# ON THE SAFETY OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH)

## THE MAXIMUM TOLERATED DOSE (MTD) IN DOGS IS 500 MG PER KG*

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Running title: Safety and Maximum tolerated Dose (MTD) of NADH

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**Summary:**

The objective of the study was to determine the maximum tolerated intravenous dose (MTD) of ßNADH (reduced form of nicotinamide adenine dinucleotide) in beagle dogs and to evaluate the potential toxicity of the established MTD in these animals for 14 days. Two male and two female dogs received 100 mg NADH/kg/day, for 4 days followed by 200 mg NADH/kg/day for 3 days, followed by 500 mg NADH/kg/day for 4 days, and 1000 mg NADH/kg/day on the final day.

At the end of the MTD phase the control animals which have received saline solution in the MTD phase were used for the fixed dose phase in which they received 500 mg NADH/kg/day for 14 days. There were no deaths.

At dose levels between 100 and 1000 mg/kg/day changes that identified the cardiovascular system as a target system were observed. There was also some evidence of an effect in the central nervous system and the adrenals. At doses of 500 mg/kg/day and above food consumption and body weight were reduced. On the basis of the observed changes, the maximum intravenous dose of NADH that was tolerated by beagle dogs was considered to be 500 mg/kg/day. In further experiments the toxicity of the stabilized orally absorbable form of NADH (ENADA®) was determined following oral administration for 14 days. The oral administration of the stabilized orally absorbable form of NADH to beagle dogs at dose levels of 20, 100, and 150 mg/kg for 14 days elicited no signs of a toxicological effect. A transitory change in stool formation was observed in intermediate and high dose males.

There were also apparent increases in adrenal, heart, kidney, liver, brain and thyroid weights, particularly in males, but none of the changes were considered to be toxicologically significant. There were **no** grosser microscopic findings suggestive of toxicity in the organs of tissues examined. Based on these findings the stabilized orally absorbable form of NADH (ENADA®) can generally be regarded as safe (GRAS).

## Introduction:

NADH (Nicotinamide adenine dinucleotide hydride) is a coenzyme essential for energy production, hence present in all living cells. This coenzyme has been used as a new therapeutic approach for PD (Parkinson´s Disease) (Birkmayer et al 1993), for depression (Birkmayer & Birkmayer, 1991) and for CFS (Chronic Fatigue Syndrome) (Forsyth et al. 1999). For more extensive, multicenter clinical trials to be approved by the health authorities in the European Union and in the US, toxicology studies are a prerequisite.

Hence the maximum tolerated intravenous dose (MTD) of NADH was determined in beagle dogs, and the toxicity of this dose level was evaluated for 14 days. The intravenous route of administration was chosen because it is a possible human therapeutic route. Dogs were selected because they are one of the non-rodent species recommended by various regulatory authorities. In addition, the toxicity of the stabilized orally absorbable form of NADH (ENADA®) (Birkmayer 1994 a, Birkmayer 1994 b) was determined following oral administration for 14 days as this form was used in the CFS study.

## Materials and Methods

### 1. Experimental design and dose levels

Maximum Tolerated Dose (MTD) for the intravenous Phase and for Fixed Dose Phase

The test substance, NADH, was a white powdered substance. When not in use, NADH was stored at about 4°C, protected from light and moisture prior to solution preparation. The control article and vehicle for the test article was physiological saline. The test and control articles were administered intravenously at a rate of 0.5 mL/minute, into the cephalic veins, alternating - where possible - between left and right.

For the MTD Phase, two groups (2 males and 2 females per group) received intravenously either the physiological saline or NADH solutions of varying concentrations (Table 1). The NADH concentrations were based on a prior rat study.

The control group received the saline control for 14 days, while the test group received the intravenous NADH in saline solution at varying doses at different times during the investigation.

| Table 1: Study Schedule | | | | | |
|---|---|---|---|---|---|
| Group Number | Group Prescription | Dose Level (mg/kg/day) | Day of Study | Animals Group | |
| | | | | Male | Female |
| 1 | Control | 0.00 | 1-14 | 2 | 2 |
| 2 | Test | 100 | 1-4 | 2 | 2 |
| | | 200 | 5-7 | | |
| | | 500 | 8-11 | | |
| | | 1000 | 12 | | |
| | | 0.00 | 13 | | |
| | | 750 | 14 | | |

On completion of the MTD Phase, the control beagles, which have received placebo in the MTD phase, were assigned to a Fixed Dose Phase. The animals were treated at 500 mg NADH/kg/day on the basis of findings from the MTD Phase. A constant dose volume of 5 mL/kg was used for both the MTD and Fixed Dose Phases of the study. The required volume was adjusted daily during the MTD Phase and twice weekly during the Fixed Dose Phase, calculated from the most recently recorded individual body weight.

