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Review

# Safety assessment of β-nitropropionic acid: a monograph in support of an acceptable daily intake in humans

George A. Burdock*, Ioana G. Carabin, Madhusudan G. Soni

*Burdock and Associates, Inc., 622 Beachland Blvd., Suite B, Vero Beach, FL 32963, USA*

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**Abstract**

Several molds, *Aspergillus, Penicillium* and to a lesser extent, *Arthrinium* can produce β-nitropropionic acid (NPA). The presence of NPA has been detected in at least four families of higher plants. Use of *Aspergillus* as an economic mould in the production of foods and the accidental contamination of foods provides for an historically lengthy and widespread exposure of humans to NPA. Despite widespread consumption of foods containing NPA, human poisoning by NPA is rare and confined to circumstances involving gross mishandling of the food products. NPA is absorbed in the gastrointestinal tract, enters the circulation and is metabolized to nitrite, although some may bind succinate dehydrogenase upon oxidation. The primary mechanism of toxicity of NPA is as a "suicide" substrate (non-competitive inhibitor) of succinate dehydrogenase, an enzyme of the mitochondrial membrane (part of Complex II) that catalyzes the oxidation of succinate to fumarate, which is manifested as pathological change in striatal areas of the brain. The physiological damage caused by NPA metabolic compromise resembles the genetic disorder, Huntington's disease. This resemblance has been extensively exploited in recent years to understand the mechanisms of neurodegeneration. There are no irreversible effects resulting from ingestion of subthreshold doses of NPA, nor is there any accumulation of NPA in the body. The $LD_{50}$ dose of NPA for mice and rats is between 60 and 120 mg/kg. In long-term studies, NPA did not exhibit carcinogenicity or chronic toxicity. The reported no observed adverse effect level (NOAEL) for NPA is 2.5 and 3.75 mg/kg/day for male and female rats, respectively. Results of mutagenicity tests are mixed, but positive assays can be traced back to the use of a single impure sample of NPA. Based on the NOAEL of the chronic rodent bioassay, an ADI of 25 μg/kg/day or 1.750 mg/day for a 70 kg human is appropriate. 

*Keywords:* NPA; Excitotoxicity; Huntington's disease; Mitochondrial toxin

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* Corresponding author. Tel.: +1-561-234-0860; fax: +1-561-234-0026.
*E-mail address:* gburdock@salvitas.com (G.A. Burdock).

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## 1. Introduction

β-Nitropropionic acid (CAS No.: 504-88-1; also called 3-nitropropionic acid, NPA, hiptagenic acid) is found in at least four families of higher plants, in insects and as a mycotoxin. NPA may be unique among toxins because of its widespread occurrence. Further, although the method of biosynthesis may vary between life forms (i.e. via aspartate or malonate) the evolutionary purpose of this substance in all species appears to be as a defence against predators.

Animal exposure to NPA is well documented as a result of the consumption by livestock of forage plants which may contain high amounts of the toxin, (e.g. as much as 26,000 ppm). Although there is no record of human consumption of these forage plants, documentation of animal toxicity resulting from forage plant is widespread. Much of the research on NPA has been driven because of the economic losses sustained from loss of pasture, as the result of NPA-containing plants, many of which had been mistakenly introduced as a source of forage.

Human exposure to NPA has apparently occurred for centuries from tree nuts in New Zealand where the nuts were eaten as a dietary staple by Maori tribesmen and from the fungi *Aspergillus* and *Penicillium* used in the production of oriental dietary staples including *miso* (fermented soybean paste) and *katsuobushi* (fermented dry bonito). Accidental poisonings of NPA have been reported as anecdotal cases of poisoning with unprocessed karaka tree nuts and from mouldy sugarcane in China.

The mechanism of action of NPA is well defined and it has been shown to act as a reversible inhibitor of fumarase and aspartase and an irreversible, non-competitive ("suicide") inhibitor of succinic acid dehydrogenase, an electron acceptor and a part of Complex II of mitochondria. The specific site of toxicity in mammals is a highly energetically consumptive portion of the brain, which undergoes irreversible change upon receiving a threshold dose. There are no irreversible effects resulting from sub-threshold doses, nor is there any accumulation of NPA in the body.

## 2. Natural occurrence of β-nitropropionic acid

The primary source of toxic aliphatic nitro compounds producing animal toxicity are glucose esters of NPA (although some free acid is present), present in the *Coronilla, Astragalus* and *Indigofera* genera of the Leguminosae family, as well as other families described in Table 1. Misertoxin, the glucoside of NPOH (β-nitropropionic alcohol), was first isolated from *Astragalus miser* var. *oblongifolius* by Stermitz, Noms, and Williams (1969), but it has been detected subsequently in a number of other species. The species listed in Table 1 have a range of NPA (as NPA, NPOH or as $NO_2$, depending on the method of analysis) from $<1$ ppm to 26,000 ppm. Further, Williams and Gomez-Sosa (1986) have identified 32 species of *Astragalus* specific to Argentina with 2000 to 13,000 ppm NPA (as $NO_2$) in the leaves and Williams (1983) has identified 27 nitro-bearing species of *Lotus*. Recently, Ebrahimzadeh,

Niknam, and Maassoumi (1999) detected nitro compounds in concentrations of 2–25 mg $NO_2$/g (2000–25,000 ppm $NO_2$) of plant in 37 of 440 (8.4%) species of *Astragalus* tested from Iran. Other authors have documented the presence of NPA-bearing forage plants on all continents and in specimens from herbaria dating from as early as 1822 (Williams, 1981). Most of the livestock losses have been attributed to consumption of crownvetch (*Coronilla varia,* Fabaceae), milkvetch (*Astragalus* spp.) and creeping indigo (*Indigofera endecaphylla*). Unfortunately, some of these plants had been introduced as sources of forage prior to the knowledge that they were capable of producing the toxin (Williams & Davis, 1982).

In *Coronilla varia,* NPA occurs esterified to glucose, although trace amounts of the free acid are present. The ester variants are the six-monoester, two diesters, cibarian and coronarian, and three triesters, coronillin, karakin and corollin. Concentrations of the esters, when measured in freeze-dried plant material, reached a maximum of 20 mg $NO_2$/g in the flowers and 12 mg $NO_2$/g in the leaves, with much lower levels found in the stems and roots. The diester, cibarian, and the triester karakin, comprised approximately two thirds of the total esters in the leaves and were present in nearly equal amounts (Gustine, 1979). NPA is also found as an ester in the glycoside *hiptagen* in the bark of the Javanese tree *Hiptage mandoblata* (Alston, Porter, & Bright 1985).

Table 1
Occurrence of 3-nitropropanoic acid in higher plants

| Family | Genus and species |
|---|---|
| Malpighiaceae | *Hiptage benghalensis* (L.) Kurz |
| | *Hiptage mandablanta* |
| | *Heteropteris angustifolia* Gris. |
| Corynocarpaceae | *Corynocarpus laevagatus* J.R. and G. Forst. |
| | *Corynocarpus similis* |
| Violaceae | *Viola odorata* |
| Leguminosae | *Indigofera spicata* Forsk. |
| | *Indigofera suffruticosa* |
| | *Indigofera endecaphylla* |
| | *Coronilla varia* L. |
| | *Astragalus candadensis* L. |
| | *Astragalus cibarius* Sheld. |
| | *Astragalus collinus* Dougl. |
| | *Astragalus emoryanus* Rydb. |
| | *Astragalus falcatus* |
| | *Astragalus flexuosis* (Hook.) Don |
| | *Astragalus hamosus* |
| | *Astragalus miser* |
| | *Astragalus robbinsii* (Oakes) Gray |
| | *Lotus corniculatus* |
| | *Lotus pedunculatus* |

Modified from Gustine, 1979.

A unique source of naturally occurring NPA (as NPOH) is as an exocrine chemical defence of the chrysomelid beetle (*Chrysomela tremulae*). The beetle secretes two $\Delta^3$-isoxazolin-5-one glucosides, one of which hydrolyzes to yield NPOH upon ingestion by the predator. The toxins are stored in extracellular vesicles and secreted onto the dorsum of the gold-metallic-coloured beetle (Pasteels, Rowell-Rahier, Braekman, Daloze, & Duffey, 1989). Feeding labelled amino acid to the beetles, Randoux, Braekman, Daloza, and Pasteels (1991) demonstrated that the nitrogen source for these glycosides and the NPA was L-aspartic acid. Pasteels et al. (1989) showed that, with aspartic acid as a beginning, the beetles convert the aspartate to β-aminopropanoic acid or N-hydroxyaspartic acid, then either of these to N-hydroxy-β-aminopropanoic acid and from there to β-nitropropanoic acid or isoxazolidin-5-one and thence to δ-3-isoxazolin-5-one (Pasteels et al., 1989).

