EFFECT OF ACUTE ALCOHOL INTAKE ON RAT THYMUS THROUGH PROTHYMOSIN ALPHA QUANTIFICATION

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ABSTRACT
EFFECT OF ACUTE ALCOHOL INTAKE ON RAT THYMUS
THROUGH PROTHYMOsin ALPHA QUANTIFICATION

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The aim of this study is to assess the effect of alcohol intake on the thymus, and thus on the maturation of T-cells, through the quantification of prothymosin alpha, a protein involved in the maturation and proliferation of thymocytes. To accomplish this purpose a new protocol for the quantification of prothymosin alpha had to be developed, and for that purpose a characterization of prothymosin alpha and its behavior on SDS-PAGE had to be performed.

After characterizing prothymosin alpha’s behavior on SDS-PAGE (appearing as a dimer at 20.78 ± 0.17 KDa, a new quantification protocol was developed. The first steps of the protocol involved well documented preliminary extraction
methods, isoelectric precipitation at pH 3.55, and finally, quantification on SDS-PAGE by Coomassie Blue staining.

The effect of acute alcohol intake was assessed through two doses (3g/kg body weight and 4.4g/kg body weight) over two incubation periods (three hours and two days), in addition to a dose of 4.4g/kg body weight over five hours.

No effect was observed for the lower dose of 3g/kg body weight; whereas a pronounced effect was detected for the high dose of 4.4g/kg body weight, over two days: a decrease in thymus/body weight percentage (46 % decrease--p < 0.05), a reduction in the cortical region of the thymus and a decrease in prothymosin alpha levels (13 %, p < 0.05). A milder effect was observed for the five hours incubation period of the high dose of 4.4 g/kg body weight.

Acute alcohol intake affects negatively the thymus gland, and thus T-cell maturation, especially above a certain threshold point that exists between 3 and 4.4 g/kg body weight. A direct effect is probable due to the observable effects of the high dose (4.4g/kg) over the short period of five hours.
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GLOSSARY

ADH: Alcohol dehydrogenase

BSA: Bovine serum albumin

HPA: Hypothalamic-pituitary-adrenal Axis

KDa: Kilo-daltons

MW: Molecular weight

NAD: Nicotinamide adenine dinucleotide

PCA: Perchloric acid

PTA: Prothymosin alpha

RP-HPLC: Reverse phase – high performance liquid chromatography

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TFA: Tri-fluoroacetic acid
Chapter 1

INTRODUCTION

The relationship between excessive alcohol intake and poor health has been suspected since early times.

The first reported impacts on health included increased morbidity from cirrhosis, gastrointestinal hemorrhage, trauma, cancer, and infection. One of the pioneer scientific studies was performed by Benjamin Rush in 1785, in which he listed tuberculosis, pneumonia, and yellow fever as complications of alcoholism (Rush, reprinted in 1943). His work was followed by a number of studies reporting similar results and adding to the list of disorders, throughout the nineteenth and twentieth centuries.

Many factors have long been associated with the high incidence of infection among alcoholics, including dulled mental function, breakdown of local barriers, and malnutrition. However, the frequency and severity of infections were so pronounced among alcoholics that a conviction has long existed among physicians that alcohol itself directly inhibits the body’s specific immune defense mechanisms (McGregor, 1986), but it was not until 1938 when Pickrell, a surgical resident at Johns Hopkins Hospital, first demonstrated a direct effect between acute intoxication in rabbits and failure to mount an acute polymorphonuclear response to experimental pneumococcal infection (Pickrell, 1938). Since that time, a wealth of evidence has accumulated regarding the acute and chronic effects of excessive alcohol consumption on the various divisions of the immune system; especially in the last two decades throughout which precise modes of action were proposed, and more advanced mechanisms were elucidated; especially at the cellular and molecular levels.
Today, alcoholism remains a serious problem and even considered a disease due to its addictive aspects. Complications arising from alcohol are being extensively studied and the different consequences of the various administration methods, whether chronic or acute, are being elaborated.

**Purpose of the study:**

The purpose of this study is the evaluation of the effect of acute alcohol intake on rat thymus through a new approach and with a new variety of doses and incubation times:

1. Study the effect of acute alcohol intake on thymus gland through rat thymus/body weight percentage, histological sections, and prothymosin alpha quantification as a proliferation/apoptosis index and thymocyte concentration index.

2. Partial characterization of prothymosin alpha and its behavior on SDS-PAGE.

3. Develop a fast protocol for prothymosin alpha quantification.
Chapter 2

LITERATURE REVIEW

2.1. Effects of Alcohol Intake on Immunity:

2.1.1. Effect of alcohol intake on the immune system in general:

The large amount of research performed has concentrated on chronic alcohol intake, only a small part dealt with acute alcohol intake.

Early studies of alcohol's influence on immune responses examined whether alcohol alters the numbers or ratios of lymphocytes and lymphocyte subpopulations found in the blood. These studies consistently showed a decrease in the numbers of lymphocytes isolated from the blood of alcoholic humans or from laboratory animals that had consumed alcohol over a period of several weeks. These lymphocytes also responded abnormally to in vitro stimulation by mitogens or antigens, suggesting an adverse effect on their capacity to react appropriately to infection (Macgregor, 1986).

Improved technology for characterizing and isolating different types of lymphocytes (B-cells, T-cells, and various T-cell subpopulations) has allowed investigators to determine alcohol's effects on the numbers and functions of lymphocyte populations. One group of researchers has found that the numbers of circulating helper T-cells and cytotoxic or suppressor T-cells were reduced in alcoholics. Alterations in circulating lymphocyte numbers could contribute to increased susceptibility to infection (Bagasra et al, 1987; Grossman et al, 1988).
On the other hand, ethanol ingestion was shown to result in a loss of lymphocytes from the thymus and spleen and impairments in lymphocyte proliferation in response to T-and B-cell mitogens. Decreased antibody-producing cell numbers in response to a T-dependant antigen also occurs in ethanol fed mice, but the response to a T-independent antigen is not affected. It is clear thus that the effect of ethanol on the immune system is fairly specific to T-cell dependant antibody responses (Jerrells et al, 1986).

It has been suggested that ethanol directly affects the lymphocyte and influences the function of this cell, perhaps through the well-known effect of ethanol on cell membrane fluidity. However, the direct effect of ethanol on cells has required that relatively large amounts (more than 1%) of ethanol be added to the cultures, and in most cases the levels used are not achievable *in vivo*. On the other hand, it was shown that physiological levels of ethanol do not negatively affect lymphocyte function, including proliferation and lymphokine production. In fact, results of many experiments performed *in vitro* would suggest that low doses of ethanol enhance lymphocyte function most likely because of increased membrane fluidity (Jerrells et al, 1990).

There is enough evidence to suggest that the immunosuppression associated with ethanol administration is due to indirect effects.

2.1.2. Neuroendocrine effects of alcohol intake:

Evidence suggests that bidirectional communication occurs between the immune and neuroendocrine systems (Macgregor, 1986). Accordingly, stimulated lymphoid cells send signals mediated by cytokines and other immune products to inform the central nervous system (CNS) about the activity of the immune system. The brain, in turn, modulates the immune response via the pituitary-
endocrine axis as well as the autonomic neural output. Thus, effective feedback communications between the CNS and the immune system are crucial to host defense responses. The neuro-endocrine-immune network is influenced by a wide variety of factors, including various stressors as well as by alcohol which can compromise the immune system and, consequently influence the outcome of diseases, including AIDS. Alcohol produces a host of neuroendocrine changes and its effects on the hypothalamic-pituitary-adrenal (HPA) axis have been extensively investigated (Watson et al, 1993).

It has been well documented that adrenal glucocorticoid hormones are potent immune suppressors and, thus, are frequently employed in the clinical setting (Capps et al, 1982). It is probable, therefore, that this neuroendocrine axis is of considerable importance in the feedback interactions between immunity and the nervous system. On the other hand, both acute and long-term studies demonstrated that alcohol facilitates the activity of the HPA axis as shown by increased circulating levels of corticosterone (Ellis, 1966; Guaza et al, 1983).

However, the mode of administration of the alcohol dose is of crucial importance. Three different modes are most commonly used: intraperitoneally, intragastrically, or through a liquid diet. Each method has its advantages and disadvantages, and the choice of the method is dependant on the purpose of the experiment.

It was found (Ogilvie et al, 1997) that the alcohol liquid diet (6.4% w/v) had no effect on the hypothalamic-pituitary-adrenal hormone secretion, when administered for 4-6 days. Whereas both intraperitoneal and intragastric administrations elevated blood ACTH level. In both cases plasma levels showed dose-related increases, which supports the concept that alcohol does not merely act as a stress, but represents a distinctive and specific signal for the HPA axis.
It is important to note that a longer exposure to alcohol, using an alcohol diet would eventually lead to elevated levels of corticosterone (Watson, 1985).

As such a possible mechanism through which alcohol intake affects the immune system indirectly has been suggested.

2.1.3. Effect of alcohol on the thymus:

The effect of alcohol on the thymus has been specifically investigated. It was reported that alcohol abuse leads to a loss of lymphoid cells from the thymus. (Jerrells et al, 1986, 1990).

Since nutrition can be an important variable in immune modulation during chronic alcohol consumption, Budec et al (1992) performed a single-dose experiment to study the direct effect of ethanol on the thymus. The single dose consisted of an intraperitoneal injection of 4g/Kg body weight of ethanol; with an incubation time of 20 hours. Their results showed a significant decrease in thymus/body weight ratio in ethanol treated rats relative to controls (about 32% decrease). Moreover, histological sections of the thymus showed that the reduction mainly affected the cortex and to a much lesser extent the medulla (See Section II for more details on the inner architecture of the thymus).

In an attempt to understand the mechanism underlying the effect of ethanol, Budec et al (1992) also included a treatment with ethanol + Naltrexone (an opioid antagonist). Their results showed that naltrexone inhibited ethanol-induced changes in rat thymus morphology, suggesting an involvement of the neuroendocrine system through brain opiate receptors.

Another study (Han et al, 1993) performed on mice used an alternative method of ethanol administration and different doses. In this study ethanol was administered intragastrically and with higher doses (5-7g/Kg body weight), over a
two-day period (as a single dose or multiple doses). Atrophy of the thymus was also observed along with a decrease in thymic cellularity, especially for the immature thymocytes. Furthermore, the use of RU 486 (glucocorticoid antagonist) blocked the depletion of thymocytes from the thymus; supporting a mechanism of action involving glucocorticoids.