During the MTD Phase, the test article NADH was administered once daily, excluding Day 13 and the day of necropsy. During the Fixed Dose Phase, the test article was administered once daily for a minimum of 14 days, excluding the day of necropsy.

Dosing of solutions of the test article were prepared daily for each group. The formulations were stable for two hours. Before dosing, the formulations were stored at ambient temperature, in the dark.

Four male and four female pure-bred beagles were obtained from Hazleton Research Products, Inc. Before delivery, they received a course of treatment at the supplier's premises for endo-parasites and were vaccinated against distemper virus, canine infectious hepatitis virus, parvo virus, rabies, *Bordetella,* parainfluenza virus, *Leptospira canicola,* and *Leptospira icterohaemorrhadiae.* Documentation provided by the supplier included date of birth, litter identification, vaccination dates, and details of treatment given.

Shortly after arrival, the beagles were inspected for ill health, re-vaccinated against distemper virus, canine infectious hepatitis virus, parvo virus, *Bordetella,* parainfluenza virus, *Leptospira canicola,* and *Leptospira icterohaemorrhadiae,* and received oral anthelminthic treatment.

The beagles were held in stock for about three weeks before the start of the MTD Phase. Towards the end of this period, their health status was reassessed and their suitability for experimental purposes confirmed.

At the start of treatment, males weighed 7.85 Kg to 12.30 Kg while the females weighed from 8.20 Kg to 9.65 Kg. All were between 9 and 11 months old.

The beagles were housed in a single room, singly during the working day and in pairs of the same sex and group overnight. The room was air-conditioned to provide a minimum of ten air changes per hour and routinely maintained at a temperature of 16°C to 22°C and a relative humidity of 40% to 80%. Fluorescent lighting was controlled automatically to give a cycle of 12 hours light (0700 to 1900 h) and 12 hours darkness.

Daily throughout this investigation, each beagle was offered 400 g of SQC Laboratory Diet A, Expanded (Special Diets Services Ltd. Witham). Any uneaten diet was removed and weighed during each afternoon, then discarded. Filtered drinking water was available *ad libitum* from an automatic watering system. The diet and water were considered not to have contained any contaminant at a level which might have affected the integrity or outcome of the study. The beagles were arbitrarily assigned to treatment groups. After allocation to a treatment group, each beagle was individually identified by a subcutaneous electronic implant. A color-coded card on each kennel gave information including study number and animal number.

2. Experimental Observations at the MTD Phase and Fixed Dose Phase

All beagles were observed in the morning, before feeding and again in the afternoon. In addition, all beagles were given a detailed clinical examination at weekly intervals. An individual record of the condition of each beagle was maintained. Additional observations were made throughout the working day as necessary, particularly between two and four hours after dosing during the MTD Phase. On days when the dose level was increased and Day 1 of the Fixed Dose Phase, the beagles were also observed between 8 and 12 hours post dosing.

Individual body weights were recorded once weekly during the pre-dose period. Body weights were recorded daily during the MTD Phase and twice weekly during the Fixed Dose Phase. The beagles were also weighed before necropsy.

Individual food consumption was determined daily throughout the study by subtracting the amount of food left or discarded from the quantity offered. Blood pressure recordings (diastolic, systolic, and mean arterial) were made from all beagles before dosing, and 10 minutes, and 1, 2, and 4 hours after dosing on Day 1 of the Fixed Dose Phase. Blood pressure was measured by cannulation of the ear artery. On Day 1 of the Fixed Dose Phase, the heart rate of each beagle was determined from the blood pressure traces.

3. Laboratory Investigations

Blood samples were obtained from all beagles pre-dose and at the end of both the MTD and Fixed Dose Phases. Blood samples were collected from the jugular vein after an overnight fast. Samples taken during the treatment periods were collected before daily dosing.