Other sources of NPA in nature include unidentified fungi isolated from zinnia (*Zinnia elegans*; Kamikawa, Higuchi, Tanniguchi, & Asaka, 1990), the fungus *Melanconis thelebola* isolated from red alder (trees) (*Alnus rubra* Bong.) and tomato plants (Evidente, Capretti, Giordano, & Surico 1992) and the fungus *Septoria cirsii*, introduced to control Canada thistle (Hershenhorn, Vurro, Zonno, Stierle, & Strobel, 1993). Of the two sources of NPA for humans, economic fungi and karaka tree nuts, the kernel of the fruit of the karaka tree (*Corynocarpus laevigatus* J.R. & G. Frost) is more restricted in its distribution, confined to New Zealand and the Chatham Islands. Although the tree is now primarily used for its decorative purposes, karaka nuts once formed the Maori tribes' staple vegetable articles of food. The nuts were prepared for eating by baking the fruit in earth ovens for several hours, washing it for a day or two to remove the poison and to loosen and remove the skin and flesh (sarcosarp), and then drying the kernels in the sun (Bell, 1974). The food value of the kernels has been reported to be fat 15.5%, protein 10.9% and carbohydrate 58.0%, while concentration of the toxin ranges from 0.025% in green berries, and 0.08% in ripe berries to 0.03% in fresh nuts (Bell, 1974). Anecdotal reports include one from the year 1871, describing toxicity in children following consumption of the unprocessed nuts, a report of toxicity in cattle recorded in 1886 and in pigs reported in 1918. There is also a report of honeybees, attracted to the flowers of the Karaka tree in spring, suffering 20% mortality (Bell, 1974), therefore indicating that the toxin may not be confined to the nuts alone. Carter (1951) described the toxin extracted from nuts as being a glucoside, 1,4,6-tris-(β-nitropropionyl) D-glycopyranose ($C_{15}H_{21}O_{15}N_3$) which could be hydrolyzed to yield three molecules of NPA.

The second source of NPA for humans offers more exposure because NPA may also be produced by eco-

nomically valuable fungi. Those fungi with the greatest historical use are *Aspergillus* sp. and *Penicillium* sp. used in starter cultures (called *tane-koji*) for fermentation of soybean paste for *miso*, fermentation of dried bonito flesh for *katsuobushi* and other products (Kinosita, Ishiko, Sugiyama, Seto, Igarasi, & Goetz, 1968; Orth, 1977). Kinosita et al. (1968) notes that *miso* is consumed on an average of 32.7 g/day by rural Japanese, and 26.1 g/day by city dwellers. No quantitative data are available for *katsuobushi,* although Kinosita et al. (1968) maintained that consumption in rural areas is "fairly large". In urban areas, use of *katsuobushi* is being supplanted by glutamate and inosinate. Processing plants manufacturing the *katsuobushi*, also produce a widely eaten appetizer called *shuto* or *shiokara*, which is made from the washed, then fermented, intestines of the bonito. The fermentation organism used is the same for *katsuobushi*. Consumption data for *shuto* are not available. Other products, which Kinosita et al. (1968) feel may be produced by the same starter cultures include soy sauce (*shoyu*) and *sake* or rice wine.

Kinosita et al. (1968) obtained starter cultures from a commercial production facility in Los Angeles and were able to isolate *Aspergillus oryzae* in Czapek medium. *Aspergillus oryzae* was also obtained from starter cultures and food samples obtained from commercial food production facilities in Japan, from rural areas where small shops maintain cultures, and from households where cultures of wild strains are kept and passed down from one generation to another. Starters were also obtained from Honolulu and fermented soybean paste was obtained from South Korea. Care was taken not to obtain food or starter samples from areas known to harbour *Aspergillus tamarii*. From 24 samples of foodstuffs, 37 strains of fungi were isolated, which were, in turn, transferred to other media and the amount of NPA determined (Table 2).

Unfortunately, Kinosita et al. (1968) did not define the term "trace" as cited in Table 2, but if we can assume that "trace" is equal to one-tenth of the lowest reported amount (0.1 mg/kg), those cultures attributed to trace amounts contained 0.01 mg/ml NPA. This being the case, and, if those cultures producing zero are excluded, then the average potential NPA production in these fermented foods is 0.17 mg/ml. Therefore, if *miso* is consumed at an average rate of 29.4 g/day, then NPA may be consumed at a rate of approximately 5.0 mg/day from *miso* alone. Disappearance data indicate that the Japanese consume approximately 9.8 l soy sauce (*shoyu*)/person/year (Uchida, 1989) and 500 ml/person/year in the USA (Personal communication, 1995). On the basis of the Japanese consumption of 2.7 ml/day (this assumes only one-tenth of soy sauce is actually consumed, with the remainder left on the utensils), the daily intake of NPA from soy sauce would be approximately 0.46 mg/day. Thus, NPA consumption from *miso* and soysauce would be approximately 5.5 mg/day.

Cheese is also a good medium for fungi, Iwasaki and Kosikowski (1973) identified NPA in five of 18 cheeses examined (e.g. Fontina, Geitmelshe Kaas, Le Sanglier, Pave De Moyan and Fontinelli).

Iwasaki and Kosikowski (1973) obtained 18 cultures (including wild cultures) from the American Type Culture Collection (ATCC), the Northern Utilization R&D Division (NRLL) and from Cornell University stocks. Of the 18, five produced detectable amounts of NPA in Nakamura's medium (Table 3). Orth (1977) conducted a similar experiment, using 16 different strains of *Aspergillus oryzae* and found that seven produced from 1.9 to 43.6 mg NPA/l of culture. Using *Aspergillus*

Table 2
Potential occurrence of 3-nitropropionic acid in *Katsuobushi* (Fermented Bonito) and *Miso* (adapted from Kinosita et al., 1968)

| Natural source | Source organism | β-Nitropropionic acid (mg/ml medium) | |
|---|---|---|---|
| | | Glucose-ammonium nitrate medium | Supplemented Czapeck Dox medium |
| Katsuobushi | *Penicillium cyclopium* | Trace | 0 |
| Katsuobushi | *Penicillium cyclopium* | Trace | 0 |
| Miso and shoyu | *Aspergillus* sp. | Trace | 0 |
| Miso and shoyu | *Aspergillus glaucus* group | Trace | 0 |
| Miso and shoyu | *Aspergillus* sp. | 0.3 | 0 |
| Miso and shoyu | *Aspergillus versicolor* group | 0.2 | 0 |
| Miso and shoyu | *Penicillium chrysogenum* | Trace | 0 |
| Miso and shoyu | *Aspergillus oryzae* | Trace | 0 |
| Miso and shoyu | *Aspergillus soyae* | 0.2 | 0 |
| Miso and shoyu | *Aspergillus soyae* | Trace | 0 |
| Miso and shoyu | *Aspergillus oryzae* | Trace | 0 |
| Miso and shoyu | *Aspergillus soyae* | 0.1 | Trace |
| Miso and shoyu | *Aspergillus soyae* | 0.4 | 0 |
| Miso and shoyu | *Aspergillus candidus* group | 1.1 | Trace |
| Miso and shoyu | *Aspergillus flavus group* | 0 | Trace |

*oryzae* ATCC 12892 and *Aspergillus oryzae* Higati, Iwasaki and Kosikowski (1973) and Penel (1977) examined the potential for these moulds to produce NPA on commonly eaten foods (Table 4). Thus, contaminated foods also provide a source of NPA. *Arthrinium* species, including *Arthrinium sacchari, saccharicola* and *phaeospermum,* have been shown to be the organisms responsible for mass poisonings in China through production of NPA in improperly stored sugarcane. These species were shown to produce as much as 1600–1700 ppm NPA in culture (Liu, Luo, & Hu, 1989, 1992). Wei, Chang, Lin, Doong, and Jong (1994) demonstrated production of NPA by strains identified by Liu and his colleagues and by other ATCC strains (Table 5). Further, Wei et al. (1994) demonstrated NPA production by *Arthrinium sacchari* in several media under different conditions of growth. *Penicillium atrovenetum,* used by Porter and Bright (1987), was shown to produce 2000 mg NPA/l of culture. Majak and Pass (1989) confirmed NPA production by *Penicillium atrovenetum*.

Thus, consumption of NPA by humans is likely through consumption of both commercially and domestically prepared foodstuffs, using fungi and through unintentional exposure from fungi-contaminated foods. This concept of long-term consumption was recited in Section 1 of the NCI (National Cancer Institute) carcinogenicity study on NPA, stating in its rationale for carcinogen testing of NPA "...because of its use in food preparations and its identification as a contaminant in foods..." (NCI, 1978).

## 3. Chemistry of β-nitropropionic acid

### 3.1. Chemical structure and description

NPA (Fig. 1) is a white crystalline solid with melting point of 65–67°C. It has the empirical formula $C_3H_5NO_4$ and has been assigned the CAS registry number 504-88-1. The official ninth Chemical Index name for NPA is Propionic acid, 3-nitro-, but several legitimate synonyms commonly used are listed in Table 6. NPA has been isolated from *Streptomyces* found in soil and occurs as a metabolite of a number of fungal species of *Aspergillus* and *Penicillium* (fungi make only NPA, not the esters as do higher plants; Gustine, 1979). These species of fungi are commonly present in several oriental fermented foodstuffs in which NPA has been identified. As described earlier, NPA has also been isolated from plants and nuts.

Table 3
Production of NPA from stock cultures (Modified from Iwasaki & Kosikowski, 1973)

| Mould | NPA (mg/l culture) |
|---|---|
| *Aspergillus oryzae* ATCC 11494 | 1 |
| *Aspergillus oryzae* ATCC 12892 | 1279 |
| *Aspergillus oryzae* ATCC 7252 | 40 |
| *Aspergillus oryzae* Higati | 111 |
| *Aspergillus flavus (oryzae)* ATCC 11500 | 17 |

### 3.2. Biosynthesis and degradation

NPA was probably the first nitro compound to be detected in nature. Gorter originally isolated NPA in 1920 as hiptagenic acid and was later correctly identified by Carter and McChesney (1949). Glucose esters of NPA have been characterized from creeping indigo, *Viola odorata*, various *Astragalus* species, crownvetch and *Lotus pedunculatus*. NPA is also produced by fungi *Aspergillus flavus, Aspergillus wentii, Penicillium atrovenetum, Arthrinium sacchari, Arthrinium saccharicola* and *Arthrinium phaeospermum* (Hamilton, Gould, & Gustine, 2000). Majak and Pass (1989) reported that biosynthesis studies were originally conducted with the astomycete fungus *Penicillum atrovenetum*, which synthesizes NPA.

$$O_2N\text{-}CH_2\text{-}CH_2\text{-}COOH$$

β-Nitropropionic Acid (NPA)

Fig. 1. Chemical structure of NPA. Note the attachment of the nitro-function on the β-carbon.