In addition, Padgett et al (2000) found a close association between corticosterone levels and a loss of lymphocytes from lymphoid organs. As such, they performed an experiment in which they compared the effects of an alcohol liquid diet on a normal group of mice and an adrenalectomized group. Their results indicated that ethanol-fed adrenalectomized mice showed much less thymic cell loss than did ethanol-fed intact animals. This experiment further supports the involvement of adrenal steroids in the effect of alcohol on the thymus.

Thymic involution due to alcohol intake can be the result of either migration of cells or a programmed cell death (apoptosis). It has been demonstrated that binge drinking of alcohol (Han et al, 1993) or in vitro exposure of thymocytes to ethanol enhance thymocyte apoptosis (Ewald et al, 1993). The exact mechanism was partially elucidated by Shao et al (1995) when they found that ethanol produced a dose-dependant increase in intracellular calcium level within thymocytes. This dose-dependant increase of ca$^{2+}$ was paralleled by the magnitude of DNA fragmentation induced by ethanol at various concentrations, which means that calcium might be involved in the signal pathway(s) leading to thymocyte cell death.

2.2. The Thymus:

The endodermally derived thymus is primarily a lymphoid organ of infancy and early childhood. At birth, it is a relatively large, well developed, bilobed, grayish
white body located in the superior part of the chest. It rests on the great vessels of the heart and extends into the root of the neck. At puberty it reaches its maximum. Then it regresses and is largely replaced by adipose tissue. Its interesting life cycle suggests that it has some undefined role in development, since it reaches its greatest development during the body's most active growth period and then begins to atrophy about the time the growth of the body levels off.

The thymus is the principal production site of T-lymphocytes: T-cell progenitors formed during hematopoiesis enter the thymus gland as immature thymocytes and mature there to become antigen-committed, immunocompetent T-cells. It is a cardinal factor in the immune defense mechanism of the body. It also produces humoral factors important to the establishment of the immune system in general.

A thin, connective tissue capsule surrounds each of the two lobes of the thymus. Thin septa from the capsule penetrate the gland to divide it into incomplete lobules uneven in size. Each lobule is organized into two compartments: the outer compartment, or cortex, is densely packed with thymocytes, whereas the inner compartment, or medulla, is sparsely populated with thymocytes (Figure 1).

The actual maturation sequence within the thymus is not known. Generally it is thought that progenitor T cells enter the thymus and begin to multiply within the cortex. At this stage there is rapid proliferation of thymocytes coupled to an enormous rate of cell death. A small subset of more mature thymocytes is then thought to migrate from the cortex to the medulla where they continue to mature and finally leave the thymus via postcapillary venules (Kuby, 1994).
Figure 1: Structure of the thymus. A section through one lobe of a postnatal thymus: The cortical (C) and medullar (M) regions are indicated (100x) (Dulbecco, 1997)
2.3. Prothymosin alpha:

The thymus provides the microenvironment for the development of bone marrow-derived lymphoid progenitors into mature T-lymphocytes. This microenvironment includes a family of peptides secreted by cells of the thymus, some of which has been successfully extracted, purified and sequenced (Cordero et al 1998). The most prominent of these peptides was Thymosin-alpha one, a 28 amino acid acidic peptide that was proven to have immunomodulatory activities.

The extraction of Thymosin alpha 1 was performed under normal conditions. However, when the extraction was performed under harsh conditions, no Thymosin alpha one (or only trace amounts) were detected. Harsh conditions consisted mainly of boiling the tissue, thus inactivating all proteolytic enzymes. These data led to the conclusion that Thymosin alpha 1 is first synthesized as a larger precursor, which afterwards is subjected to a specific cleavage mechanism. This precursor was later isolated and characterized by Haritos et al (1984a). They were able to purify a polypeptide of approximately 112 amino acids, and named it: “Prothymosin alpha” (PTA).

PTA was shown to have same immunomodulatory effects as Thymosin alpha one, only to reveal in later researches a much more complex and ubiquitous functions based on its wide tissue distribution, nuclear localization and peculiar structure.

2.3.1. Distribution and Localization of PTA:

PTA was first extracted and purified from the thymus gland. However, it has been shown that it is a ubiquitous protein present in many other tissues; its highest levels being in the thymus gland (Haritos et al 1984b). Furthermore, its major intrathymic source was found to be the thymocyte population, since its
concentration in purified thymocytes was in the range of that in the whole thymus (Franco et al. 1989) and that PTA mRNA levels were found to be 50-folds higher in thymocytes than in stromal cells (Gomez-Marquez et al., 1989).

Intracellularly, attempts to locate PTA were first hampered by the fact that it is poorly immunogenic and no monoclonal antibodies with high titer or specificity do exist. The close similarities between most of the alpha-thymosins were also a disadvantage for its specific localization. However, the presence of a putative nuclear migration sequence near the C-terminus of prothymosin alpha suggested its nuclear localization (Gomez-Marquez, 1988). This hypothesis was supported by following researches. The first and most significant among these consisted of microinjecting bovine PTA into the cytoplasm of *Xenopus laevis* oocytes, followed by separate monitoring of nuclear and cytoplasmic concentrations. It was shown that prothymosin alpha migrated to the nucleus at a rate comparable to that of histone H1 (Watts et al., 1989). The nuclear localization sequence was further specified to involve residues 87-88 and 101-104 (Rubstov et al., 1997).

This nuclear localization opened the door for following studies to elucidate the role of PTA based on its interactions with the various macromolecules occupying the nucleus.

2.3.2. Biochemical Characterization of PTA:

Prothymosin alpha is one of the most acidic proteins known having a high content of glutamic and aspartic acids (about 50 %) resulting in an isoelectric point of 3.55. In addition, its primary structure is highly unusual, since aromatic and sulfur amino acids are totally absent; consequently it does not absorb at 280 nm.
The complete amino acid sequence of rat PTA has been established, first based on conventional amino acid sequencing (Haritos et al, 1985) and afterwards by cDNA cloning (Frangou-Lazaridis et al, 1988) (See Appendix p. 90-91).

The physical state of PTA in solution is a controversial issue. Gel filtration experiments at neutral pH resulted in an apparent molar mass that is five times greater than that calculated from the amino acid sequence (Haritos et al 1984a, 1989). When PTA was eluted with HCOOH/pyridine (pH 2.8), it showed an apparent molar mass that is three times its calculated molar mass (Haritos et al, 1987). On the other hand, sedimentation equilibrium measurements under identical conditions gave a molar mass of 12,800 g/mol, a value being nearly that expected for the monomeric PTA molecule (Haritos et al, 1989).

When PTA was studied on SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), more complications emerged. First, it was reported to appear as a band of relative molecular mass of 13 KDa (Watts et al, 1990), or 15 KDa (Palvimo et al, 1990), supporting the theory of monomeric nature of PTA in solution. Two years later a study performed by Cordero et al (1992) to elucidate this anomalous behavior led to the conclusion that under no conditions of high temperature, SDS and beta-mercaptoethanol, does PTA appear as a monomer. Instead, their results showed a band of relative molecular mass of 20.6 KDa ± 5%, that they have hypothesized to be a stable dimer of PTA.

Today, this controversy remains unsettled. It is proposed, however, that PTA has a monomeric nature in vivo and it tends to aggregate when it is manipulated (Pineiro et al, 2000).
2.3.3. Functions of PTA:

The functions of PTA fall into two major categories: An immunomodulatory and an intracellular ubiquitous role.

Upon its discovery and characterization, PTA was suspected to have hormonal functions, as the precursor of Thymosin alpha one, one of the most prominent thymic hormones. However, this hormonal function was thrown into doubt, firstly by the demonstration using cDNA cloning that there is no signal peptide for secretion in the primary translation product (Goodall et al 1986, and Eshenfeldt et al 1986), and also from the observation of a broad tissue distribution for the protein (Haritos et al 1984). Although PTA was detected in human blood, its presence was due to its high concentrations in circulating leukocytes; and the trace amounts found in human plasma were suggested to result from injured macrophages (Panneerselvam et al, 1987).

The immunomodulatory effects of PTA (Pineiro et al, 2000) are diverse. Rat PTA was early demonstrated to be protective against *Candida albicans* infection in mice (Haritos et al, 1985b). Daily inoculation with PTA in both young and aged rats showed increased humoral and cell-mediated immune reactions (Maric et al, 1991a, 1991b). The most outstanding *in vivo* assays were those where the anticancer activity of prothymosin alpha was described in an experimental tumor model. These assays revealed multiple effects of PTA such as the induction tumoricidal peritoneal macrophages, enhancement of natural killer and lymphokine-activated killer activities in splenocytes, induction of the production of interleukin-2 and tumor necrosis factor, and when administered simultaneously with tumoral cells, induction of tumor specific cytotoxic (CD-8+) and helper (CD-4+) T-cell activation (Baxevanis et al, 1994, 1995).
The intracellular functions of PTA on the other hand are rather ubiquitous and not related specifically to the immune system. The biochemical properties, wide distribution, expression patterns and nuclear localization revealed some of these functions.

PTA was first discovered in the thymus, and afterwards found in other tissues like the spleen, lung, kidney, liver and brain (Haritos et al, 1984b). Its mRNA was further detected in the ovary, heart, muscle, intestine, cerebellum, and testis (Eschenfeldt et al, 1986; Clinton et al, 1989). This wide distribution refuted the theory that PTA is only an immunomodulatory peptide.

Several pieces of evidence supported the involvement of PTA in chromatin organization: its high concentration in the nucleus, its acidic nature which resembles some chromatin/chromosomal proteins, and its ability to bind histone H1 in vitro (Segade et al, 1999).

Recently, different research groups have focused their work on the activity of PTA in relation to chromatin. PTA was found to bind selectively to the linker histone H1 through its acidic domain, by in vitro (Papamarcaki et al, 1994), as well as by vivo studies (Keretzou et al, 1998). Furthermore, PTA is present in amounts equivalent to those of H1 in mammalian cells (Surlati et al, 1990). These analyses suggested a putative role for PTA in fine tuning the stoichiometry and/or mode of interaction of H1 with chromatin.

Furthermore, since histone H1 binds to the nucleosome core particle, sealing the entry and exit points of the DNA and is involved in higher-order chromatin packing, it was proposed that PTA participates in chromatin decondensation allowing replication to proceed. (Gomez-Marquez et al, 1998) (Figure.2).
Figure-2: Schematic representation of the proposed role of PTA in the decondensation of chromatin through its interaction with histone H1. According to this model, the interaction of PTA with H1 provokes the unfolding of the 30-nm chromatin fibers facilitating the occurrence of biochemical processes that need chromatin decondensation to take place. (Segade et al, 1999).