(a) Hematology

Blood was collected into EDTA anticoagulant and the following parameters were measured:

- Hemoglobin Concentration
- Mean Cell Volume
- Red Blood Cell Count and Indices (including Mean Cell Hemoglobin, Mean Cell Hemoglobin Concentration, Packed Cell Volume)
- Total and Differential White Blood Cell Count
- Platelet Count

Further blood samples were collected into 3.13% trisodium citrate anticoagulant and prothrombin and activated partial thromboplastin times measured according to Babson and Babson (1974).

(b) Clinical Chemistry

Blood was collected into lithium heparin anticoagulant and the following parameters were measured:

- Aspartate Aminotransferase (Kommissionen für Enzymdiagnostik und Standardisierung, 1972)
- Alanine Aminotransferase (Kommissionen für Enzymdiagnostik und Standardisierung, 1972)
- Gamma Glutamyl Transferase (Szasz et al., 1974)
- Alkaline Phosphatase (Kommissionen für Enzymdiagnostik und Standardisierung, 1972)
- Potassium (Eisenmann et al., 1957)
- Glucose (Schmidt, 1981)
- Total Bilirubin (Pearlman et al., 1974)
- Total Protein (Spencer et al., 1977)
- Albumin/Globulin Ratio
- Calcium (Moorehead et al., 1974)
- Urea (Gutmann et al., 1974)
- Creatinine (Jaffé, 1886)
- Albumin (Spencer et al., 1977)
- Total Cholesterol (Allain et al., 1974)

4. Pathology

The beagles were killed by an intravenous injection of sodium thiopentone, following an overnight period without food. The beagles were exsanguinated, a full internal and external examination was made under the general supervision of a pathologist, and all lesions were recorded. The necropsies for each phase of the study were carried out on a single day.

(a) Organ Weights

The following organs were dissected free from fat and other contiguous tissue and weighed before fixation:

- Adrenals
- Brain (including Brain Stern)
- Heart
- Kidneys
- Liver
- Lungs
- Ovaries
- Pancreas
- Pituitary
- Prostate
- Spleen

- Testes and Epididymides
- Thymus
- Thyroids
- Uterus

(b) Histology

Samples of the following tissues were fixed in 10% neutral buffered formalin, with the exception of the eyes and optic nerves which were fixed in Davidson's Fluid:

- Adrenals
- Aorta
- Brain (including Brain Stern)
- Caecum
- Colon
- Duodenum
- Epididymides
- Eyes (With Optic Nerves)
- Femur
- Gall Bladder
- Heart
- Ileum

- Injection Sites
- Jejunum
- Kidneys
- Lachrymal Glands
- Liver
- Lungs (with Mainstem Bronchi)
- Lymph Nodes (Mandibular and Mesenteric)
- Esophagus
- Ovaries
- All Gross Lesions
- Pancreas
- Pituitary
- Prostate
- Rectum (with Anus)
- Salivary Gland (Submandibular)
- Sciatic Nerves
- Skeletal Muscle (Quadriceps)
- Skin and Mammary Gland
- Spinal Cord (Lumbar, Cervical, Thoracic)
- Spleen
- Sternum (with Bone Marrow)

- Stomach
- Testes
- Thymus
- Thyroids (with Parathyroids)
- Tongue
- Trachea
- Urinary Bladder
- Uterus (Corpus and Cervix)
- Vagina

Tissues specified above from all fixed dose beagles were embedded in paraffin wax BP, sectioned at a nominal thickness of 5 µm, stained with hematoxylin and eosin, and evaluated using light microscopy.

5. Experimental design and dose levels for the oral form of NADH

The test article ENADA® were white tablets, containing 5 mg of NADH per tablet. The ENADA tablets had the following composition: βNADH (reduced form of Nicotinamide Adenine Dinucleotide) 5 mg (Supplier: Oriental Yeast), sodium bicarbonate (2.92 mg), Sodium ascorbate (0.3 mg), mannitol (49.55 mg), magnesium stearate (0.9 mg), methacrylate (Eudragite®) (7,1 mg), microcrystalline cellulose (1.18 mg). Except for NADH all other ingredients were supplied by Merck A.G., Darmstadt,

Germany. The ENADA® tablets were produced by Merck Darmstadt at its production plant Merck Spittal, Austria.