Table 4
Production of NPA by *Aspergillus oryzae* ATCC 12892 in commonly eaten foods

| Substrate | NPA (mg/kg) |
|---|---|
| Cheese | 248 |
| Soybean | 12 |
| Peanut | 15 |
| Cheese[a] | 111 |
| Cheddar cheese | 12 |
| Yellow sweet potato | 1 |
| White potato | 5 |
| Banana | 20 |

(Modified from Iwasaki & Kosikowski, 1973; Penel, 1977)
[a] Produced by *Aspergillus oryzae* Higati.

Table 5
*Arthrinium* Spp. shown to produce NPA (Wei et al., 1994)

| Fungi | ATCC strains | Maximal NPA conc. (μg/ml) |
|---|---|---|
| *Arthrinium sacchari* | 76289 | 716 |
| | 76290 | 295 |
| | 76981 | 1716 |
| *Arthrinium phaeospermum* | 24357 | 1620 |
| | 76291 | 1593 |
| *Arthrinium terminalis* | 24358 | 692 |
| *Arthrinium aureum* | 56042 | 1211 |
| *Arthrinium sereaenis* | 76309 | 1527 |

The incorporation of radiolabelled compounds into NPA by growing cultures of *Penicillum atrovenetrum* was investigated by Shaw (1967). The author concluded that both the amino group and the carbon skeleton of aspartic acid are on a direct pathway to NPA. Shaw and McCloskey (1967) also explored the incorporation of $^{15}N$- and $^{18}O$-labelled substrates into NPA by growing cultures of *Penicillum atrovenetum*. They found that ammonium ion was used for the synthesis of the nitro group in preference to nitrate. The label from [$^{18}O$]-potassium nitrate was not incorporated into the nitro group. The amino group of aspartic acid was utilized in preference (approximately 2:1) to ammonium ion for the synthesis of the nitro group. [3-$^{14}C$]- and [4-$^{14}C$]-aspartic acids were incorporated equally well into NPA. Dilution of the label was small in spite of low efficiency of incorporation. L-[4-$^{14}C$]-Aspartic acid, but not the corresponding D-isomer, was incorporated into the nitro compound. These studies supported the earlier conclusions that both the amino group and the carbon skeleton of aspartic acid are on a direct pathway to NPA. Label from tartaric acid, which promotes NPA synthesis, was not incorporated into the nitro compound (Shaw & McCloskey, 1967). Baxter et al. (1992) also examined the origin of NPA in the fungus *Penicillum atrovenetrum* using a combination of stable isotope methods. The hypothesized biosynthetic pathway involved conversion of L-aspartate to nitrosuccinate via oxidation of the amino group followed by decarboxylation of the nitrosuccinate to NPA. Decarboxylation of the nitrosuccinate presumably gives rise to an *aci* intermediate, which tautomerizes to afford NPA. The authors also reported that the biosynthesis of NPA appears to occur by very different routes in fungi as opposed to higher plants, as also reported by Candish, La Croix, and Unrau (1969). The latter demonstrated that malonate and malonylhydroxamate were likely precursors of NPA by using radiochemical studies with whole plants and cuttings of *Indigofera spicata*. Gustine (1979) had earlier suggested that malonate may play a role in nitro-aliphatics of *Iindigofera spicata* and demonstrated that $^{15}N$-labelled nitropropanol was incorporated into cibarian ester in *Astragalus cibarius*.

Shaw and DeAngelo (1969) concluded that NPA synthesis is probably not directly associated with the metabolism of inorganic nitrogen compounds and that an organic pathway for the formation of the nitro group is more likely. Faix, Gustine, and Wright (1978) studied the influence of growth temperature on the concentration of NPA in various plant parts of crownvetch forage. NPA increased with increasing growth temperature and the most consistent increase occurred in the stem tips and leaflets. The author concluded that higher growth temperatures can increase NPA production in crownvetch forage and the overall yield can be a function of the leafiness of the forage.

Porter and Bright (1987) reported that *Penicillum atrovenetum* abruptly and reproducibly secretes large quantities (2 g/l) of NPA on the fifth day after inoculation into medium. Moulds may also degrade NPA. Becker (1967) found the rate of oxidation of NPA to nitrate by cultures of *Aspergillus flavus* to be linear. For each micromole of NPA assimilated by the mould, 1 μmol of nitrate was detected. NPA was readily oxidized at pH 3.8, but not at pH 6.8. In the presence of glucose, nitrate was not detected until after all the glucose had been utilized. The effect of glucose is attributed to its inhibition of NPA uptake and also to its role as an electron donor for a nitrate reductase (Becker, 1967).

Gruner, DeAngelo, and Shaw (1972) reported that an enzyme system, found in *Penicillum atrovenetum* extracts, catalyzes the degradation of NPA to nitrite, nitrate and unidentified carbon compounds. They had evidence to indicate that more than one enzyme is involved in the degradation and that this system may be responsible for the production of nitrite and nitrate by growing cultures of *Penicillum atrovenetum*.

Table 6
Synonyms for propanoic acid, 3-nitro-

| |
|---|
| 3-Nitropropanoic acid |
| 3-Nitropropionic acid |
| Propionic Acid, 3-Nitro |
| BNP |
| BRN1759889 |
| EINECS 208-003-0 |
| Bovinocidin |
| Hiptagenic acid |
| NCI-C03076 |
| beta-Nitropropionic acid |
| NPA |
| Propanoic acid, 3-nitro- (9CI) |

CHEMID (2000).

### 3.3. Quantitation of NPA

Several approaches for the determination of NPA in a variety of substrates have been developed. One of three techniques is generally employed: thin-layer chromatography (TLC), gas–liquid chromatography (GLC), or high-performance liquid chromatography (HPLC).

#### 3.3.1. Thin layer chromatography

Thin-layer chromatographic methods are relatively straightforward, as extensive removal of interfering substances is generally not necessary; many of the interferences from the sample matrix are well separated from, and do not mask the NPA spot. Moskowitz and Cayle (1974) determined NPA in crude biological extracts by TLC. The sample is extracted with ether at pH 2.0–2.5 and evaporated to dryness. The residue is dissolved in 1.0 ml acetone and spotted on silica gel

plates for either one- or two-dimensional TLC. This approach can detect as little as 30 μg in a large excess of contaminating materials. Several different solvent systems can be used. Paterson (1986) used TLC on silica gel with a toluene-ethyl acetate-90% formic acid eluting solvent. The authors determined and compared the retention factors of 107 secondary metabolites (including NPA) of 304 *Penicillium* strains. Benn, McDiarmid, and Majak (1989) determined NPA by silica gel TLC using a solvent system of chloroform–acetone containing 1% water. The compound is visualized with diazotized *p*-nitroaniline spray. Evidente et al. (1992) employed TLC on silica gel with a *n*-butanol-acetic acid-water eluting solvent. The NPA spot was developed with iodine and/or by spraying with 0.5% ninhydrin in acetone, followed by heating at 110°C for 10 min.

*3.3.2. Gas chromatography*

Gas chromatographic methods are generally very sensitive. Removal or clean-up of sample matrix and other interferences is critical, otherwise the signal from trace amounts of the compound of interest is generally covered up or rendered difficult to detect. Gilbert, Penel, Kokowski, Henion, Maylin and Lisk (1977) described a sensitive GLC method for NPA, based on extraction, isolation and derivatization of the compound to its pentafluorobenzyl derivative. Determination was by electron affinity GLC. The method was applied to mould filtrates and cheeses with limits of detection of approximately one and 3 ppm, respectively.

*3.3.3. High-performance liquid chromatography*

Of the three techniques, high-performance liquid chromatography is the newest. It has the potential for determining a wide variety of substances, as it does not require the volatility needed for GLC, and is usually far more sensitive than TLC. HPLC is also capable of tolerating larger amounts of interferences than does GLC. Muir and Majak (1984) separated and determined NPA in plasma by reverse-phase HPLC on 15 and 30 cm octadecylsilane on silica columns protected by guard columns. Elution was performed isocratically with 0.15% orthophosphoric acid (adjusted to pH 2.0) at 1 ml/min. The columns were cleaned with a 0–30% methanol gradient between injections of plasma samples. NPA was quantified using an external standard and a variable wavelength detector set at 210 nm. Plasma samples were obtained by low-speed centrifugation of heparinized whole blood treated with cold 0.6 N perchloric acid and chilled. The supernatant of the centrifuged sample was used for analysis. Frisvad and Thrane (1987) developed a general method for the analysis of mycotoxins and other fungal metabolites. The method is based on HPLC with an alkylphenone retention index and photodiode-array detection combined with TLC in two different eluents. The retention characteristics of NPA and 181 other mycotoxins and related compounds were reported.