Another line of evidence emerged relating PTA to proliferation. Experiments on HL-60 cells showed that enhanced expression of PTA accelerates the proliferation rate of these cells, decreasing the G1 phase of the cell cycle. Until now, few genes have been described as candidates for a direct regulation by the Myc protein, a transcription factor that has a key role in the regulation of mammalian cell proliferation. PTA is one of the clearest Myc targets, being not only related to proliferation, but directly involved in that process as an early
component in the proliferation events triggered by $myc$ genes (Rodriguez et al, 1998).

Additional reports point towards the same conclusion. Thus it has been reported that conditional expression of N-$myc$ in human neuroblastoma cells increases the expression of PTA accelerating progression into S-Phase (Hidefumi et al, 2001a), as well as for $emyc$ (Vareli et al 1995), and that overexpression of PTA is related to rat hepatic carcinogenesis (Wu et al 1997).

On the other hand, when human myeloma cells were incubated with PTA antisense oligodeoxynucleotides, cell division was inhibited. Evidently, the transient deficit of prothymosin alpha postpones mitosis. Treatment with PTA antisense oligomers also results in a small (10%) reduction in total protein synthesis; consequently, an effect on mitosis mediated through a deficiency in one or more other proteins must be considered (Gomez-Marquez et al, 1989).

The correlation between PTA levels and proliferation focused studies on the values of PTA in tumoral processes because of their intense proliferative activity. And tissue content of PTA was proposed as a prognosis factor for several types of cancer.

2.3.4. PTA levels in relation to thymocyte maturation:

In a direct relevance to this study, a research was performed to correlate PTA expression with the maturation levels and the proliferation state of thymocytes. In this study Gomez-Marquez et al (1988) found that PTA mRNA levels were highest in large thymocytes in comparison with small nonmature thymocytes, bone marrow pre-T-cells, and spleen lymphocytes. Furthermore, this heterogeneous population of large thymocytes was divided into two groups: mature and immature thymocytes. When these two populations were tested for PTA expression levels, immature cells (undergoing continuous divisions) contained three-fold more PTA mRNA than postmitotic mature thymocytes.
These data indicate a differential transcriptional activity of the PTA gene during the maturation of T-cells in vivo, increasing from pre-T-cells to immature thymocytes and decreasing in mature thymocytes and circulating lymphocytes.

2.3.5. Prothrombin alpha and Apoptosis:
As PTA has been proven to play an essential role in the proliferation of cells, its relation to the programmed cell death has also been a subject of research.
Recent studies showed that PTA cleavage occurs in cells triggered to undergo apoptosis by a variety of cell death inducers and may therefore represent a general feature of apoptotic cells. Caspase fragmentation of PTA appears to be an early event detectable as early as 2 hours post induction of apoptosis (Evstafieva et al, 2000).

2.3.6. Methods of extraction and quantification of PTA:
The first reliable extraction of PTA was reported by Haritos et al (1984), in which extensive boiling of the tissue was required as the first step of the isolation procedure. The need for boiling was due to the necessity of a total inactivation of proteolytic enzymes. All previous methods produced Thymosin alpha-1 instead of PTA, due to the breakdown of the latter during the extraction procedure.
The following steps included several purifications on Sephadex S-200, C4 and C18 Reversed-phase HPLC columns.

An alternative procedure used 5% PCA as the extraction buffer, benefiting from the denaturing capacity and the selective solubility of PTA in 5% PCA (along with few other proteins). This extraction was also followed by several chromatographic purification steps (Watts et al, 1990).
Several RIA and ELISA techniques have been reported for the estimation of PTA levels, with high sensitivity (Haritos et al, 1985b; Loidi et al, 1997; Costopoulou et al, 1998). However, these methods detect segments of the peptide; mostly the N-terminus which is Thymosin alpha-1. This causes cross reactivity problems due to the above mentioned close similarities of the different alpha-thymosins. This problem may not be of major importance if we are testing plasma levels of Thymosin alpha-1, but it should be considered when testing PTA (or Thymosin alpha-1) levels in thymic extracts; due to the high amounts of the other alpha-thymosins in the thymus gland itself. Another problem is the low immunogeneity of PTA, resulting also in some difficulties in producing high titers of monoclonal antibodies to be available commercially. Thus the search continues for new quantification methods.
Chapter 3

Prothymosin alpha characterization and development of a new protocol for its quantification.

3.1. Animals:

Male Sprague-Dawley rats were used in all of the experiments. The rats were raised in the LAU animal room, in which the temperature is constantly controlled to 22-23 °C and a 12 hour light-dark period is maintained. The diet consisted of standard rat chow.

Rats used in all the experiments of this study were 45-60 days of age, in order to obtain the largest size of the thymus gland. However, members of the same experiment were age matched in that their ages differed in no more than 2-3 days; due to the pronounced effect of age on the thymus gland.

3.2. Preliminary Extraction Procedure:

The preliminary steps for prothymosin alpha extraction were the same as those described by Hartos et al (1984a): Thymic tissue was removed from rats within five minutes of slaughter, and quickly frozen in liquid nitrogen and stored at -80 °C until processed.

Upon processing, frozen thymic tissue was pulverized under liquid nitrogen and directly put into boiling sodium phosphate buffer (pH 7.0) and boiling was continued for five minutes. The boiling was intended to inactivate all proteolytic enzymes and thus preserve prothymosin alpha from degradation.

The suspensions were then cooled and homogenized on ice with a Polytron homogenizer (Glas-Col Homogenizer) for three minutes at top speed. The
homogenate was then centrifuged in a microcentrifuge (Biofuge 13R, Heraeus Sepatech) at 13,000 rounds per minute (rpm) for 20 minutes. The clear supernatant contained prothrombin alpha (Haritos et al, 1984a). The supernatant was then subjected to several selective and non-selective precipitations. The precipitates were analyzed on SDS-PAGE.

3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

3.3.1. Principle:

In SDS-PAGE, a porous gel, where the pore size is similar to the size of the protein molecules, is used and the effective pore size is inversely related to acrylamide concentration in the polymerization mixture. In a gradient polyacrylamide gel, acrylamide concentration increases as the proteins migrate from the origin resulting in the separation of a wide range of protein sizes into sharper bands than is usually possible with single concentration gel (Hames, 1990).

Proteins are visualized by staining with Coomassie Blue, which has range of sensitivity of 0.2-0.5 micrograms up to 15-20 micrograms (Hames, 1990). Proteins have different chromogenicity with Coomassie Blue, since it binds to different extent with various proteins. This is why it is essential to run standards in each gel and to construct a standard curve for the protein to be assayed.

3.3.2. Stock solutions:

1. **Sample buffer** (two fold concentrated): 20 ml glycerol was added to 1.5 g Tris dissolved in 35 ml of distilled water and pH adjusted to 6.75 with 12 M HCl following which 10 ml of 2-mercaptoethanol, 4 g of SDS and 2
mg of bromophenol blue were added and diluted with distilled water to a final volume of 100 ml.

2. **3 M Tris buffer (pH 8.8):** 36.3 g Tris was dissolved in about 80 ml distilled water; the pH was adjusted to 8.8 with 12 M HCl. Distilled water was added to a final volume of 100 ml. This solution is stable for several weeks at 4°C.

3. **0.5 M Tris buffer (pH 6.8):** 6.02 g Tris was dissolved in approximately 90 ml distilled water. The pH was adjusted to 6.8 with 12 M HCl and the final volume was made up to 100 ml with distilled water. This solution is stable for several weeks at 4°C.

4. **Urea/SDS/dithiothreitol solution:** 19.2 g urea, 12.3 mg dithiothreitol and 0.08 g SDS were dissolved in 25 ml of distilled water and the final volume was made up to 40 ml.

5. **1.5 % ammonium persulphate solution:** 0.15 g of ammonium persulphate was dissolved in 10 ml of distilled water.

6. **Electrode buffer (pH 8.3):** 42.6 g glycine, 9.18 g Tris and 3 g SDS were dissolved in 3 L distilled water.

7. **Protogel:** 30 g acrylamide and 0.8 g N,N'-bisacrylamide were dissolved in approximately 70 ml of distilled water; the final volume was adjusted to 100 ml.
3.3.3. Procedure:

3.3.3.1. Preparation of samples for electrophoresis: Sample buffer (two-fold concentrated) was added to the precipitated protein mixture and vortex mixed. As the protein mixture is mostly acidic, the sample buffer-containing bromophenol blue- turned greenish yellow. Therefore, and to prevent an anomalous migration of the proteins, 1μl of diluted NaOH was added, turning the sample buffer to its blue color. The sample buffer containing the protein mixture was then pipetted into Eppendorf tubes and heated at 95°C for 5 minutes to denature the proteins. The tubes were then centrifuged at 13,000 rpm for 4 minutes to separate any insoluble material.

For the characterization experiments, molecular weight markers (Amersham Pharmacia) were dissolved in sample buffer according to the supplier's instructions, aliquoted and stored at -70°C. Just before use, the molecular weight aliquots were thawed, and treated as for samples described above. 20 μl of molecular weight marker solution were loaded into one of the gel lane with a gel loading tip.

3.3.3.2. Preparation of SDS-PAGE: Two types of gels were prepared in different experiments according to the original method of Laemmli (1970). Linear gradient (10-20%) polyacrylamide gels and one concentration (15%) polyacrylamide gels were utilized according to the conditions of the experiment; gradient gels are more suitable for preliminary characterization experiments, since they have a wider spectrum of separation.

For the 10-20 % gradient gels the volumes of the constituents used are represented in table - 1 (for one gel slab).
<table>
<thead>
<tr>
<th>Table – 1 : Gel mixtures for 10-20% Gradient Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 M Tris</td>
</tr>
<tr>
<td>Protogel</td>
</tr>
<tr>
<td>Urea/SDS/DTT</td>
</tr>
<tr>
<td>1.5% APS</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Partial Volume</td>
</tr>
<tr>
<td>Total Volume</td>
</tr>
<tr>
<td>10%</td>
</tr>
<tr>
<td>1.54 ml</td>
</tr>
<tr>
<td>2.10 ml</td>
</tr>
<tr>
<td>1.22 ml</td>
</tr>
<tr>
<td>0.29 ml</td>
</tr>
<tr>
<td>8.39 ml</td>
</tr>
<tr>
<td>2.25 g</td>
</tr>
<tr>
<td>13.54 ml</td>
</tr>
<tr>
<td>30.64 ml</td>
</tr>
</tbody>
</table>

Each of the two solutions: the 10% and the 20% solutions were prepared separately, according to the volumes presented in table-4. The next step was done as quickly as possible: 11.5 µl of tetramethylene-diamine (TEMED) were added to each of the two solutions.