When not in use, the test article was stored at ambient temperature (10°C to 30°C), in the dark and protected from moisture. The vehicle for the tablets was gelatin capsules. Control animals received empty capsules. The ENADA tablets were administered orally by capsules. Doses to the nearest 5 mg (whole tablet) were administered.

Dose levels of 0, 20, 100, and 150 mg/Kg/day were chosen. The high dose was selected because it was considered to be the maximum that could be practically administered repeatedly over 14 days. This was achievable only by administering half the dose in the morning and half later in the day.

An intermediate dose level was chosen for comparison with the same dose administered intravenously in this study. The low dose was a multiple of the intended therapeutic dose. Individual doses were adjusted weekly according to the latest body weight. The test article was administered twice daily for a minimum of 14 days, excluding the day of necropsy.

Capsules were prepared weekly for each individual. The requested number of tablets for each individual were counted, then put into the minimum number of capsules practicable (approximately 30 tablets per capsule) and assigned to the beagle by

placing in a labeled pot. Before dosing the capsules were stored at ambient temperature in a sealed container.

Twelve male and twelve female pure-bred beagles obtained from Hazleton Research Products, Inc. were used for the oral application. Before delivery, the beagles received the same treatment at the supplier's premises as the dogs for the MTD phase.

At the start of treatment, males weighed 8.65 Kg to 10.95 Kg and females weighed 7.05 Kg to 9.90 Kg and were between 7 and 9 months old.

6. Observations at the oral subchronic toxicity experiments

All beagles were observed in the morning, before feeding, and again in the afternoon. In addition, all beagles were given a detailed clinical examination at weekly intervals. An individual record of the condition of each beagle was maintained. Additional observations were made throughout the working day as necessary. Individual body weights were recorded weekly during throughout the study, and before necropsy individual food consumption was determined daily throughout the study by subtracting the amount of food left or discarded from the quantity offered.

The eyes of all beagles were examined pre-dose and in Week 2. The examination was carried out using a Keeler indirect ophthalmoscope. A mydriatic agent (1 % tro-

tropicamide) was instilled into the eyes before the examination. Electrocardiographic (ECG) recordings (leads I, II, and III, aVR, aVL, aVF) were taken from all beagles predose and in Week 2, three hours after the afternoon dose. The heart rate and PR, QRS, QT, and QTc intervals were determined from lead II, using an automated ECG recorder Blood pressure recordings (diastolic, systolic, and mean arterial) were made from all beagles before the morning dose and three hours after the afternoon dose in Week 2. Direct measurements were made from the ear artery.

Laboratory and Pathology Investigations were performed identically to the ones described for the MTD and Fixed Dose Phase.

7. Statistical Evaluation

Data were processed, where appropriate, to give group mean values and standard deviations. The start of treatment body weights, all body weight gains, food consumption intervals, clinical chemistry and hematology variables were analyzed using two-way analysis of variance (ANOVA) (Snedecor et al. 1980). Pairwise comparisons, for each sex separately, were made using Dunnett's test (Dunnett 1955), apart from predose variables for which pair-wise comparisons were made using a protected t-test (Snedecor et al. 1980). The t-test is protected in the sense that the results are reported only if overall ANOVA is significant ($p<0.05$).

For each variable measured during the treatment period and for each sex separately a regression test (Armitage 1971) was performed to determine whether there was a linear relationship between increasing dose and response. In case of a significant result ($p<0.05$) and any of the pair-wise comparisons were also significant. A precedence was given to the pairwise tests and the regression result was not reported.

Levene's test (Levene 1960) for equality of variances across groups, between sexes and for any interaction was also performed. If it was considered necessary, especially when these tests showed evidence of group effects or a sex-group interaction ($p<0.02$), the data were re-analyzed using non-parametric methods for each sex separately.

The methods used were the Kruskal-Wallis (Lehmann 1975) ANOVA, and the Terpstra-Jonckheere test (Jonkheere 1954) for dose response; for pre-dose variables, only the ANOVA was reported. It should be noted that the Wilcoxon test for pairwise comparisons was not reported in all cases. Even in the most extreme case, the Wilcoxon test can not achieve significance because of the small number of animals in each group. If the Levene's test showed evidence of differing variances between the sexes only ($p<0.02$), then a one-way ANOVA, regression test and Dunnett's test were performed for each sex separately as considered necessary. For pre-dose variables, the ANOVA and protected T-Lest were performed.