## 4. Biological data

### *4.1. Biochemical aspects*

*4.1.1. Mechanism of action of NPA*

As stated earlier, the primary mechanism of toxicity of NPA is as a "suicide" substrate of succinate dehydrogenase [succinate:(acceptor) oxidoreductase, EC 1.3.99.1], an enzyme of the mitochondrial membrane (part of Complex II) that catalyzes the oxidation of succinate to fumarate. Toxicity is manifested as pathological change in striatal areas of the brain. NPA also induces reversible inhibition of fumarase and aspartase (an enzyme not present in humans). Other enzymes affected include isocitrate lyase and possibly rat brain acetylcholinesterase (Ludolph, He, Spencer, Hammerstad, & Sabri, 1991). Administration of NPA results in clinical symptoms similar to those observed in Huntington's disease (Brouillet et al., 1993a, 1993b; Ludolph et al., 1991; HD). NPA produces selective lesions in the striatum, which appear to involve secondary oxidative stress following loss of adenosine triphosphate (ATP). Its toxicity also appears to be age-dependent. Because of the specificity of the toxicity, its systemic injection has been extensively used as an animal model of HD. NPA preferentially affects the basal ganglia (Vecsei, Diho, & Kiss, 1998).

*4.1.1.1. NPA as a "suicide" substrate.* The isoelectronic form of NPA can be converted, at physiological pH, to the highly reactive dianion, which irreversibly inhibits succinate dehydrogenase (Hamilton et al., 2000). This has been proposed as the biochemical basis of NPA toxicity. Following in vitro experiments with rat liver mitochondria, Alston, Mela, and Bright (1977) concluded that NPA is an isoelectronic analogue of succinate and that: (1) When rat liver mitochondria oxidize succinate in the presence of 3-nitropropionate carbanion, the rate of $O_2$ consumption decreases exponentially to a zero value. This pattern is duplicated by subsequent additions of mitochondria. The dependence of the apparent first-order rate constant for enzyme inhibition, as well as the number of enzyme turnovers completed before inhibition, on the concentrations of 3-nitropropionate carbanion and succinate, are those expected for an active site-directed and irreversible inhibitor. (2) The inactivated enzyme is not resuscitated by centrifugation and washing of the mitochondria, in contrast to malonate-treated enzyme and malonate protects against irreversible inhibition. (3) The inhibitor species is 3-nitropropionate carbanion and no external nucleophile

is required for inhibition. (4) The respiratory rates, respiratory control ratios and adenosine diphosphate (ADP)/O ratios obtained with nicotinamide adenine dinucleotide (NAD)-linked substrates are unaffected by 3-nitropropionate carbanion. These results show that 3-nitropropionate carbanion is a highly specific, time-dependent and irreversible inhibitor of succinate dehydrogenase. By analogy with the reaction of nitroethane with D-amino acid oxidase, the data are consistent with the hypothesis that the carbanionic inhibitor forms a covalent N-5 adduct with the active site flavin. Therefore, toxicity of NPA is due to the irreversible blockage of the Krebs cycle by 3-nitropropionate carbanion (Alston et al., 1977).

This mechanism of NPA action was confirmed by the experiments of Coles, Edmondson, and Singer (1979). These investigators used purified, soluble preparations of (succinate dehydrogenase) SDH from beef heart and a stoichiometric amount of 3-nitropropionate dianion. They found that the inhibition of SDH developed slowly and nearly complete inactivation occurred. 3-Nitroacrylate, the expected product of dehydrogenation by the enzyme, inactivates the SDH rapidly and irreversibly. Several lines of evidence suggested that the oxidation product, 3-nitroacrylic acid, reacted with an essential-sulfhydril (SH) group at the substrate site. Prior treatment with NPA prevented the binding of $^{14}C$-labelled oxalacetate at the substrate site and conversely, prior binding of oxalacetate to the enzyme prevented the irreversible inactivation by a two-fold excess of NPA. Inactivation of the enzyme by NPA also prevented the alkylation of one-SH group by N-ethyl[$^{14}C$]-maleimide. The-SH group in question is located in the 70,000 dalton subunit and is known from prior studies to be the combining site of succinate and of oxaloacetate. It is suggested that the inactivation step involves a nucleophilic attack by this essential-SH group on the double bond of 3-nitroacrylate (Coles et al., 1979).

*4.1.1.2. Localization within the brain.* Erecinska and Nelson (1994) described their observations of the mechanism of toxicity as it relates to the central nervous system (CNS). The first observation is that a fall in [CrP]/[Cr] is a very early and sensitive indicator of a mismatch between the energy supply and demand. Although the majority of striatal neurons have a low level of electrophysiological activity, the striatum receives a rich blood supply and exhibits a rather high metabolic rate; therefore, it is sensitive to mitochondrial toxins such as NPA. Additionally, because in younger animals the rate of energy use appears to be lower than in adults, degenerative changes may affect adults more. Stimulation of lactate synthesis is another early indicator of the mitochondrial dysfunction. A second observation is that a fall in energy generation results in a decrease in [guanosine triphosphate (GTP)]/[guanosine diphosphate (GDP)] — subtle reductions in this ratio substantially curtail protein production due to effects at the level of initiation. Therefore, even a small block in ATP generation might lead to a cell's inability to maintain a normal contingent of protein and explain rapid neuronal death. The third observation concerns the metabolism of amino acids and generation and release of the potent excitotoxin, glutamate, by glutaminase. This release explains why, even at the early stages of inhibition of the respiratory chain, glutamate might exert an excitotoxic action; it also provides an explanation for the apparent effectiveness of the glutamate receptor blockage in the treatment of consequences from inhibition of mitochondrial function. In addition to glutamate, the level of $\gamma$-aminobutynic acid (GABA) also increases internally and externally and should be detectable as an early indication of mitochondrial dysfunction (Erecinska & Nelson, 1994).

The conclusions of Erecinska and Nelson (1994) are consistent with those of Spencer, Ludolph, and Kisby (1993) who cited experiments with mouse cortical explants treated with NPA showing decreased energy levels and development of comparable patterns of neuronal pathology; these changes are attenuated by prior treatment with glutamate antagonists (MK-801, kynurinic acid; Kim et al., 1999). These findings strongly suggest that NPA blocks ATP production and thereby renders nerve cells susceptible to the excitotoxic effects of glutamate neurotransmitter (Spencer et al., 1993). Dautry et al. (2000) studied the N-acetylaspartate depletion in rats and primates treated with NPA. The authors reported that early N-acetylaspartate depletion reflects a reversible state of neuronal dysfunction preceding cell degeneration. These results suggest that in vivo quantification of N-acetylaspartate by 1H-magnetic resonance spectroscopy may become a valuable tool for assessing early neuronal dysfunction, and the effect of potential neuroprotective therapies in neurodegenerative disorders (Dautry et al., 2000).

*4.1.1.3. Role of NPA in neurotoxicity.* As NPA administration in animals produces clinical symptoms similar to HD, during the last 5 years approximately 2000 articles on NPA-induced neurotoxicity have been published; citation of all the articles is beyond the scope of this paper. Instead, a narrative summary is provided. The mechanism by which NPA exposure leads to neuronal degeneration is not well understood. Experimental studies in mice and rats have shown that administration of NPA produced brain injury consistent with an excitotoxic mechanism observed in ischemia/hypoxia (Gould & Gustine, 1982; Hamilton & Gould, 1987a). Because glutamate receptor antagonists provided protection from NPA-induced toxicity, while extracellular glutamate levels were not changed (Ludolph et al., 1992), it has been suggested that NPA-induced neuro-

degeneration is a consequence of secondary excitotoxic mechanisms, i.e. indirect activation of glutamate receptors (Riepe, Hori, Ludolph, Carpenter, Spencer, & Allen 1992; Zeevalk, Derr-Yellin, & Nicklas, 1995). Additionally, recent studies have suggested involvement of reactive oxygen species and oxidative stress in the NPA-induced neurotoxicity (Beal et al., 1995; Binienda & Kim, 1997; Fu, He, Zhang, & Zhang, 1995). Lesions produced by systemic administration of NPA increased the production of hydroxyl-free radicals in the striatum, as assessed by the conversion of salicylate to 2,3- and 2,5-dihydroxybenzoic acid, and NPA neurotoxicity was attenuated in copper/zinc superoxide dismutase transgenic mice (Beal et al., 1995). Recently, Binienda, Simmons, Hussain, Slikker, and Ali (1998) reported depletion of glutathione levels and induction of antioxidant enzyme activities after NPA administration, suggesting conditions favourable for oxidative stress. Alexi, Hughes, Faull, and Williams (1998) reported that metabolic compromise with NPA causes neurodegeneration that involves three interacting processes: energy impairment, excitotoxicity and oxidative stress. This triplet of co-operative pathways of neurodegeneration helps to explain NPA regional selectivity of neurotoxicity to basal ganglia.

Sato et al. (1997) demonstrated that apoptosis occurred in the rat striatum after intraperitoneal injection of NPA and that apoptotic neuronal death was the main part of the striatal damage. Apoptosis, induced by NPA in the striatum, might be related to excitotoxicity, but in vitro studies do not support this hypothesis (Pang & Geddes, 1997). Kim et al. (1999) suggested that apoptotic neuronal death, initially involved in striatal damage, caused by mild failure, created more energy failure with subsequent NPA treatment, eventually leading to neuronal necrosis. In in vitro experiments, cells might be lacking organized glutamatergic input. Kim et al. (1999) demonstrated that removal of the corticostriatal glutamate pathway reduced superoxide production and apoptosis induction in the denervated striatum of decorticated mice after NPA treatment. Also, N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 prevented apoptosis in the striatum after NPA treatment for 5 days, whereas non-NMDA receptor antagonist was ineffective. Kim et al. (1999) also evaluated the initial type of neuronal death by NPA treatment for different durations, from one to 5 days. In early striatal damage, apoptotic neuronal death initially occurred after NPA treatment. These results show that excitotoxicity related to oxidative stress initially induces apoptotic neuronal death in mouse striatum after treatment with NPA.