Then, the 10 % and the 20 % gels were poured into two different chambers in a gradient forming apparatus (Coleparmer, USA). The 20 % solution was placed in the mixing chamber and the 10 % solution in the reservoir chamber. The mixing chamber outlet was connected to a peristaltic pump (Watson Marlow 503U, U.K.) discharging between the glass plates of the electrophoresis apparatus (Hoefer SE 600 series with buffer saver). As the gels were poured into the chambers, stirring commenced and the valve outlet of the mixing chamber was opened. Simultaneously, the peristaltic pump and the valve between the two chambers was opened, forming a 10-20 % concentration gradient. The gel was then overlaid with water saturated butanol and left for 45 min to allow polymerization to take place. After 45 min the water saturated butanol was
discarded and the gel top rinsed several times with distilled water. Remaining water droplets were removed with a filter paper.

For the 15 % polyacrylamide gel the volumes of the constituents used is represented in table-2 (for one gel slab).

In the 15 % gel no gradient formation is needed and as such all the constituents were mixed in one flask and 11.25 μl of TEMED was added and the solution was directly poured between the glass plates of the electrophoresis apparatus, forming a gel slab of on concentration (15%). Also the gel was allowed to polymerize for 45 min, after being overlayed with water saturated butanol.

<table>
<thead>
<tr>
<th>Table - 2: Gel composition for 15% Polyacrylamide Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 M Tris</td>
</tr>
<tr>
<td>Protogel</td>
</tr>
<tr>
<td>Urea/SDS/DTT</td>
</tr>
<tr>
<td>1.5% APS</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Total Volume</td>
</tr>
</tbody>
</table>

The Stacking Gel: Whether for the gradient gels or for the one concentration gels, the stacking gel was prepared in the same manner: the volumes of the constituents are presented in table-3.
Table - 3: Gel Composition for the Stacking Gel (For 2 gel slabs)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3 M Tris</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Protogel</td>
<td>1.85 ml</td>
</tr>
<tr>
<td>Urea/SDS/DTT</td>
<td>0.30 ml</td>
</tr>
<tr>
<td>1.5% APS</td>
<td>1.15 ml</td>
</tr>
<tr>
<td>Water</td>
<td>7.50 ml</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>14.55 ml</strong></td>
</tr>
</tbody>
</table>

After mixing the presented constituents in the same flask, 11.25 μl were added and the stacking gel poured on top of the resolving gel. Immediately, a twenty-well comb was inserted and the gel left for polymerization. After 30 min, the upper and lower electrode chambers were filled with electrode buffer and the comb was carefully removed.

3.3.3.3. Electrophoresis: After loading the samples, the lid of the upper chamber was fitted and the water cooling system switched on. The power supply was turned on and electrophoresis was carried out at 30 mA for one gel or 60 mA for two gels until the bromophenol blue marker reached the resolving gel after which the current was raised to twice the value reported above. 4 hours later, the, power was switched off, the gel removed and stained with Coomassie Blue.

3.3.3.4. Staining of the proteins in the gel: The protein staining solution was prepared by dissolving Coomassie Blue R-250 (0.1 % w/v) in water:methanol:glacial acetic acid (5:5:2 by volume). The solution was filtered (Whatman No.1 filter paper) to remove any insoluble material before use. The gel slabs were put in an automated rocker staining/destaining machine (Processor
Plus--Amersham Pharmacia Biotech). The program included rinsing with distilled water for about a minute (to remove excess SDS), followed by staining for 6 hours and afterwards destaining for about 9 hours, with a final rinse with distilled water. Gel slabs were then placed between two transparencies and scanned using a desktop scanner.

3.4. Identification of a band as prothymosin alpha

3.4.1. Different Precipitation methods
As mentioned above, the clear supernatant obtained from the preliminary extraction was subjected to various precipitation and analyzed by SDS-PAGE (10-20 %), in order to identify a well separated band as prothymosin alpha.

3.4.1.1. Acetone Precipitation: The first precipitation trial was a non selective acetone precipitation, in which 6 volumes of ice cold acetone were added to the supernatant obtained from the above mentioned extraction. The mixture was left undisturbed at −20°C for more than two hours. After which it was centrifuged at 5,000 rpm for 20 min (Sigma 2K15) and the precipitate resuspended in SDS-PAGE sample buffer.

3.4.1.2. Isoelectric Precipitation: A more selective precipitation was performed by adding 10 volumes of tri-sodium citrate buffer titrated to pH 3.55, which is the isoelectric point of prothymosin alpha. It is a known fact that proteins tend to aggregate and to precipitate if the pH is near their isoelectric point. Thus, this selective precipitation would only precipitate proteins having their isoelectric around 3.55. This combination (supernatant + buffer) was left to stand for 30 minutes at 4°C, before it was centrifuged at 5000 rpm for 20 minutes. Also the precipitated proteins were resuspended in sample buffer.
3.4.1.3. Double Selectivity Precipitation: A well documented characteristic of PTA is that it is one of the very few proteins soluble in 5% PCA (Watts et al, 1990). To take advantage of this characteristic, it was combined to the fact that PTA precipitates at pH 3.55. As such a precipitation at pH 3.55 was performed. However, the precipitate was not resuspended in SDS-PAGE sample buffer, but it was redissolved in 1 ml of 5% PCA. All the proteins found in the precipitate that are soluble in 5% PCA went into solution. Then it was centrifuged at 13,000 rpm to precipitate all insoluble proteins. The supernatant was afterwards subjected to a non-selective precipitation with ice cold acetone. This procedure has a double selectivity; that of the isoelectric point and that of the solubility in 5% PCA.

The precipitate was made in duplicates the first resuspended in SDS-PAGE sample buffer and analyzed on SDS-PAGE; whereas the second was analyzed on Reverse Phase-HPLC.
3.4.2. Results of the three precipitations (Figure 3)

<table>
<thead>
<tr>
<th>Lane-1</th>
<th>Lane-2</th>
<th>Lane-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone Precipitation</td>
<td>Isoelectric(3.55) Precipitation</td>
<td>Double Selectivity Precipitation</td>
</tr>
</tbody>
</table>

Figure 3: Results of the three precipitation methods: Lane-1: Acetone precipitation; Lane-2: Isoelectric precipitation; and Lane-3: Double Selectivity Precipitation.

The results obtained in figure 3 show that acetone precipitation yielded a compact lane with an abundance of protein bands that cannot be distinguished separately. All the proteins found in the supernatant of the extract were precipitated and visualized on SDS-PAGE. No separate band can be observed for prothymosin alpha. Whereas the isoelectric precipitation yielded a smaller number of proteins.
and these are proteins with isoelectric points near 3.55. As such fewer bands are observed in the corresponding lane. Finally, the double selectivity precipitation showed only one band corresponding to the protein that is at the same time of isoelectric point near 3.55, and soluble in 5% perchloric acid. These are two characteristics of prothymosin alpha.

3.4.3. Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC):

For a more precise confirmation that the band obtained from the double selectivity precipitation is actually prothymosin alpha, it was run on RP-HPLC versus a standard.

3.4.3.1. Procedure:

The precipitated protein was resuspended in 0.1% TFA, and analyzed using RP-HPLC on a Waters Nova-Pak C-18 column (3.9 x 150 mm). Injection was performed on a 50 µl manual HPLC injector (Rheodyne). The mobile phase consisted of a gradient of 20-40% acetonitrile in water containing 0.1% TFA. The gradient was achieved over 30 minutes, through two pumps (Waters 510). The flow rate was 1 ml/min. Standard bovine PTA (Thymoorgan, Germany), the extracted sample (the duplicate of the sample showing only one band on SDS-PAGE), or a mixture of both were run to determine the identity of the extracted protein. The eluted proteins were detected by their UV-absorption at 214 nm. The absorbance was measured on a UV-Visible detector (Waters 486, Tunable Absorbance Detector), and the chromatogram was recorded on a SHIMADZU C-R6A Chromatopac.
3.4.3.2. Results: The elution time was 19.2 min, corresponding to about 21.5 % Acetonitrile concentration (Figure-4).

**Figure-4**: Chromatogram of Prothymosin alpha: a) Standard PTA; b) Sample of the double selectivity precipitation; c) A mixture of (a) and (b).
The chromatogram shows a peak at about 19 min, this peak represents PTA. The purified standard was run first and then the precipitate of the double selectivity precipitation, and finally a mixture of both.

3.5. Molecular weight confirmation:

3.5.1. Procedure: To determine the molecular weight of the protein obtained and to see if it conforms to the molecular weight documented in the literature for prothymosin alpha, it was run on SDS-PAGE (both on “one concentration” gels and “gradient” gels) along with molecular weight markers (Amersham Pharmacia).

The molecular weight determination was done according to the protocol described in the book of “Protein Methods” (Bollag et al, 1996). The $R_i$ values were calculated for the sample and for the molecular weight markers as follows:

\[
R_i = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}
\]

Then the $\log_{10}$ of the known protein molecular weights was plotted as a function of their $R_i$ values. And after measuring the $R_i$ value of the sample, its molecular weight was calculated, by extrapolation from the graph (Figure-5), and algebraically by finding the equation of the two nearest points on the graph.
Figure-5: Graph of $\log_{10}$ of the molecular weights of the markers versus their Rf values.

The equation calculated for this specific graph was: $Y = -1.2625X + 5.041$. From which relative molecular weight of PTA was calculated.

These two methods were applied for 7 gels, 4 of which were one concentration (15%) gels and the other three were gradient (10-20 %) gels.
3.5.2. Results:
The relative molecular weights obtained are represented in table-4:

| Table-4: Calculated Molecular Weights for PTA |
|-----------------|----------------|
| Gel-1           | 20.89          |
| Gel-2           | 20.84          |
| Gel-3           | 20.18          |
| Gel-4           | 21.53          |
| Gel-5           | 20.32          |
| Gel-6           | 20.63          |
| Gel-7           | 21.07          |
| Mean ± SEM      | 20.78 ± 0.17   |

With PTA having about 13 KDa as its molecular weight, estimated from amino acid sequencing, the molecular weight that has been calculated conforms with the theory that PTA is found as a dimer in SDS-PAGE conditions, presented by Goredero et al (1992), in which PTA was found to appear as a band at 20.6 KDa ± 5%.