Regarding Hematology, basophil counts were not analyzed because more than 30% of the data points had the same value. The percentage neutrophil, lymphocyte, monocyte, and eosinophil counts were analyzed in addition to the absolute values.

All organ weights were analyzed using analysis of covariance (ANCOVA) (Armitage 1972) and Dunnett's test, using the necropsy body weight as a covariate. This analysis depends on the assumption that the relationship between the organ weights and the covariate is the same for all groups, and the validity of this assumption was tested. Levene's test for equality of variances across the groups was also performed for all organ weights, and in all cases gave no evidence of heterogenous variances Statistically significant differences between test and control groups are annotated in the tables, and those considered to be biologically significant are described in the text.

**Results**

All Beagles survived the phases of the study to which they were assigned.

1. Maximum Tolerated Intravenous Dose (MTD) Phase and Fixed Dose Phase

1.1 Clinical observations

During dosing at all dose levels administered during the MTD Phase the treated Beagles frequently became subdued, and the gums often became pale. At dose lev-

els of 200 mg/kg/day and greater, the Beagles frequently had tremors immediately after dosing and occasionally during dosing. From day 10 onwards, in Beagles receiving 500 mg/kg/day and greater, the pads of the feet became pale after dosing, and those of the males were also cold to touch.

During dosing at 750 and 1000 mg/kg/day the beagles often had warm ears and a dry nose. After dosing the ears were cold and the beagles were described as being restless and having an arched back or hunched posture. The respiratory rate of female number 4088 increased during dosing at both levels. The respiratory rate of both males increased after dosing at 1000 mg/kg/day. However, after dosing at 750 mg/kg/day this was only exhibited by one animal. The beagles also had an awkward gait, ataxia and were unsteady on their feet after dosing at these levels. All treated beagles vomited during the MTD Phase, but the incidence showed no clear relationship with dose. Other signs exhibited by treated beagles included salivation, retching, lip licking, soft or mucoid feces which were often pale or yellow, red eyes, and the appearance of the third eyelid during dosing.

On several occasions, one male (number 4084) was clearly agitated and vocalized during dosing. On days 3 and 4 the infusion was suspended for a short time during which the signs regressed, but once the infusion was continued similar signs were again exhibited. On days 5 and 6 the beagle was not dosed, but on day 7 the beagle was returned to dose at 200 mg/kg/day.

The clinical signs observed during the Fixed Dose Phase were similar to those seen during the MTD Phase.

Treated beagles became subdued, and had pale gums, cold (and less frequently warm) ears, blood shot eyes, dry nose and increased respiratory rate during dose administration. After dosing all beagles had tremors and the pads of their feet were cold. All beagles except one male vocalized and occasionally appeared agitated during dosing.

Other signs seen included salivation, lip licking, red eyes, awkward gait, increased heart rate, panting and the appearance of the third eyelid during dosing. Only one female (number 4087) vomited during the Fixed Dose Phase.

There were no other clinical observations that were associated with the administration of the test article.

During the MTD Phase, at dose levels of up to 200 mg/kg/day there was little change in the body weight of treated beagles, but at doses of 500 mg/kg/day and greater, the weight of these beagles decreased. On day 13, the day after the administration of 1000 mg/kg/day, the body weights of the males were 4 and 6% lower and the weights of the females were 6 and 8% lower than before treatment started on day 1. The body weights of all controls on day 14 were within 2% of their weights on day 1. On day 14 of the Fixed Dose Phase the weight of female number 4086 was 3% lower and the weight of female number 4087 was 6% lower than on day 1. The weights of both males were slightly lower (2%) on day 14 than on day 1.

The food consumption of treated beagles in the MTD Phase, at dose levels of 500 mg/Kg/day and greater tended to be slightly low compared with pre-dose and control values. Neither of the males ate more than 260 g/day during treatment at these levels, whereas pretreatment both beagles ate up to 400 g/day. Similarly, during the same period females ate up to 210 g/day, compared with up to 400 g for number 4085 and 370 g for number 4088 pre-treatment. At lower dose levels the food intake was similar to that pre-treatment.

Throughout the Fixed Dose Phase, the food consumption of one male (number 4081) and both females was generally lower than pre-dose. Prior to treatment the male frequently consumed all of the food offered (400 g) and the females ate up to 370 or 380 g.