#### *4.1.2. Absorption, distribution, metabolism and excretion*

Because of the economic impact of nitroaliphatic containing forage, a number of studies have been conducted to assess the factors involved in absorption. For example, Majak, Pass, Muir, and Rode (1984) found that miserotoxin was rapidly hydrolyzed to 3-nitropropanol in the rumen of cattle dosed with timber milkvetch (*Astagalus miser* var. *serotinus*). The aglycone showed a rapid rate of disappearance from the rumen with an average half-life of 1.24 h. Rapid absorption of NPOH from the rumen was shown by plasma levels of NPA and inorganic nitrite, but conversion of NPOH to NPA was not observed to any significant extent in the rumen. James, Hartley, Williams, and Van Kampen (1980) concluded that the difference in toxicity between NPA and NPOH is related to the rate of its absorption from the gastrointestinal tract. While, misertoxin is hydrolyzed to glucose and NPOH in ruminants, it is hydrolyzed to glucose and NPA under the acidic conditions of the stomach in monogastric animals (Mosher, Krishnamurti, & Kitts, 1971). Hamilton et al. (2000) reported that, in non-ruminants, NPA esters can be rapidly hydrolyzed by mammalian tissue esterases to release NPA, while NPOH is oxidized by hepatic alcohol dehydrogenase to NPA.

Pass, Majak, Muir, and Yost (1984) infused 3-nitropropanol (NPOH) into the rumen (30 mg/kg), abomasum (10 mg/kg) or small intestine (10 mg/kg) of sheep. The authors found that the NPOH was readily absorbed and converted into NPA. The reticulo-rumen was the major site of absorption for the miserotoxin aglycone but the abomasum and the small intestine also had the capacity to absorb NPOH. When NPA was injected into different regions of the alimentary tract, the reticulo-rumen was also the major site of absorption. Absorption of NPA or NPOH from the small intestine was much more rapid than from the abomasum. Plasma levels of NPA and inorganic nitrite were higher after dosing with NPOH than with NPA, indicating a more rapid rate of uptake of the aglycone (Pass et al., 1984).

After absorption from the digestive tract, NPOH is rapidly converted to NPA by a non-reversible reaction. This has been demonstrated in sheep, cattle and rats with the oxidation probably occurring in the liver (Majak & Pass, 1989). Degradation of NPA by ruminal microorganisms in cattle was found to be enhanced when the diet was supplemented by protein. The protein supplement increased nitropropanol degradation by 37–44% and promoted in vitro cellulose digestion (Majak, 1992).

#### *4.1.3. In vitro metabolism*

Alston, (1981) proposed that NPOH is oxidized to 3-nitropropanal (NPAL) by equine alcohol dehydrogenase (ADH) and that this decomposes to nitrite and acrolein, the proximate toxin which irreversibly binds succinate dehydrogenase (SDH). To test this hypothesis, McDiarmid, Majak, and Yost (1986) used equine alcohol dehydrogenase to oxidise NPOH and found the products to be NPA (12%) and nitrite (50%).

An intermediate generated in this series was thought to be 3-nitropropanal. Benn et al. (1989) further examined the oxidation of NPOH by ADH using HPLC. The intermediate product NPAL, was synthesized and it was also trapped in the reaction mixture as the semi-carbazone. At neutral pH, NPAL spontaneously decomposed to nitrite and acrolein but in the presence of ADH, NPAL was partially oxidized to NPA (Benn et al., 1989). Pass, Muir, Majak, and Yost (1985) examined this conversion of NPOH to NPA and found that it was suppressed by prior administration of ethanol or 4-methylpyrazole to inhibit alcohol dehydrogenase. Administration of ethanol or 4-methylpyrazole before NPOH treatment protected rats from intoxication. However, if the alcohol dehydrogenase inhibitors were given after the nitroalcohol, toxicity still developed. Administration of the aldehyde dehydrogenase inhibitor, diethyldithiocarbamic acid, had little effect on the conversion of NPOH to NPA and did not alter the toxicity of NPOH. Pass et al. (1985) concluded NPOH and NPA are equally toxic to rats, but that NPOH is toxic due to its rapid conversion to NPA.

The mechanism for conversion of NPOH to NPA and subsequent detoxification by ruminal bacteria was examined by Majak and Cheng (1981). They noted that although NPA has been detected in seven genera, representing four different families of higher plants, NPOH is found only in a group of *Astragalus* species of the family Luguminosae. Bound forms of NPA and NPOH are usually isolated, with NPOH occurring as the β-glucoside misertoxin and NPA as the mono-, di-, or tri-ester of glucose. Of the 33 strains of rumen bacteria tested by Majak and Cheng (1981), five degraded both NPOH and NPA under anaerobic conditions; another five strains degraded only NPA. The nitroacid was metabolized at a faster rate than the nitroalcohol by both pure cultures of rumen bacteria and mixed rumen micro-organisms. Nitrite was detected during incubation of NPOH and NPA with resting cells but not with growing cultures of active strains of rumen bacteria. Nitrite was metabolized much faster than the nitrotoxins by both pure cultures of rumen bacteria and mixed rumen micro-organisms. These results suggest that the nitro moiety of NPA or NPOH is metabolized to inorganic nitrite, which is reduced to ammonia by rumen micro-organisms, thereby resulting in its detoxification (Majak & Cheng, 1981).

Anderson, Rasmussen, and Allen (1993) explored the concept that rates of detoxification reactions are critical to acquisition of tolerance to nitroaliphatic-containing plants. Anderson et al. (1993) examined the detoxified end products of NPA and NPOH by ruminal organisms. They found that the rates of disappearance of NPA and NPOH varied somewhat between samples of ruminal fluid, but were approximately 0.4 and 0.1 μmol/ml of ruminal fluid per hour, respectively; rates with three-fold-concentrated cells from rumen fluid were correspondingly higher. Ruminal microbes from both cattle and sheep, reduced the nitro groups in situ, NPA was converted to β-alanine (which was, in turn, converted to other products) and NPOH was converted to 3-amino-1-propanol (87% conversion). Addition of sulfide and ferrous ions, to suspensions of ruminal microbes, increased the rate of NPOH reduction approximately three-fold, but rates of NPA reduction were not similarly increased (Anderson et al., 1993).

### 4.2. Toxicological studies

#### 4.2.1. General

Toxicity of nitroaliphatic compounds has had reasonably wide-spread coverage in the literature. However, there are a number of inconsistencies in the results obtained. These inconsistencies stem from an uncertainty of the qualitative and quantitative nature of the nitroaliphatic substance administered in the studies and the ability of ruminants to detoxify the NPA.

In studies with nitroaliphatic substance containing plants, the plants were often dried or frozen and stored for various periods of time, or extracted in various solvents prior to use. Williams and James (1976) showed that the amount of nitroaliphatic substances in the plants diminished with time and storage. Further, the amount of nitroaliphatic substances varies with the growing conditions of the plants and the portion of the plant used; that is, the leaf will differ in the concentration from the flower, stem, seed or root (Aylward, Cort, Haydock, Strickland, & Hegarty 1987; Gold & Brodman, 1991) and mature leaves have a concentration different from immature leaves (Cooke, 1955). The difficulty stemming from these observations is two-fold: first, studies were often expressed only in terms of the quantity of plant administered to the animal as opposed to the amount of $NO_2$ present in the sample and, second, quantitative analysis (of $NO_2$, NPA, etc.) was not conducted on the substance immediately prior to administration. Also, as noted in the National Cancer Institute report on the bioassay study, NPA itself was determined not to be stable in feed (NCI, 1978).

Further, it was often difficult to determine from the study description, exactly which nitroaliphatic substance the animals received, i.e. whether as the glucoside (misertoxin) or as NPA, NPOH or what combination of the three. The form of the toxin is important because, as will be discussed, animals vary in their ability to hydrolyze the toxin from the glucoside and thus make it available for absorption and toxicity. Additionally, since the ultimate toxin is NPA, much depends on the ability of the animal to transform NPOH to NPA.

Further, there are indications that more than one toxin may be present in some higher plants, since Hutton, Windrum, and Kratzing (1958) demonstrated that, when

seeds of *Indigo endecaphylla* were fed to mice, liver toxicity was produced, but the seeds did not give a positive reaction for NPA and that NPA-free extracts of leaves still produced the hepatotoxicity. Ludolf et al. (1991) noted that not all species of *Astragalus* contain NPA or NPOH and that others may be selenium-accumulating and some may produce 8α,β-indolizidine-1α,2α,8β triol, the likely toxic constituent in locoweed poisoning.

Another source of inconsistencies arises from the use of NPA produced from a *synthetic* process involving β-propiolactone, a mutagen and carcinogen, which may have been present as a contaminant (Hansen, 1984). This contaminant would not be present from naturally derived NPA, as biosynthesis takes place via a different route. NPA, used in the NCI study, was manufactured *via* the β-propiolactone process and had an impurity level of approximately 5% (NCI, 1978).

