pH to 4 with phoshoric acid. Make up to 2 liters.

Filter with 0,45 µm millipore filter.

7.8



Reactions in chemoluminescence determination of ATP using a luminometer.

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