The only occasions during treatment when consumption approached similar levels was when the food was left available to the beagles overnight to encourage eating. In Week 2 of the Fixed Dose Phase the amount eaten by male number 4082 also decreased. Previously, this beagle almost consistently ate all 400 g offered, but in Week 2 consumption became as low as 250 g/day.

The systolic and mean arterial blood pressure was lower one hour after dosing on day 1 of the Fixed Dose Phase than before dosing in all beagles except for one female (number 4086) for which the values were similar to those before dosing. How-

ever, there was no consistent pattern of change in blood pressure at the other time points at which measurements were taken.

The heart rates of all beagles except one male (number 4082) on day 1 of the Fixed Dose Phase were higher at all time points when measurements were taken after dosing when compared with the predose rates. The heart rate of dog number 4082 generally remained similar to the pre-treatment rate.

1.2 Laboratory Investigations

(a) Hematology

On day 15 of the MTD Phase, the treated beagles showed changes in a number of the hematology parameters measured compared to the pre-dose values. However, similar changes were generally apparent in controls, suggesting that the changes were not associated with treatment.

On day 14 of the Fixed Dose Phase, the hemoglobin concentration, red blood cell count and packed cell column in male number 4081 and female number 4086 were high, but the mean cell hemoglobin and mean cell hemoglobin concentration were low compared with pre-dose and day 15 of the MTD Phase values.

Similar changes were evident in female number 4087 except for the hemoglobin concentration which was higher than at the end of the MTD Phase but lower than pre-dose.

The other parameters measured were considered to be unaffected by treatment.

(b) Clinical Chemistry

On Day 15 of the MTD Phase the activities of alkaline phosphatase in all treated beagles and alanine aminotransferase in one female (number 4085) were higher than pre-treatment and control values. The creatinine concentrations in one male (number 4085) and both treated females, and the cholesterol concentrations in all treated beagles were also high compared with pre-treatment and control values. Other changes apparent in treated beagles were generally also evident in the controls.

P*re-dose:* the activities of aspartate aminotransferase and alanine aminotransferase in beagle number 4087 were unusually high. The beagle was re-bled before dosing on day 1 of the study and both parameters were considered to be within the ranges normally expected.

On Day 14 of the Fixed Dose Phase the plasma calcium concentration in all beagles was high compared with pre-dose and day 15 of the MTD Phase values. The activity of alkaline phosphatase in one female (number 4086) was higher than on both previous sampling occasions, and the activity of this enzyme in one female (number 4087) was also higher than on day 15 of the MTD Phase, but lower than pre-treatment.

The total protein and cholesterol concentrations in all beagles were higher, but the total bilirubin concentration in both females was lower than pre-dose and day 15 of the MTD Phase.

1.3 Pathology Investigations

(a) Organ Weights

At termination of the MTD Phase, the relative adrenal weights of the beagles were generally higher than normally expected for beagles of this age and strain at this laboratory. Two beagles (male number 4083 and female number 4088) had relative brain and lung weights which were considered to be slightly higher than normally found in beagles of the same age and strain. The relative heart weight of both females was also considered to be high.

At termination of the Fixed Dose Phase of the study, all beagles were also found to have relative adrenal weights higher than normally expected. The relative lung and heart weights of one male (number 4081) and the relative lung weight of one female (number 4087) were considered to be high. The weights of the other organs were considered to be unaffected by treatment.

(b) Macroscopic Pathology

Most tissues were unremarkable at the necropsies of both maximum tolerated dose (MTD) and Fixed Dose Phase beagles.

Macroscopic observations at injection sites were consistent with those expected with repeated venepuncture. All other findings were consistent with the expected pattern of background observations in dogs of this strain and age.

(c) Microscopic Pathology

The majority of microscopic findings were infrequent, of a minor nature, and consistent with the expected background pathology in dogs of this strain and age and were not considered to be related to test article administration.

The only unusual microscopic findings were a mixed, mainly mononuclear, inflammatory cell perivascular cuffing of blood vessels in the medulla oblongata of the brain in all beagles together with a focus of inflammatory cells in the thalamus of one female.

The microscopic findings at injection sites (low grade dermatitis, phlebitis/periphlebitis and subcutaneous hemorrhage) were considered to be consistent with the physical procedure of repeat intravenous injections with no evidence of local irritation or other toxicity of the test article.