Therefore, much of the data involving feeding plant stuffs to animals, as well as much of the data involving administration of synthetic NPA, should be considered to be only qualitative.

### 4.2.2. Acute (single dose) toxicity studies

Acute toxicity studies (Table 7) show a fairly consistent $LD_{50}$ in rats given NPA or NPOH, either intraperitoneally or orally, to be in the range of 60–80 mg/kg, with Bell (1974) reporting that 100 mg/kg was quickly fatal. The subcutaneous $LD_{50}$ is approximately half the amount for other routes (30 mg/kg). Hamilton (1986) was successful with the 30 mg/kg dose in the production of brain lesions for study, but the progression of these animals through the stages of toxicity (Section 4.2.3) and subsequent deaths of the animals, indicated this dose was quite close to the $LD_{50}$.

Toxicity in mice was fairly consistent for an oral or subcutaneous dose, with a range of approximately 165–250 mg/kg, although refined sugarcane toxin had an $LD_{50}$ slightly less at 68–100 mg/kg. An approximate lethal dose, reported by Schafer and Bowles (1985) at 1600 mg/kg, is at least a factor of 20 greater than reported by other authors. No explanation of this outlier is determinable from the original publication.

Bossi, Simpson, and Isacson (1993) explored the age-dependent nature of NPA toxicity and produced 50% deaths at 30 mg NPA/kg in rats 11–14 weeks of age, but no deaths in groups of animals 3-6 weeks of age or 7–10 weeks of age. Tan et al. (1990) also explored this phenomenon, but found no difference in the $L_{D50}$ between "young" and "old" mice with a single dose (Section 4.2.3.2). In a situation analogous to NPA toxicity, Beal et al. (1993a) produced age-dependent striatal lesions that were significantly greater in 4- and 12-month old animals than in 1-month-old animals with malonate, a reversible inhibitor of SDH.

Penel (1977) reported results inconsistent with other investigators, with no deaths within 5 days of a single oral dose of NPA at 125 mg/kg, but 100% deaths at 200 mg/kg and higher. However, when these animals were

Table 7
Single dose studies with NPA or NPOH

| Species | Route/dose | Substance | Findings | Reference |
|---|---|---|---|---|
| Rat | Oral | NPA | No deaths at 125 mg/kg, 100% mortality at 200, 250 and 500 mg/kg. Histological changes in liver, lungs and kidneys | Penel, 1977 |
| Rat | Oral | NPA | 60 mg/kg animals subdued, 100 mg/kg quickly fatal | Bell, 1974 |
| Rat | Oral | NPOH | $LD_{50}$ = 77 mg/kg | Majak, Pass and Madryga 1983 |
| Rat | Intraperitoneal | NPA | $LD_{50}$ = 67 mg/kg (95% CL = 63-72) | Pass et al., 1985 |
| Rat | Intraperitoneal | NPOH | $LD_{50}$ = 61 mg/kg (95% CL = 51-70) | Pass et al., 1985 |
| Rat | Subcutaneous 30 mg/kg | NPA | Group 1 ages 3–6 weeks, 0 deaths; Group 2 ages 7–10 weeks, 0 deaths; Group 3 ages 11–14 weeks 50% deaths; Group 4 ages 16–20 weks 64% deaths | Bossi et al., 1993 |
| Rat | Subcutaneous | NPA | $LD_{50}$ = 22 mg/kg | Gould et al., 1985 |
| Rat | Subcutaneous | NPA | 30 mg/kg brain lesions | Hamilton, 1986 |
| Rat | Subcutaneous | NPA | Brain lesions 10 mg/kg X 1–4 days or 30 mg/kg single dose | Hamilton and Gould, 1987a, 1987b |
| Mouse | Oral | NPA | $LD_{50}$ for young mice = 221 mg/kg (95% CL = 166–295) $LD_{50}$ for old mice = 205 mg/kg (95% CL = 169–249 No significant difference between the two age groups | Tan et al., 1990 |
| Mouse | Oral | NPA | $LD_{50}$ = 221 mg/kg (Range 166–295 mg/kg) | Tan et al., 1989 |
| Mouse | Oral | Refined sugarcane toxin | $LD_{50}$ = 100 mg/kg in males $LD_{50}$ = 68.1 mg/kg in females | Liu et al., 1989 |
| Mouse | Oral | NPA | Approximate lethal dose = 1600 mg/kg | Schafer and Bowles, 1985 |
| Mouse | Subcutaneous | NPOH | $LD_{50}$ = 190 mg/kg | Gould et al., 1985 |
| Mouse | Injection | NPA | Lethal dose 300 mg/kg | Drummond, Gustine, and Phillips, 1975 |
| Pigeon | Gavage | NPA | Lethal dose 60–70 mg/kg (Range of 38–80 mg/kg) | Bell, 1974 |

again dosed at 125 mg/kg, following 5 days abstinence from NPA, all died within 75 min of administration. Histopathologic findings from the study were also at odds with other investigators who reported histological changes in the liver, lungs and kidneys, but no changes in the brain; the latter organ exhibiting consistent pathological change in other studies. Kitchin, Brown, and Kulkarni (1993) dosed rats with 10 mg NPA/kg and found no changes in hepatic ornithine decarboxylase, serum alanine aminotransferase (ALT) activity or $P_{450}$ compared with controls, indicating no hepatic damage.

Penel (1977) also reported that the pathological findings were consistent with nitrite poisoning. Gould, Wilson, and Hamar (1985) and Hamilton, Gould, Wilson, and Hamar (1984) also noted the formation of methaemoglobin in rats and mice, but these levels were below the threshold for lethality. Further, James et al. (1980) demonstrated that, although methaemoglobinaemia occurred in cattle fed *Astragalus* (up to 33% is formed in the blood in acute poisoning) and although this formation may contribute to the respiratory distress of the animal, death still occurs, even if the formation of methaemoglobin is prevented by administration of methylene blue.

Binienda et al. (1998) studied the acute effects of NPA on activities of endogenous antioxidants in the rat brain. Rats were administered 30 mg/kg NPA subcutaneously and sacrificed at 30, 60, 90 and 120 min after injection. Catalase activity was increased in hippocampus after 90 min of treatment. Cytosolic copper/zinc superoxide dismutase (SOD) and mitochondrial Mn-SOD levels were increased in the frontal cortex at 120 min. The activity of glutathione peroxidase and levels of reduced glutathione were decreased in the hippocampus at 120 min. The authors concluded that the depletion of glutathione and induction of antioxidant enzyme activities after NPA exposure suggested conditions favourable to oxidative stress. In an earlier, similar type of study, Binenda and Kim (1997) reported that acute administration of NPA increases free fatty acid in frontal cortex and hippocampus of rats, providing a substrate for free radical formation. A single dose of NPA (30 mg/kg, s.c.) to adult male SD rats resulted in progressive hypothermia, with a loss of 3°C or more in core body temperature by 3 h after dosing (Nony, Scallet, Rountree, Ye, & Biniendra, 1999).

Using an electron spin resonance technique, Fu et al. (1995) observed increase in free radical signals in rat livers at 15, 30 and 45 min after oral administration of 80 mg/kg NPA. The activities of liver superoxide dismutase and glutathione peroxidase, as well as the content of malondialdehyde, were significantly increased in rats treated with 80 mg/kg NPA. Klivenyi et al. (1999) studied the susceptibility of mice, with a disruption of the glutathione peroxidase gene to NPA. Systemic NPA administration resulted in significantly greater striatal damage and increases in 3-nitrotyrosine concentration in glutathione peroxidase knock-out mice, as compared with wild-type control mice. The authors concluded glutathione peroxidase plays an important role in detoxifying increases in oxygen radicals after NPA administration.

Alexi, Faull, and Hughes (2000) studied the variable toxic effects of systemic injection of NPA in rats. Controlled animal studies show variable susceptibility to NPA toxicity, yet the reasons remain elusive (Alexi et al., 2000; Fukuda, Deshpande, Shimano, & Nishino, 1998; Guyton, Hantraye, Dolan, Palfi, Maziere, & Brouillet, 1997). Although, it is clear that there is an age-related susceptibility to NPA toxicity in experimental animals, there is still variability within each age group as well as between age groups (Beal, Brouillet, Jenkins, Henshaw, Rosen, & Hyman, 1993b; Brouillet et al., 1993b). A single intraperitoneal dose of NPA (30 mg/kg) caused mortality in 21.7% of rats within 16 h of injection (Alexi et al., 2000). After 7 days, the in situ brain activity of SDH, the biochemical target of NPA, was severely decreased. Although all animals showed a severe decrease in SDH activity, most of the animals did not develop neurological damage, as assessed by histochemical staining. The authors claimed that rapid (1 day) decline in brain SDH activity induces neurological damage in the striatum, whereas a gradual (7 day) decline does not. This distinction occurred despite the absolute levels of decline in SDH which were almost identical. The rapid decline in vulnerable rats was associated with a striatal pocket of fully depleted SDH activity and neurological lesioning, which was associated both with the pocket and with the peripheral region surrounding the pocket. The fast decline in SDH activity in vulnerable rats was followed by an induction of TUNEL-label (terminal deoxytransferase-mediated dUTP-biotin nick end labelling) and cell death, whereas the slower decline in resilient rats was not.