3.6. Effects of different SDS-PAGE conditions:

3.6.1. Procedure:
Since prothymosin alpha appeared as a dimer of molecular weight 20.78 KDa, further investigation was carried out to complete the characterization of PTA on SDS-PAGE. As such, various conditions were created to study the stability of PTA as a dimer.
A large piece of thymic tissue, obtained by pooling several thymus glands, was
pulverized as a whole under liquid nitrogen. The extraction procedure was as
mentioned earlier. The clear supernatant obtained after boiling, homogenization
and centrifugation was distributed equally to 9 tubes and treated for isoelectric
precipitation. Each 3 tubes where used to test the effect of the increase in the
concentration of one substance.
The conditions included increasing the concentrations, in the sample buffer, of:

1. SDS: 4, 8, and 16 %
2. Urea: 1, 4, and 8 M
3. β-mercaptoethanol: 10, 20, 30 %

3.6.2. Results:

The banding pattern remained the same at all conditions, as for SDS (Figure-6),
for Urea, and for β-mercaptoethanol.
Under no conditions did prothymosin alpha appear as a monomer, which means
that it is stable as a dimer. (Only the results for SDS test are shown since the
other two tests showed exactly similar results).
3.7. Standardization of the protocol

The procedure used in the preliminary extraction of prothymosin alpha, combined with the semi-selective isoelectric precipitation and the quantitative aspect of Coomassie Blue staining in SDS-PAGE can form a well organized protocol for PTA quantification. However for this protocol to be quantitative, it needed some standardization.
3.7.1. The temperature needed for proteolytic enzyme inactivation:

3.7.1.1. Procedure: It is well documented (Haritos et al, 1984) that boiling for 5 min is needed for inactivation of most proteolytic enzymes and hence the preservation of PTA. However it has been proposed that placing the tissue at 65°C for 15 min would be sufficient to inactivate proteolysis. To test this hypothesis, extractions were performed at:

1. 60°C for 15 min
2. 80°C for 10 min
3. 90°C for 10 min
4. Boiling for 5 min

3.7.1.2. Results: There was a significant increase in the band density from 60 to 80°C. No significant increase from 80 to 90°C and again a significant increase from 90°C to boiling (Figure-7). These data confirm further that the band under consideration is preserved by boiling, in conformity with Haritos et al (1984). And as such the step of boiling for 5 min is essential to the protocol of quantification of PTA.

![Image](image_url)

**Figure-7:** The increase in PTA yield with the increase in the temperature treatment. Lane-1: 60°C; Lane-2: 80°C; Lane-3: 90°C; and Lane-4: Boiling.
3.7.2. Choice of the extraction solution:

Two solutions are most widely used in the extraction of proteins, especially prothymosin alpha; and these are Sodium Phosphate Buffer (pH 7.4) and normal saline (0.9%). Both were tested in parallel and yielded the same result. As such normal saline was chosen as more appropriate, since it is cheaper and easier to prepare.

3.7.3. Standardizing the periods needed for isoelectric precipitation:

3.7.3.1. Procedure: The isoelectric precipitation consists of mixing the clear supernatant (obtained from thymic homogenization and centrifugation) with 10 volumes of tri-sodium citrate (pH 3.55) and leaving them at 4°C. The next step being the centrifugation in order to precipitate the aggregated proteins. In order not have errors due to variable times of incubation and centrifugation, both these steps were tested.

1. For the same extraction, 4 different incubation times were tested: 10, 20, 40, and 60 min.
2. For the same extraction, 4 different centrifugation times were tested: 10, 20, 40, and 60 min.

3.7.3.2. Results:

10 min were found sufficient in both steps in order to precipitate all of the protein found in the supernatant. However, to keep a margin of safety 20 min was considered the optimal time for incubation and centrifugation.
3.7.4. The final form of the protocol:

1. Thymic tissue is well pulverized under liquid nitrogen.
2. Pulverized tissue is weighed and directly placed in boiling saline in a ratio of 100mg/2ml. And kept to boil for 5 min with quick vortexing once or twice.
3. The solution is then cooled on ice.
4. Then it is homogenized (also on ice) with a Polytron Homogenizer for three minutes.
5. The homogenate is then centrifuged at 13,000 rounds per minute for 25 min.
6. The clear supernatant is removed and placed in a clean tube and 10 volumes of pre-chilled tri-sodium citrate buffer (pH 3.55) are added.
7. The mixture is left to stand without disturbance at 4°C for 20 min.
8. The mixture is afterwards centrifuged at 5,000 rounds per minute.
9. The supernatant is decanted and the precipitate resuspended in SDS-PAGE sample buffer, and stored at – 20°C until assayed by electrophoresis.
Chapter 4

ALCOHOL TREATMENT AND ITS EFFECTS

4.1. Materials and Methods

4.1.1. Experimental Design:

Four experiments were performed to test the effect of acute alcohol intake on the thymus. These four experiments included two types of doses labeled lower dose and higher dose. The lower dose consisted of 3 g ethanol/kg body weight and the higher dose of 4.4 g ethanol/kg body weight. In both cases, the effect was studied over a very short period (3 or 5 hours) and for a longer period of 48 hours.

Ethanol was administered through intraperitoneal injections. The concentration of ethanol in the injected solution was 20 \% (w/v), to reduce the effect of ethanol on soft tissues. All control animals received an equal volume of 0.9 \% saline to eliminate the placebo effect. Moreover, to reduce the possibility of infections, solutions were prepared using autoclaved water, under sterile conditions, and sterile syringes were used for the injections.

Prior to the injections, rats were weighed and the proper volume for injection was calculated for each rat so that it will receive exactly the same amount of ethanol per kilogram of body weight.

For the two days incubation period injections were given between 9:00 and 9:30 a.m. and animals sacrificed between 10:00 and 11:00 a.m.
For the 3 hours incubation period injections were given between 9:00 and 9:30 a.m. and animals sacrificed between 12:00 and 12:30 p.m.

Rats were sacrificed by cervical dislocation and quickly dissected (Figure-8) and all thymic tissue carefully removed within 5 min of slaughter. The thymus was washed with saline solution and blot dried.

After weighing the thymus, a small piece was cut and placed in 4% formaldehyde for histological studies and the rest of the thymus directly placed in liquid nitrogen for instant freezing. At the end of each experiment, the tissues were removed from the liquid nitrogen bucket and stored at −80°C until processed.

Specific experimental conditions were as follows:

**Experiment-1:**

In experiment-1, three groups of animals were treated.

1. **Group control** (n=8): received saline injections.
2. **Group-I** (n=8): received an injection of 3g ethanol/kg body weight and were sacrificed after 3 hours.
3. **Group-II** (n=8): received an injection of 3g ethanol/kg body weight on the first day and another on the second day; they were sacrificed on the morning of the third day.

**Experiment-2:**

1. **Group control** (n=7): received two saline injections over two days; and were sacrificed on the third day.
2. **Group Alcohol** (n=7): received two 4.4g ethanol/kg body weight injections over two days and were sacrificed on the third day.
Experiment-3:
1. Group control (n=6): received one saline injection and were sacrificed after three hours.
2. Group alcohol (n=8): received one 4.4g/Kg injection and were sacrificed after three hours.

Experiment-4:
1. Group control (n=6): received one saline injection and were sacrificed after 5 hours.
2. Group Alcohol (n=6): received one 4.4g/kg injection and were sacrificed after 5 hours.
Figure 8: The dissected animal as it appeared in every surgical procedure performed in various experiments. After removal of the anterior part of the thoracic cage, the thymus gland becomes conspicuous at the upper end of the mediastinum.
4.1.2. Blood Alcohol Levels:

Blood alcohol levels achieved after each intraperitoneal injection were tested for both doses: 3g/Kg and 4.4 g/Kg body weight.

Tail-tip blood samples were collected after 90 minutes and 180 minutes, post-injection, measurements were performed spectrophotometrically with an alcohol dehydrogenase kit (Sigma Diagnostics., St Louis MO).

4.1.2.1. Principle:

\[
\text{Ethanol + NAD} \xrightarrow{\text{ADH}} \text{Acetaldehyde + NADH}
\]

Alcohol dehydrogenase (ADH) catalyzes the oxidation of alcohol to acetaldehyde with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH. The consequent increase in absorbance at 340 nm is directly proportional to alcohol concentration in the sample.

4.1.2.2. Reagents and Equipment:

1. NAD-ADH single assay vial: NAD 1.5 μmoles, ADH (yeast), 150 units, and buffer salts.
2. Glycine buffer reagent: Glycine 0.5 mol/l, pH 9.0.
3. Ethanol Standard Solution: Ethanol 0.08 % (w/v).

Spectrophotometric measurements were performed using a Helios spectrophotometer (Thermospectronic-Unicom unlimited).
4.1.2.3. Procedure:
1. Blood samples collected were directly centrifuged and 10 µl of plasma were used for the assay.
2. 3 ml of Glycine buffer reagent were added to each NAD-ADH vial, which was capped and inverted gently several times to dissolve contents.
3. 10 µl of plasma were then added and mixed gently by inversion.
4. Solutions were allowed to incubate for 10 minutes at room temperature after which absorbance was measured at 340 nm.

10 µl of deionized water instead of the 10 µl of sample were used as a blank.

4.1.2.4. Calculations:
Ethanol concentrations were calculated relative to the standard ethanol solution containing 0.08 % (80mg/dL) by the following formula:

\[
\text{Alcohol, mg/dl} = \frac{\text{Absorbance at 340 nm of the sample}}{\text{Absorbance at 340 nm of the standard}} \times 80
\]

4.1.3. Histological Study:

The histological study of the thymus was performed according to the general procedure presented by Davenport in his book “Histological and Histochemical techniques” (1960). However, this procedure is a general haematoxylin and eosin staining procedure; as such, some steps were slightly modified to suit the purpose of thymus staining.
1. Pieces of thymus glands, not more than 5 mm thick, were carefully cut. (Care was taken, that all the pieces collected from different animals were always from the posterior end of the right lobe).