**2. Oral application**

2.1. Clinical observations

All beagles survived to their scheduled termination. On Days 3 to 7 of the treatment, all high dose males passed loose or liquid feces. In Week 2, these beagles continued to pass loose feces, but less frequently than in Week 1. The feces of intermediate dose males were also occasionally loose, whereas the feces passed by control and low dose beagles appeared normal throughout the study.

Females occasionally passed loose feces, but the incidence showed no relationship with dose. There were no other clinical observations that were attributed to treatment.

Treated and control beagles showed little change in body weight over the treatment period. Therefore, body weight was considered to be unaffected by test article administration.

During treatment, the mean food consumption of all treated female groups was generally lower than that of controls (up to 23% lower at the low dose, up to 27% lower at the intermediate dose and up to 22% lower at the high dose levels). However, similar inter-group variations were apparent pre-treatment. The food intake of treated males was generally similar to that of the controls.

No treatment-related ocular changes were evident during ophthalmoscopic examination.

The electrocardiography traces did not reveal any changes that were associated with the oral administration of stabilized NADH.

The systolic and mean arterial blood pressures of treated males in week 2 were generally high before dosing compared with control values. However, three hours after dosing, there was little difference between the blood pressures of treated and control males. In females there did not appear to be any effect of treatment on blood pressure.

## 2.2 Laboratory Investigations

(a) Hematology

In Week 2 there were **no** changes in any of the hematology parameters measured that could be attributed to treatment.

(b) Clinical Chemistry

Inter-group differences in the clinical chemistry parameters in Week 2 were generally apparent before the start of treatment and were therefore considered to be unrelated to treatment.

(c) Urine Analysis

From the samples collected in Week 2 it was evident that the composition of the urine remained unchanged by the test article.

## 2.3 Pathology

(a) Organ Weights

In males there was a small but dose-related increase in adjusted mean liver weight. The means were 7, 8, and 12% higher than the control mean for low, intermediate and high dose males respectively. The adjusted mean liver weight of the high dose females was 5% higher than the control mean.

The adjusted mean brain weights in low dose males, intermediate dose females and high dose males and females were high compared with the control means. The means for low dose males and intermediate dose females were 5% higher and the means for high dose males and females were 7 and 9% higher, respectively, than the control means.

The adjusted mean adrenal weights of all treated male groups were higher than the control mean (11, 8, and 21 % higher, low to high dose) and the adjusted mean thyroid weight of high dose males was 38% higher than the control mean.

The adjusted mean kidney (combined) weights of the intermediate and high dose males were 7 and 6% higher, respectively, than the control mean.

The weights of all other organs weighed were unaffected by treatment.

(b) Macroscopic Pathology

Most tissues and organs were unremarkable on gross examination. Certain findings, such as red focus in lung, urinary bladder, stomach, caecum and colon, occurred in treated groups compared to a zero incidence in controls. These were generally isolated occurrences and considered change/agonal events. There **were no findings suggestive of treatment-related toxicity.**

(c) Microscopic Pathology

Histopathology findings were generally infrequent and within the spectrum associated with young healthy dogs. Findings were generally comparable between control and treated groups. Urinary bladder cystitis occurred in some treated females, compared to a zero incidence in controls, but this was considered to be procedure-related rather than a direct effect of treatment. There was **no evidence of a dose-related increase in the incidence or severity thereof.**

Any further findings without comparable counterparts in controls were generally isolated occurrences and considered change/agonal events. There were **no** further findings of an unusual nature or incidence in the organs or tissues examined.

## Discussion

The intravenous administration of NADH to beagle dogs at dose levels in the range of 100 and 1000 mg/Kg/day resulted in a number of findings that demonstrated an effect on the cardiovascular system. Clinically these effects included pale gums, pale and cold pads of the feet, bloodshot eyes, and changes in the temperature of the ears. The subdued behavior of the beagles during dosing may also have been associated with cardiovascular changes.

Data from the Fixed Dose Phase showed that blood pressure was reduced 1 hour after the administration of 500 mg/Kg/day and there was an increase in heart rate for at least four hours after dosing.

The heart weights of both MTD Phase females and one Fixed Dose Phase male were higher than normally expected. Although this may have been an indication of an adaptive response to increased workload, there were no histopathological findings in the Fixed Dose Phase male to support this.