James et al. (1980) dosed cattle and sheep with nitroaliphatic bearing plants, NPA or NPOH at both single and multiple intervals. The authors gathered various species of *Astragalus (pterocarpus, canadensis, falcatus* or *emoryanus*) from the field, then dried, ground and stored the material frozen. At time of use, the plant material was mixed with water and administered by stomach tube. A single dose of dried *Astragalus pterocarpus* to a cow (200 mg of $NO_2$/kg body weight) resulted in no effect, but when followed with two doses of NPA (10 mg/kg) over a 6-day period, the animal exhibited rapid respiratory rate, CNS depression and incoordination. At an unspecified period of time later, when this same animal was dosed four times over 17 days with another species of *Astragalus* (3 mg $NO_2$/kg body weight), the animal exhibited general body weakness and incoordination and was killed on the day of the last dose. Another cow, dosed once with *Astragalus canadensis* (275 mg of $NO_2$/kg body weight), exhibited

frothing at the mouth and generalized weakness. A third cow, dosed once with *Astragaus falcatus* (350 mg of $NO_2$/kg body weight) exhibited weakness and died.

Although it would seem from the data (James et al., 1980) that the no effect level is 200 mg $NO_2$/kg, there are many variables involved in the intoxication of ruminants. Susceptibility of ruminants to NPA toxicity rests on several factors, i.e. on the ability of the ruminant to absorb the intact misertoxin followed by subsequent hydrolysis of the NPOH and reduction to NPA, ruminal hydrolysis of the NPOH from the glycoside followed by absorption and reduction, and reduction to the hydrolyzed NPOH to nitrite. A clearer picture emerges, with additional experiments by James et al. (1980), wherein cattle were dosed (orally) once with NPA (15 mg $NO_2$/kg body weight) or NPOH (15 mg $NO_2$/kg body weight). In this case, both animals experienced incoordination, nervousness, weakness and stiffness and eventually died. Two sheep treated in a similar manner with NPA at 20 and 30 mg $NO_2$/kg body weight also died (James et al., 1980).

Hong, Castillo, Rivero, and Somanathan (1990) assessed the effects of oral administration of 1.0, 3.1 or 10 mg/kg of NPA on blood pressure and heart rate recordings from single-kidney hypertensive mongrel dogs. Measurements were taken at 0, 1, 2, 4, 6 and 8 h after drug administration. The investigators noted a dose-dependent decrease in maximal systolic and diastolic arterial blood pressures approximately 2 h following drug administration. Recovery from the antihypertensive effect of the nitro compound was slow and symptoms disappeared completely 8 h after ingestion of the NPA. A slight, but long-lasting decrease of heart rate was observed after administration of 1.0 and 3.1 mg/kg; however, the ingestion of 10 mg/kg of the compound provoked a modest and brief tachycardia (described by the authors as reflex tachycardia, common with nitrite-type anti-hypertensives; Hong et al., 1990). Castillo, Valencia, Reyes, and Hong (1993) noted a mild, but consistent decrease of both systolic and diastolic arterial blood pressure after oral NPA administration during 4 weeks of administration to renal hypertensive dogs.

Ether-extracted juice from creeping indigo (*Indigofera endecaphylla* Jacq.), associated with cattle poisonings, was force-fed to day-old chicks in 0.5 ml aliquots and 1 ml was fed to chicks that were 4 days old. The day-old chicks died within 1 min, the 4-day-old chicks died in 3–4 h. Synthetic NPA (1 ml of a 0.2% solution) was force-fed to several 2-week-old chicks. Chicks receiving synthetic NPA died within 0.5 h (Cooke, 1955). Yin, Yu, Chen, and Ma (1992) irradiated suckers of crownvetch and produced new varieties with lower NPA content. The mortality rate of chicks given diets containing 10% dry meal of the new varieties was lower by 20–40%.

*4.2.3. Subacute (multiple dose) toxicity studies*

*4.2.3.1. Studies in rats.* Pass, Majak, and Yost (1988) injected rats intraperitoneally with 10, 20 or 25 mg NPA/kg twice daily for up to 4 days. The authors noted that growth was decreased in rats at 20 and 25 mg/kg; food and water intake decreased at all dose levels. Neurological signs of hind-limb paresis, progressing to paralysis, were evident within 2 days in the most severely affected rats. The severity of the symptoms was related to the dose of NPA given. Of the 12 animals given NPA at 25 mg/kg, 10 died and two recovered; two of six rats at 10 mg/kg developed slight hind-leg incoordination but did not die. Administration of thiamin hydrochloride (i.p. at 10 mg/kg daily for 4 days) did not alleviate toxicity (Pass et al., 1988).

Hamilton (1986) characterized the stages of NPA toxicity. Sprague Dawley (SD) rats were injected subcutaneously with either a single dose of 30 mg/kg NPA in saline or 10 mg/kg/day over 1–4 days. Hamilton noted that most rats at the high dose immediately became recumbent, while others at the high and lower doses (10 mg/kg), exhibited increased spontaneous motor activity and/or uncoordinated gait. This is likely due to a release of excitatory neurotransmitter, described earlier. Intoxication was roughly divisible into three stages, characterized by somnolence in Stage I, uncoordinated gait with stereotypical paddling and rolling movements in Stage II and ventral or lateral recumbency in Stage III. Length of time to a specific stage was inversely proportional to dose. Morphologic lesions in the brains were bilateral and symmetrical, affecting the caudate-putamen, the hippocampus (including the dentate gyrus) and the thalamus. The lesions were common to rats in both dose regimens and the severity was proportional to the length of time spent in what the author described as the most advanced stage of symptoms (Stage III, recumbency), but not with dose. Of 40 rats on study which became recumbent, all had injuries of the caudate-putamen and 63% had injury at all three sites. Brain damage was similar, but not identical to rats with hypoxic brain damage from other causes (ischemia and hypoglycaemia; Hamilton & Gould, 1987b). The rats with typical brain lesions were not hypotensive (systemic blood pressure, $139 \pm 11$ mmHg), or hypoxemic (PaO2, $95.5 \pm 7$ mmHg). That is, neither hypotension nor hypoxaemia were necessary for the development of morphologic lesions (Hamilton et al., 1984).

Hamilton (1986) also reported no evidence of selective effects on neuronal subpopulations in the caudate-putamen or thalamus but, in the hippocampus, the neurons in the granule cell layer were relatively resistant to injury in comparison with the neurons in the pyramidal cell layer. No gradients of susceptibility were observed in the pyramidal cell layer. Ultrastructurally, neuronal alterations ranged from chromatin clumping, with increased cytoplasmic lucency, to severe cellular

shrinkage or swelling, with marked mitochondrial swelling (high amplitude swelling). White matter alterations included axonal swelling and axonal splitting of myelin lamellae. Vascular changes included perivascular deposits of proteinaceous material, presumably from leakage of serum proteins, variable electron lucency of endothelial cell cytoplasm, an apparent increase in pinocytotic vesicles, rare platelet thrombosis of capillaries and rare intravascular blebs of luminal plasma membrane (Hamilton, 1986; Hamilton & Gould, 1987b). Histochemical staining of frozen sections of the brain indicated that SDH was reduced in a uniform manner throughout. Nishino et al. (1997) reported that NPA-induced striatal damage was associated with astrocyte cell death and dysfunction of the blood–brain barrier. In a more recent study, Nishino et al. (2000) hypothesized that the striatum specific lesion by NPA is due to cumulative insults characteristic of the striatum, including glutamatergic excitotoxicity, dopaminergic toxicity and vulnerability of the lateral striatal artery and high activity in the glutamate-transporter.

In a range-finding experiment for a chronic study, NPA was added to rat feed in concentrations ranging from 100 to 900 ppm. Five males and five females were tested at different doses and controls were used. All animals were dosed for 6 weeks, then observed for 2 weeks. In male rats, mean body weight gain was 77% of controls at 100 ppm (5 mg/kg/day), 59% at 150 ppm (7.5 mg/kg/day) and 57% at 250 ppm (12.5 mg/kg/day). All males at 500 (25 mg/kg/day) and 900 ppm (45 mg/kg/day) died. In females, mean body weight gain was 97% of controls at 100 ppm, 87% at 150 ppm, 71% at 250 ppm and 62% at 500 ppm. Two females died at 250 ppm, four at 500 ppm and five at 900 ppm. On histologic examination, testicular atrophy with spermatogenic arrest was found in male rats and malacia in the midbrain in both sexes given doses of 150 ppm and above. On the basis of these results, doses for the chronic study were set at 25 and 50 ppm for males, and 50 and 100 ppm for females (NCI, 1978).

Borlongan, Koutouzis, Freeman, Hauser, Cahill, and Sanberg (1997) reported that manipulating the number of injections of NPA can result in either increased nocturnal spontaneous locomotor activity (hyperactivity) or nocturnal akinesia (hypoactivity). Two intraperitoneal injections (one injection every 4 days) of NPA (10 mg/kg) resulted in hyperactivity, while four or more injections of NPA produced hypoactivity.

Nishino, Nakajima, Kumazaki, Fukuda, Muramatsu, and Deshpande (1998) studied the gender differences in the vulnerability of the lateral striatal artery after systemic NPA administration. Subcutaneous injection of NPA (20 mg/kg once a day for 2 days) induced striatal selective lesions in half of the male rats associated with motor symptoms, while female rats were resistant. The motor and histological disturbances were highly sex-dependent. Castration had little effect, but ovariectomy enhanced vulnerability. Replacement therapy with testosterone increased, while estradiol or tomoxifen suppressed the vulnerability in ovarictomized females. Authors suggested the therapeutic use of estradiol and tamoxifen.