2. The cut pieces were fixed in 4% formaldehyde overnight at 4°C.

3. After fixation, the pieces were stored in 70% alcohol at 4°C until processed. Further dehydration included 3 hours in 96% alcohol and 1 hour in pure alcohol. (The original procedure indicated dehydration, directly after fixation, for 6 hours in 96% alcohol and 6 hours in pure alcohol. As such, the modification in the dehydration times included an elongated incubation time in 70% alcohol and a decrease in the incubation times in 96% alcohol and especially in pure alcohol, thus preventing the hardening of the thymic tissue.)

4. The dehydrated tissue was then placed in xylene for about 1 hour, which was enough to clear the tissue. (Also here a modification of the usual overnight incubation time with the clearing agent was considered, since an elongated incubation time with xylene would also lead to the hardening of the thymic tissue.)

5. The tissue was then placed in melted paraffin in the oven at 50-52°C, for three changes, each of one hour.

6. The piece of tissue was embedded with the surface to be cut facing downward; and directly put in salted ice water to harden. The fast cooling is needed to prevent formation of air bubbles in the wax around the tissue. (The usual embedding box has a cubic shape which would take us too much time...
to trim the wax around the tissue. Therefore a special embedding box was designed (Figure-9).

![Image](image.png)

**Figure 9:** This box is made of aluminum and once sealed with adhesive tape, it would have two cylindrical compartments of varying diameters (2.1 cm and 1.2 cm); the tissue would be placed at the bottom of the small compartment and the melted wax poured over it. Once hardened, the tape would be removed and the box opened and only fine trimming is needed before the block becomes ready for microtome cutting.

7. The block was then attached to the wax block holder of the microtome, with the embedded tissue facing the knife. It is important that the surface to be cut should be in the same plane as the traverse of the blade across the block.

8. Sections of 5-10 micra were cut. And then floated on water bath at 45°C.
9. After flattening out, the sections were lifted onto albuminized glass slides, on which a thin film of a solution of egg albumin and glycerol in a 1:1 ratio was made. After excess water was removed, sections were heated in an oven at 50 to 55°C for at least one-half hour.

10. Sections were afterwards removed from the oven, allowed to cool, and brought to water through two changes of xylene, each of 5 minutes duration. Then with two changes of absolute alcohol each of 3 minutes duration, and two changes of 95% alcohol, each of 3 minutes duration, and then placed in tap water.

11. First staining was with Harris' haematoxylin 3 to 5 minutes. Differentiation was in acid alcohol and sections were left to develop a blue color, 5 to 10 minutes in tap water.

12. Sections were counterstained in 0.5% eosin in 70% alcohol for 2 to 3 minutes. Excess eosin was removed by placing in 95% alcohol to which a few drops of eosin have been added and left for half a minute.

13. Sections were dehydrated in two changes of 95% alcohol rapidly and left in absolute alcohol, two changes, each of 3 minutes.

14. Sections were cleared in xylene, two changes, each of 3 minutes, and mounted in balsam.

Sections were viewed on a Nikon Eclipse TE 300; inverted light microscope (Nikon Corp. Japan) and their impressions saved.
4.1.4. Estimation of total protein content (Lowry Assay):

A widely used quantitative assay for determining protein content in a solution (Lowry et al., 1951).

4.1.4.3. Protocol (Scopes, 1982)

Solutions

1. Solution A, 100 ml
   - 0.5g CuSO₄·5 H₂O
   - 1 g Na₃C₆H₅O₇·2H₂O
   Add distilled water to 100 ml
   Solution may be stored indefinitely at room temperature.

2. Solution B, 1 liter
   - 20 g Na₂CO₃
   - 4g NaOH
   Add distilled water to 1 liter.
   Solution may be stored indefinitely at room temperature.

3. Solution C, 51 ml
   - 1 ml Solution A
   - 50 ml Solution B

4. Solution D, 20 ml
   - 10 ml Folin-Ciocalteu phenol reagent (Sigma)
   - 10 ml distilled water
• Assay

1. Bring sample solution to 0.5 ml with distilled water: 100 μl of the homogenate supernatant (as mentioned above), were mixed with 400 μl of distilled water.

2. 2.5 ml Solution C were added.

3. The solution was vortex mixed and left to stand at room temperature for 5 - 10 minutes.

4. Then 0.25 ml Solution D was added and vortex mixed.

5. After 20 - 30 minutes, absorbance at 750 nm was read, using a Helios spectrophotometer (Thermospectronic-Unicom Unlimited).

A standard curve (Figure-10) was constructed using a serial dilution of BSA (Bovine Serum Albumin) and its linear equation was worked out to be:

\[
Y = 0.0045X.
\]

After reading the absorbance obtained for each sample, its protein concentration was calculated according to this equation.
Figure 10: The standard curve for Lowry assay, obtained from the serial dilution of BSA.
4.1.5. Quantification of PTA:

Prothymosin alpha quantification was performed according to the protocol developed in chapter-3. As various proteins have different chromogenicity by binding differently to Coomassie Blue, and as different gel and staining conditions might vary slightly, standard concentrations of the standard PTA (Thymoorgan, Germany) were included in each gel.

The standards were 10, 20, and 40 µl of sample buffer solution containing, respectively, 2.5, 5, and 10 µg of PTA. A standard curve (Figure-11) was constructed for each gel and the linear equation found. From this equation the density of a sample PTA band was transformed into µg of PTA.

Thymic tissue was treated according to the protocol in a proportion of 2 ml for each 100 mg. However, the clear supernatant, prior to the isoelectric precipitation was aliquoted into 0.5 ml aliquots. This was due to the fact that the amounts of PTA found in 100 mg of thymic tissue, and subsequently in 2 ml of the homogenate, were larger than what can be estimated by the range of sensitivity of Coomassie Blue. The amounts of PTA found in 0.5 ml of the supernatant were in the range of the standard curve. The values were calculated for 0.5 ml and then multiplied by 4 to yield the correct amounts found in 2 ml and hence in 100 mg of tissue (The values that are presented later on are in µg PTA/100 mg thymic tissue).

4.1.6. Statistics:

All means are presented (as indicated), as means ± SEM. Comparison of data sets were performed with two-tailed, homoscedastic (2 sample equal variance) Students t test.
Figure 11: PTA Standard curve constructed for one gel. A similar curve was constructed for every gel and from its linear equation; PTA levels were calculated for each sample.
4.2. Results:

4.2.1. Blood Alcohol Levels:

Calculated blood alcohol levels are presented in table-5. Five rats were found sufficient, as the alcohol levels were in conformity with those found in the literature (D'Souza et al, 1995).

<table>
<thead>
<tr>
<th>Table-5: Blood Ethanol Levels (mg/dl)</th>
<th>After 90 min</th>
<th>After 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For 3g/Kg:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat-1</td>
<td>271.1</td>
<td>281.5</td>
</tr>
<tr>
<td>Rat-2</td>
<td>264.8</td>
<td>262.2</td>
</tr>
<tr>
<td>Rat-3</td>
<td>302.6</td>
<td>300.7</td>
</tr>
<tr>
<td>Rat-4</td>
<td>270.6</td>
<td>273.0</td>
</tr>
<tr>
<td>Rat-5</td>
<td>279.0</td>
<td>284.0</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>277.6 ± 6.64</td>
<td>280.3 ± 6.37</td>
</tr>
<tr>
<td><strong>For 4.4g/Kg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat-1</td>
<td>369.6</td>
<td>359.6</td>
</tr>
<tr>
<td>Rat-2</td>
<td>360.0</td>
<td>379.6</td>
</tr>
<tr>
<td>Rat-3</td>
<td>400.0</td>
<td>411.1</td>
</tr>
<tr>
<td>Rat-4</td>
<td>370.5</td>
<td>380.0</td>
</tr>
<tr>
<td>Rat-5</td>
<td>375.0</td>
<td>377.0</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>375.0 ± 6.71</td>
<td>381.5 ± 8.3</td>
</tr>
</tbody>
</table>
4.2.2. Results for experiment-1: The lower dose of 3g ethanol/Kg body weight.

4.2.2.1. Histology: (Figure 12)

There was no marked effect on the histological aspects of the thymus in response to the low dose of ethanol (3g/kg body weight).

Figure 12: Experiment-1: Lower dose of 3g/kg body weight for two days: a) Group Control; b) Alcohol Group (3g/Kg body weight for two days). (Haematoxylin and Eosin; 100X)
4.2.2.2. Thymus/Body percentage:
There was no significant effect of 3g ethanol/kg body weight on the thymus/body weight percentage, neither over 3 hours, nor over two days. The thymus/body weight percentage for the control group was 0.258 ± 0.014 %; for the three hours period it was 0.255 ± 0.015 %; and for the two days incubation period it was 0.248 ± 0.02 % (Mean ± SEM). (Figure.13)

**Figure.13:** Thymic/body weight percentages for experiment-1: 3 g/kg body weight. Group-I: incubation time of three hours; Group-II: incubation time for two days.
4.2.2.3. Total protein content:
There was no change in the total protein content of the thymus for the 3 g/kg body weight neither for 3 hours, nor for the two days incubation periods. For the control group the total protein content was $1.30 \pm 0.02$ mg protein/100 mg thymic tissue; for the three hours incubation period it was $1.32 \pm 0.01$ mg, and for the two days incubation period it was $1.29 \pm 0.01$ mg. (Mean ± SEM) (Figure.14).

![Graph of protein content](image)

**Figure.14:** Total protein content for experiment-1: 3 g/kg body weight. Group-I: incubation time of three hours; Group-II: incubation time for two days.
4.2.2.4. PTA Levels:

PTA Levels were not significantly affected by the 3 g/kg body weight dose. PTA levels for the control group were $31.25 \pm 0.63 \mu g \text{PTA/100 mg thymic tissue}$, $31.06 \pm 0.44$ for the three hours incubation period, and $30.66 \pm 0.33$ for the two days incubation period (Mean $\pm$ SEM) (Figure.15).

![Bar chart showing PTA levels](image)

**Figure.15:** PTA levels for experiment-1: 3 g/kg body weight. Group-I: incubation time of three hours; Group-II: incubation time for two days
4.2.3. Results for Experiment-2:

At 4.4 g/kg (high dose), pronounced effects have been observed at all levels.

4.2.3.1. Histological Studies

A significant reduction in the cortical region was observed in the group treated with 4.4 g ethanol/kg body weight for two days (Figure-16).