An increase in adrenal weight was evident in beagles from both phases of the study. Changes in the adrenals could be related to the increase in heart rate or may have been a response to stress, but no histopathological changes in the adrenals were found.

There was also evidence of an effect on the central nervous system. At dose levels of 200 mg/Kg/day and above the beagles had tremors after dosing and the relative brain weights of two MTD Phase beagles were considered to be higher than normally expected. Microscopic findings in Fixed Dose Phase beagles included an inflammatory cell perivascular cuffing of blood vessels in the medulla oblongata of the brain. However, these histopathological findings could not be unequivocally attributed to treatment.

Treatment with NADH at dose levels of 500 mg/Kg/day and higher resulted in a reduction in food intake, with a corresponding decrease in body weight.

There were apparent changes in the activities of alkaline phosphatase and alanine aminotransferase, and in the plasma concentrations of creatinine, cholesterol, calcium, total protein, and total bilirubin, but the significance of these changes was unclear. Based on the MTD of 500 mg/kg for a 10 kg heavy dog a MTD for a 70 kg heavy human individual can be calculated to be 35000 mg or 35 grams. Taking this remarkably high dosage into account, NADH can generally be regarded as safe (GRAS).

The oral administration of ENADA ® / NADH to beagle dogs dose levels of 20, 100, and 150 mg/Kg/day resulted in very few changes that could be attributed to the test article.

The feces of males treated at 150 mg/Kg/day became loose or liquid after 3 days of treatment but this regressed during Week 2. There was some evidence of a similar, but less marked, change in males treated at 100 mg/Kg/day. However, **no** corresponding histopathological changes were found in the gastro-intestinal tract. Females were unaffected. The weights of the adrenals, heart, and brain appeared to be increased. This was consistent with the findings from a previous intravenous study in dogs, but **none** of the associated cardiovascular changes, clinical observations or pathological findings seen in the previous study were evident in this study.

**None of these changes were considered to be of toxicological importance.**

The significance of the apparently increased kidney and thyroid weights of intermediate and/or high dose males was unclear. In the 14 days of the investigation, beagle dogs received dose levels of 20, 100, and 150 mg/Kg/day of NADH tablets. **There were no deaths. Body weight was unaffected and so was food consumption.** Electrocardiographies did **not** show any treatment related changes. Blood pressures were unaffected by the treatment, the hematology parameters were **not** affected and so were the clinical chemistry parameters. The composition of the urine was unchanged.

The oral administration of NADH to dogs at dose levels of 20, 100, and 150 mg/Kg/day elicited apparent increases in adrenal, heart, kidney, liver, brain and thyroid rate, particularly in the male. But none of these changes were considered to be of toxicological relevance.

It may be argued the absence of any treatment related toxic effect could be due to the possibility that NADH is not absorbed in the intestinal tract. Two studies have demonstrated that the stabilized orally absorbable form of NADH (ENADA®) is absorbed. NADH passes the intestinal mucosa undegraded (Mattern, 1996). In addition oral application of one 5 mg tablet ENADA / NADH lead to an increase of NADH in the brain cortex of rats (Rex et al. 2002). This study implies that NADH is not only absorbed but also passes the blood brain barrier. In the meantime, the stabilized orally absorbable form of NADH (ENADA®) has been tested in 2 independent blind placebo controlled trials in people suffering from jet lag (Kay et al. 2002) and sleep

deficiencies (Moline et al. 2002). Both conditions cause remarkably cognitive impairment prolonged reaction time and more errors in visual perception. After one dose of oral NADH (20 mg) the improvement in cognitive functions was significantly better than compared to placebo but also better than before jet lag or sleep deprivation. Another aspect is the rather high concentration of NADH in human organs particular those which need a high amount of energy such as the heart, the brain and the muscle. A mammalian heart contain 90 mg per kg, brain and sceletal muscle 50 mg per kg (Klingenberg et al. 1960). In other words, a 100 gram piece of red raw meat contains the same amount of NADH as one tablet ENADA® / NADH 5 mg.

An oral dose of 150 mg NADH per kg amounts to a dose of 10500 mg. This dose is equivalent to 2100 (two thousand one hundred) tablets of ENADA® - NADH. This number confirms and extends NADH to be generally regarded as safe.

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