**Figure-16:** Thymus sections for experiment-2: 4.4g/kg body weight- for two days. a) Group Control; b) Alcohol Group (4.4 g/kg body weight for two days) (Haematoxylin and Eosin; 100X).
4.2.3.2. Thymus/Body weight percentage:
At the high dose of 4.4 g/kg body weight the thymic/body percentage decreased by 46 % (p < 0.05). For the control group it was 0.354 ± 0.010 % and 0.191 ± 0.007 % for the 4.4 g/kg body weight injection for two days incubation period. (Figure.17)

![Bar chart showing thymic/body weight percentages for control and alcohol groups.]

Figure.17: Thymic/body weight percentages for experiment-2: 4.4 g/kg body weight for two days incubation period.
4.2.3.3 Total protein content:

The total protein content of thymic tissue increased under the 4.4 g/kg body weight, with an incubation of two days, for about 14% (p < 0.005). For the control group it was found to be $1.17 \pm 0.04$ mg protein/100 mg thymic tissue and for the alcohol group it was $1.36 \pm 0.04$ (Mean ± SEM) (Figure-18).

![Figure 18: Total protein content for experiment-2: 4.4 g/kg body weight for two days incubation period.](image-url)
4.2.3.4. PTA Levels:

Also a decrease of about 13% was observed in PTA levels for the 4.4 g/kg body weight treatment (p < 0.05). For the control group it was found to be 27.69 ± 0.19 μg PTA/100 mg thymic tissue and 24.09 ± 0.31 for the alcohol treated group (Mean ± SEM) (Figure-19).

**Figure.19:** PTA levels for experiment-2: 4.4 g/kg body weight for two days incubation period.
4.2.4. Results for experiment-3: High dose of 4.4g ethanol/kg body weight over 3 hours period.

4.2.4.1. Thymus/Body proportion:
No effect was observed for the 3 hours treatment with 4.4 g ethanol/Kg body weight. For the control group, thymus/body weight percentage was found to be \(0.255 \pm 0.097\) and for the alcohol group it was \(0.256 \pm 0.005\) (Mean ± SEM) (Figure-20).

![Bar graph showing thymus/body weight percentages for control and alcohol groups.](image)

**Figure 20:** Thymic/body weight percentages for experiment-3: 4.4 g/kg body weight for three hours incubation period.
4.2.4.2. Total Protein Content:

Also the total protein content was not affected under the influence of the 4.4 g/kg body weight dose for the incubation period of three hours. The total protein content for the control group was 1.17 ± 0.02 mg/100 mg thymic tissue and for the alcohol treated group it was 1.22 ± 0.01 (Mean ± SEM) (Figure-21).

Figure.21: Total protein content for experiment-3: 4.4 g/kg body weight for three hours incubation period.
4.2.4.3. PTA Levels:
Prothymosin alpha levels were not affected after 3 hours of the 4.4g/kg injection. For the control group the levels were 38.33 ± 0.93 µg PTA/100 mg thymic tissue and 39.47 ± 0.74 for the alcohol treated group (Mean ±SEM) (Figure.22).

**Figure.22:** PTA levels for experiment-3: 4.4 g/kg body weight for three hours incubation period.
4.2.5. Results for experiment-4: 4.4 g ethanol/kg body weight, over 5 hours period.

4.2.5.1. Thymus/Body Proportion:
After 5 hours, the high dose of 4.4g/kg affected the thymus/body weight percentage; there was a 23.3 % decrease (p<0.05). For the control group thymic/body weight percentage was 0.116 ± 0.007 and for the alcohol treated group it was 0.089 ± 0.004 (Mean ± SEM) (Figure- 23).

![Graph showing thymic/body weight percentages](image)

**Figure.23:** Thymic/body weight percentages for experiment-4: 4.4 g/kg body weight for five hours incubation period.
4.2.5.2. Total Protein Content:

There was a slight increase in total protein content due to 5 hours of the 4.4g/kg dose; the increase was only about 3% but with statistical significance ($p < 0.05$). Total protein content for the control group was found to be $1.26 \pm 0.01$ mg protein/100 mg thymic tissue and for the alcohol treated group it was found to be $1.30 \pm 0.01$ (Mean ± SEM) (Figure-24).

![Figure 24: Total protein content for experiment.4: 4.4 g/kg body weight for five hours incubation period.](image-url)
4.2.5.3. PTA Levels:
Prothymosin alpha levels were decreased by 5% (p < 0.05). It was found to be
28.76 ± 0.24 μg PTA/100 mg thymic tissue for the control group and 27.33 ±
0.28 for the alcohol treated group (Mean ± SEM) (Figure-25).

Figure 25: PTA levels for experiment-5: 4.4 g/kg body weight for five hours
incubation period.
Chapter 5

DISCUSSION

5.1. Introduction:

The discussion of the results obtained is divided into two main parts, based on the ramifications of the topics treated within this study.

The preliminary and basic subject of this research was the effect of “alcohol intake on immunity”, and specifically the effect of acute alcohol intake, as a single dose or as two successive (within two days period) doses that are enough to elevate blood ethanol level, to a point causing intoxication. This state of intoxication has various implications on the different body systems, of which one of the most important and one of the most affected: the immune system.

Many parameters involved in the immune response have been found to be strongly affected by alcohol intake. However, a small area remains partly shadowed, as the number of studies concentrated on the effect of alcohol intake on the maturation of T-cells, as thymocytes within the thymus gland, remains limited. This study was mainly designed to cast a light on this specific topic to point out that studying the effect of alcohol on immunity has to include the important aspect of T-cell maturation along with all the other parameters commonly studied in this field such as the levels and subtypes of circulating lymphocytes, T-cell activation, cytokine production, cell mediated immunity, humoral immunity and antibody formation.

Many “traditional” methods were proposed to be utilized in this study; however, the search was ongoing to find a new parameter that would be reliably connected to the health of the thymus and to the T-cell maturation process. This parameter
was found to be “Prothymosin alpha”, a protein discovered in the early eighties (Haritos et al 1984,a) and that has been shown to be an important factor in various T-cell immunological processes, specifically in the maturation of T-cells (Gomez-Marquez et al, 1988). In addition, a strong correlation has been well documented relating prothymosin alpha to cell proliferation (Gomez-Marquez et al, 1989), to the extent that it is currently proposed as a prognosis factor for cancer, in the sense that its levels in certain tissue can be used as a “proliferation index”.

As such the levels of prothymosin alpha in the thymus were chosen to be the parameter through which the effect of acute alcohol intake on the maturation of T-cells can be assessed. However, a small obstacle was the unavailability of commercial materials or fast custom protocols that can be applied in this study. Consequently a new goal was set and that is to develop a new fast and easy to perform protocol for the direct estimation of prothymosin alpha levels in thymic tissues.

Quantification on SDS-PAGE of the band corresponding to prothymosin alpha was proposed, however another problem was faced and that is the anomalous behavior of prothymosin alpha on gel electrophoresis and the controversy about its status in solutions, specifically those containing SDS. Here also another step was added to this research.

A final scheme for this study became: to elucidate the behavior of prothymosin alpha on SDS-PAGE, then to develop and standardize a simple protocol for its quantification and finally to apply this protocol (along with some traditional methods) in the study of the effect of acute alcohol intake on the maturation of T-cells.
5.2. Prothymosin alpha characterization and the development of a new protocol for its quantification:

5.2.1. Characterization of PTA:

Prothymosin alpha was first extracted and characterized by Haritos et al (1984). Since that time, a large amount of work has been put to elucidate the nature and functions of PTA. Some of the issues have been resolved; others are still a subject of controversy (Pineiro et al, 2000).

One of the most controversial problems is the behavior of PTA on SDS-PAGE. The problem has two aspects; first it is not well established if PTA appears as a monomer or as a dimer on denaturing gels (SDS-PAGE). The second is its relative molecular weight on denaturing gels, which is directly related to the extent of binding between PTA and SDS. Some publications reported that PTA appears as a monomer at 15 KDa (Palvimo et al, 1990), others reported it to appear, also as a monomer, but at about 13 KDa (Watts et al, 1990).

A detailed study was performed by Cordero et al (1992) that particularly characterized PTA on SDS-PAGE. They reported that under no conditions of denaturing gel electrophoresis have they obtained a band representing PTA as a monomer. Instead they have reported that PTA appears as a band at 20.6 KDa ± 5 %, supporting the theory of dimerization.

This dimer was tested for stability and was found to be resistant to SDS dissociation and further reduction by β-mercaptoethanol.

Recent reviews (Pineiro et al, 2000) are still skeptical about the behavior of PTA on SDS-PAGE, and consider it to be a controversial issue.

As such the first aim of this study was to detect the band representing PTA (in order to develop a protocol for the quantification).
The clear supernatant resulting from centrifugation, after the homogenization step, contained PTA. The choice of two of PTA's well documented characteristics (its isoelectric point of 3.55 and its solubility in 5% perchloric acid) and combining them in a single protocol enhanced the selectivity of the isolation procedure. The end product appeared as a single band on SDS-PAGE. When the relative molecular weight of this band was calculated it was found to be 20.78 ± 0.17 KDa (Mean ± SEM), which conforms to the value reported by Cordero et al (1992), (20.6 KDa ±5%).

The combination of these characteristics pointed out to prothymosin alpha. Further evidence was needed. As such a duplicate of the single band obtained on SDS-PAGE was run on RP-HPLC, along with a standard of bovine prothymosin alpha: HPLC results confirmed the identity of the band as PTA.

To complete the characterization of prothymosin alpha on SDS-PAGE a variety of denaturing conditions were created to test the stability of the dimer. These conditions included increasing the concentrations of SDS, Urea, and β-mercaptoethanol. None of these conditions was able to break the dimerization bonds of PTA.

The importance of these results is at several levels. First, it was confirmed that PTA appears as a dimer on SDS-PAGE, under all denaturing conditions, supporting the work of Cordero et al (1992). Second, the procedure of isoelectric precipitation, followed by re-solubilization with 5% PCA, could represent a new method for the isolation of PTA, replacing the several chromatographic steps usually used for this purpose. And third the band identified as PTA, can now be used in the development of the quantification protocol.
5.2.2. Development of the protocol:

The most widely applied procedures for PTA quantification include ELISA or RIA protocols, in which the antibodies (for PTA) are not available commercially and have to be prepared in the laboratory by injecting animals with the target protein and, after the proper incubation period, extract the serum and purify the polyclonal or monoclonal antibodies. This procedure takes more than 6 months and thus it is too sophisticated and time consuming (especially for the purpose of this study). In addition, assays based on antibody-antigen recognition and binding are specifically troublesome for prothrombin alpha, and the difficulties are encountered on many levels:

1. Prothrombin alpha is not an immunogenic protein, especially because of its small size and the small amount of variability that exists in its primary structure among species. Therefore there are difficulties encountered on the level of producing specific antibodies for PTA.

2. Prothrombin alpha is one of many in a large family of proteins called alpha thrombins, which have a great deal of similarity among each other. This fact reflects negatively on any procedure relying on the specificity of antibodies to detect prothrombin alpha, due to all the cross reactivity that might occur during the application of these assays.

All these facts show the need for a new protocol that would be easy to perform, fast, and that has a low relative cost.

After the proper characterization of prothrombin alpha's behavior on SDS-PAGE, and the detection of a single band confirmed to represent this protein, a new protocol was developed on the basis of the needs mentioned above.

The protocol proposed is a combination of the known preliminary steps of prothrombin alpha extraction, a semi-selective isoelectric precipitation step, and finally quantification through quantitative Coomassie Blue staining.
Standardization of the protocol was performed at each step. Starting from the first step where the extraction solution was chosen to be normal saline and 100 mg of tissue where placed in exactly 2 ml of this boiling saline for each sample tested. Even though the boiling step was emphasized in earlier publications (Haritos et al, 1984), to conserve PTA from proteolytic degradation, it was further tested to elucidate its quantitative implications. The results showed that an increase in temperature from 60 to 80, 90, and 100°C reflected in a gradual increase in prothymosin alpha amounts for the same amount of thymic tissue. This shows that temperatures lower than boiling are not sufficient to inactivate the total of the proteolytic enzymes or any proteolytic enzyme totally. This confirms the necessity of the boiling step, especially in a quantitative procedure.

The next steps of homogenizing the boiled tissue and centrifuging at 13,000 rpm were carried out as reported by Haritos et al (1984) without further testing, since these are routinely performed in the laboratory.

After the centrifugation step a certain volume (usually 0.5 ml) of the clear supernatant is mixed with 10 volumes of the tri-sodium citrate buffer (pH 3.55). Here, two important parameters had to be standardized as they are directly related to the protein yield; and these are the incubation period and the centrifugation period.

The last step in the protocol was the SDS-PAGE and the densitometry quantification of the protein. As the quantitative nature of Coomassie Blue differs from protein to another according to the nature of the protein which reflects on the binding extent of Coomassie Blue to the protein, it was essential to run a standard of PTA along with the sample, in each gel.

After standardizing all the steps of the new protocol it became ready to be applied in the next section, to evaluate PTA levels in the thymus.
It is important to note that this protocol does not replace RIA or ELISA assays. It is not as sensitive as these assays, since Coomassie Blue can only detect down to 0.1 μg of a certain protein. This range of sensitivity is adequate for thymic levels of PTA, but in experiments that need to detect lower amounts (or even lower variations) of PTA, an alternative protocol has to be set.

5.3. Effect of acute alcohol intake on the thymus:

The effect of acute alcohol intake on the rat thymus and hence on the maturation of T-cells was studied by observing the proportional changes of thymus/body weight percentage, the histological changes in the inner architecture of the thymus gland, and finally by measuring the amounts of prothymosin alpha in thymic tissues relative to total protein content.

The effect of a single intraperitoneal injection of ethanol on rats (4 g ethanol/kg body weight) was reported by Budic et al (1992). It was shown that after 20 hours incubation the percentage of thymus/body weight was reduced by about 32 %, and after histological investigations the reduction was found to be mainly in the cortical region, where the thymocytes are mostly concentrated.

Two doses were investigated in this study: a lower dose of 3g ethanol/kg body weight and a higher dose of 4.4 g/Kg. For each of the doses the effect was investigated over a short period of 3 hours; and over a longer period of two days, during which two injections were given to the rat; one for each day to maintain a state of intoxication. In addition a third incubation period of 5 hours was tested for the higher dose.
The results showed that the lower dose of 3g/kg did not have any pronounced effect on the thymus, neither for the thymus/body weight proportion, nor for the histology of the thymus, nor for the total protein content and PTA concentration. These results are true for the 3 hours incubation and for the two days incubation periods.

However, pronounced effects were observed for the high dose of 4.4g/kg over the two days incubation period, milder effects for the 5 hours but no effect for the 3 hours incubation period.

An important aspect of the results obtained, both for the high dose and for the lower dose is the consistency of these results among the different parameters studied. In such a way that when there is a decrease in thymus/body weight (such as in the case for the higher dose), a reduction of the cortical region can be also observed on the histological level. And when we do not have a reduction in the cortical region, at the histological level (the lower dose), there is no decrease in thymus/body weight.

The proper interpretation of the changes in prothymosin alpha levels, relative to the total protein content, is of extreme importance. A decrease in PTA levels within the short period of incubation would indicate a direct effect of ethanol on prothymosin alpha. Such a decrease would not be accompanied by a change in the total protein content of the thymus. Furthermore, a decrease in PTA levels over two days, without a change in the general histology of the thymus and without a change in total protein content would also indicate a direct effect of ethanol on PTA. However, the results obtained in this study do not conform to any of the above mentioned. Which does not support a direct effect of ethanol on PTA.
A decrease in PTA levels (which occurs at the high dose, for the two days incubation period), accompanied by a reduction in the cortical region and a slight increase in the total protein content can be interpreted as follows: Prothymosin alpha is an intracellular protein (intra-nuclear, to be specific) (Gomez-Marquez et al, 1988; Watts et al, 1989), and is mainly concentrated in the thymocyte population of the thymus (Gomez-Marquez et al, 1989). 4.4g/kg ethanol treatment causes a reduction in the thymocyte population, indicated by a reduction of the cortical region; as PTA is mainly concentrated in thymocytes, its levels would naturally decrease. Moreover, when the thymocyte population decreases in the thymus, there would be an increase in the fiber proportion, which explains the slight increase in total protein content.

As such even though there was no direct correlation between ethanol treatment and prothymosin alpha levels, the measurement of PTA levels was shown to have a very important indication. These measurements came in conformity and supported the results obtained from the histological studies, to explain that the decrease in thymus/body weight percentage was due to a loss of thymocytes, and not any other thymic subpopulation. Here, the levels of PTA were not used as a proliferation index, but rather as a thymocyte concentration index.

The interpretation of the effects of different doses, as for the 3 g/kg, 4.4 g/kg body weight reported in this study, in addition to the effects reported by Budec et al (1992) for the 4g/kg body weight, indicate that a certain threshold exists for the effect of alcohol on the thymus.

An intraperitoneal injection of 3 g/kg body weight would elevate blood ethanol levels to about 280 mg/dL, which is a considerable amount; however, no pronounced effect is observed. The 4.4g/kg body weight injection would elevate the levels to 380 mg/dL and here severe effects were observed. Even at 4g/kg body weight (as reported by Budec et al, 1992), pronounced effects were observed. Which would lead to the conclusion that a certain threshold exists at
some point between the 3 g/kg and the 4 g/kg doses. Ethanol levels that exceed this point become harmful to the thymus gland.

The mechanisms of action of ethanol in immunosuppression have been extensively investigated. Ethanol is known to cause thymocyte apoptosis. Both a direct mechanism of action (Shao et al., 1995) and an indirect mechanism involving the hypothalamic-pituitary-adrenal axis (Watson et al., 1993) have been proposed. Many researches supported the fact that ethanol injections raise plasma ACTH, which would result in an increase in corticosterone levels, which are strong immunosuppressant, especially at the thymic level.

The data obtained in this study do not oppose the involvement of the HPA axis in the mechanism of ethanol immunosuppression; however, they propose an additional direct effect of ethanol on thymocytes.

At the high dose of 4.4 g/kg body weight no effect was observed after 3 hours and a large effect observed over 48 hours. However, the important observation was for the 5 hours incubation period for this high dose, where a significant decrease was observed for the thymus/body weight percentage (23.3% decrease- p < 0.05) and a slight, but statistically significant (p < 0.05), decrease in PTA levels (5 %), in addition to a 3 % increase in total protein content. These data show that after 5 hours, the effects of thymocyte apoptosis become observable, which means that apoptosis has started even earlier. This short time of action supports the idea of direct effect of ethanol on thymocytes, without contradicting further effect by the HPA axis.

5.4. Conclusions:

Acute alcohol intake was shown to affect the thymus, and thus the maturation of T-cells; however the pronounced effect was observed for the high dose of 4.4
g/kg body weight in a two days period; and no pronounced effect was observed for 3 g/kg body weight for two days period.

These results indicate the presence of a certain threshold in acute alcohol intake, above which it becomes extremely dangerous. Further investigation, with an expanded spectrum of doses and incubation periods, is required to elucidate this finding and to clarify the underlying mechanism.
Bibliography:


Appendix

The complete cDNA sequence for rat prothymosin alpha:

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1  gccatctttg eatttgctcc cgggtggtgc toegcgtcag eagccaaagcc
   aacctatcgg
61  ccgacccacc ggctcctctc caaagcggga ctctcggcta gcttatctcg
  gccaagagtc
121 ctcgaaactc gacatatattc taeccgtaga ccagacaccg ggctgctcccc
   accatgtcag
181 aecgcggcagt ggacaccacg tcgagataca ccaccaagga cttgaaggag
   aagagaagag
241 ttgtggagga ggacagagaat ggaagagacg cacctgccaa tgggaacgct
   caaaatgagg
301 aaaaaattgga gcagggaggt gacaatgagg tagatagaag agaggaagaa
   ggtggggagg
361 aagaggagga ggagagaagaa gggtgtgttg aggaagaaga tggagatgaag
   gatgagagag
421 ctcgggcttc tacgggcaag cggtgatctg aggatgtagaa ggtatgtgat
   gtggagacca
481 agagcagaa ggaagctgat gagagtacctg aagcagccaa agaggaaagct
   aaccttacgc
541 acggtgacct attcaccttc cactcctcgt ctcaagatctt aacggtggtc
   accttgagat
601 agagaagcag gcccccgcgga cccactcagg gtgcacccac acatgacatg
   cegctctctac
661 accacaaaa cacaacatga ttgcacatgg agagaaaaga acagaacctcc
   agggctctttt
721 ttatctaaa aatcttttaaa ggaattggttgg attttattaa ctaagcataat
   tggacagca
781 ttaataaaaatt cgacaca
```
And the deduced amino acid sequence:

"MSDAAVDTSSSEITTKDLKEKKEVVEEAENGDAAPANGNAQNEENGEQEADEVENDEEGGEEEEEDGDEDEEAEAPTGKRVAEDDEDDVETKKQKTDEDD"