Ouary et al. (2000) studied the strain differences in response to NPA in rats. Based on the results of pilot studies, three different strains of rats, SD, Lewis and Fisher 344, were treated initially with 7 mg/kg/day NPA, the dose was increased by 15% every day until all animals died and the dead animals were recorded every day before NPA injection. Within 7 days, all rats from all strains died, except one of the SD strain. Doses leading to first deaths in Fisher, SD and Lewis rats were 9.3, 12.2 and 38.0 mg/kg/day, respectively. This indicated that Fisher, SD and Lewis rats exhibited high, intermediate and low vulnerability to NPA, respectively. Survival curves showed a more heterogeneous response to NPA toxicity in SD rats than observed in Fisher and Lewis rats. These differences between SD and Lewis rats were further confirmed in a protocol of subcutaneous NPA intoxication using osmotic minipumps, where doses up to 36–45 mg/kg/day for five days were necessary to induce striatal lesions in Lewis rats as compared with 12–14 mg/kg/day for 5 days in SD rats. These results suggested that vulnerability to NPA may depend on genetic factors, which could also influence the physiological response to stress (Ouary et al., 2000).

Guyton et al. (1997) examined motor abnormality in rats treated with NPA. Subacute intraperitoneal administration of NPA at 15 mg/kg/day produced dramatic motor symptoms associated with extensive neuronal loss and gliosis in the lateral striatum, as well as severe hippocampal degeneration in 50% of rats. In contrast, subcutaneous treatment of rats with 10 mg/kg/day NPA for 1 month led to more subtle excitotoxic-like lesions, selective for the dorsolateral striatum and more closely resembling Huntington's disease[1] striatal pathology in humans. Rats with Huntington-like lesions showed spontaneous motor symptoms, including mild dystonia, bradykinesia and gait abnormalities, which were barely detectable on visual inspection, but could be identified and quantified by computerized video analysis. In animals treated for 1 month, the degree of striatal neuronal loss was significantly correlated with the severity of spontaneous motor abnormalities, as is the case in HD.

*4.2.3.2. Studies in mice*. In an attempt to find chemicals with repellant capacity, Schafer and Bowles (1985) screened some 933 chemicals for approximate lethal

[1] Huntington's disease is a chronic, progressive, or degenerative disorder beginning usually between the ages of 30 and 50 years, characterized by choreic movements in the face and extremities, accompanied by a gradual loss of the mental faculties ending in dementia

dose (ALD) and the dose promoting food reduction in mice. For the ALD, the animals were dosed and observed for three days. The authors reported an ALD in deer mice to be a previously unreported high of 1600 mg/kg. The dose of NPA, which approximated the $LD_{50}$ when mixed with seeds and fed to animals on a subacute basis, was 613 mg/kg. The authors appeared to have exceeded doses given under similar conditions by a factor of at least 20. The text does not indicate the basis of this difference.

Mosher et al. (1971) fed dried timber milkvetch (TMV) to mice weighing 25–30 g. The mice exhibited symptoms within 36 h, with death following approximately 24 h thereafter. Consumption of 7 g TMV/100 g body weight, over a period of two to 12 days, was adequate to kill mice. There was at first a loss of equilibrium; the mice rolled over frequently and were generally unsteady on their feet. They assumed a characteristic arching of the back, body temperature dropped significantly and heart rate slowed markedly. Internally, there were massive haemorrhages on the mucosal lining of the stomach, a finding not reported by other investigators. Findings were generally the same in rats (Mosher et al., 1971) with symptoms of poisoning evident at 36 h and deaths approximately 24 h later. The occurrence of gastric haemorrhages and other symptoms were similar to the mouse experiments. The authors also noted a significant increase in SGOT and serum isocitrate dehydrogenase (ICD). In an experiment to determine the toxic principal, mice were injected intraperitoneally with deproteinized blood and intestinal contents of rats fed timber milkvetch, TMV water extract or NPA. Mice fed protein-free filtrate of intestinal contents of rats died in two hours. Mice fed protein-free filtrate of blood of TMV given rats died within 4 h. Mice on TMV water extracts (orally, daily for 5 days) died in 5 days. Neutralized NPA (2 mg i.p.) killed mice in 3 h (Mosher et al., 1971).

Although, Bossi et al. (1993) found that, in rats, mortalities in response to NPA correlated with age, Tan et al. (1990) found the opposite with mice undergoing chronic administration. Following repeated daily gavage administration, the "chronic" (30 day) $LD_{50}$ was significantly ($P < 0.04$) higher in old (138 mg/kg 133–144) compared with young (49 mg/kg; 47–51) mice. However, although aged mice demonstrated no increased resistance to acute toxic effects of NPA on spinal cord neurons (i.e. glycogen accumulation), they could tolerate an almost three-fold increase in the dose of chronically administered NPA. Spinal cord intraneuronal glycogen accumulation was not seen in mice acutely fed 2.5 times the $LD_{50}$, but was present following chronic feeding of 0.2 or 0.4 of the $LD_{50}$ NPA (Tan et al., 1990).

Beal et al. (1993b), exploring the relationship between age and striatal lesions, used subcutaneously implanted Alzet7 pumps for subacute systemic administration of NPA. The NPA was administered at a dose of 20 mg/kg for 5 days. The investigators found that subacute systemic administration produced age-dependent bilateral striatal lesions. "Chronic" administration of NPA, over 1 month, produces selective striatal lesions that replicate many of the characteristic histologic and neurochemical features of HD (Beal et al., 1993b). Brouillet et al. (1993b) examined the effects of age on striatal lesions produced by local administration of NPA to rats. An in vivo chemical shift in magnetic resonance imaging showed marked increases in striatal lactate concentrations that significantly correlated with increasing age. Histologic and neurochemical studies showed a striking age dependence of the lesions, with four and 12 month old animals much more susceptible than 1-month-old animals. Continuous systemic administration of low doses of NPA, for 1 month, resulted in striatal lesions showing growth-related changes in dendrites of striatal spiny neurons using the Golgi technique. Recently, Page, Besret, Jain, Monaghan, Dunnett, and Everitt (2000) reported that, while producing striatal lesions which bear a similarity to those seen in Huntington's disease, the consequences of NPA for striatopallidal and striatonigral efferent projections do not reflect the reported neurodegenerative changes seen in an HD brain.

In the mouse range-finding study for the NCI (NCI, 1978) bioassay of NPA, the NPA was added to the mouse feed in concentrations ranging from 150 to 800 ppm. Five males and five females were tested at the different doses and controls were used. All animals were dosed for 6 weeks, then observed for 2 weeks. In males, mean body weight gain of groups receiving 150 or 600 ppm (21–85 mg/kg) were not affected. An early weight depression was observed at 800 ppm, but these animals recovered to control levels. Mean body weights of females were not affected at any dose tested. One male died at 600 ppm, and another at 800 ppm. Hydronephrosis was found in nine mice, but the incidence was not dose-related. On the basis of these findings, the low and high doses for males and females in the chronic studies were set at 75 and 150 ppm (10 and 21 mg/kg), respectively.

*4.2.3.3. Studies in rabbits.* Hutton et al. (1958) gavaged two rabbits with synthetic NPA for 22 and 34 days at dose levels approximating 53 and 84 mg/kg/day, respectively. The first animal lost approximately 200 g body weight and the second lost approximately 100 g body weight over the duration of the feeding. The animals were described as "lively" at the end of the experiment and there were no remarkable histopathological changes. In another study, two rabbits were fed dried *Indigofera endecaphylla* at a dose of 7.73 or 7.40 mg NPA/kg/day. Both animals died and liver pathology indicated fine nodular cirrhosis. Continuing this series

of studies, Hutton et al. (1958) reported groups of 2–3 rabbits fed a combination of meal and different strains of *Indigofera endecaphylla* from 5 to 32 days. One animal, surviving only 5 days, exhibited acute liver degeneration and necrosis. The remaining animals survived from 14 to 32 days and all exhibited diffuse nodular cirrhosis. All animals lost body weight during the course of treatment. In a follow-up study, rabbits fed seeds of *Indigofera endecaphylla* exhibited similar hepatic damage, but the seeds were negative for NPA. The investigators concluded that, since the animals fed NPA had greater survival and different pathology from those fed plant parts with comparable or less amounts of NPA, the operative toxin in *Indigofera endecaphylla* was not NPA.

*4.2.3.4. Studies in other mammals.* Shenk, Wangness, Leach, Gustine, Gobble, and Barnes (1976) treated weanling voles with crownvetch mixed into the diet, with 0.47% of the diet being NPA. Intake and weight gains were decreased as compared with controls on alfalfa. The initial clinical indications of toxicity were decreased activity and hunched-up appearance. Symptoms appeared within 2 days and all animals died. In a follow-up study, voles were fed crownvetch, which had from 0.008 to 0.15% NPA. One hundred per cent mortalities occurred at the highest dose and a no effect level was not achieved. The exact doses could not be determined since the method for estimation of aliphatic nitro compounds was not specific for NPA. These same investigators also fed crownvetch to pigs, which lost weight and showed serious signs of incoordination and staggering from day 8 to 15. The condition was first noticeable in the rear quarters and, at later stages, seemed to affect the entire body. The pigs also had an increased packed cell volume and decreased glucose compared with controls, but this likely only reflected decreased eating and drinking. There was no change in plasma urea nitrogen. On day 11, the pigs were returned to alfalfa diets and, after several days, the symptoms disappeared and regained lost weight.

James et al. (1980) gathered various species of *Astragalus (pterocarpus, canadensis, falcatus* or *emoryanus*) from the field, then dried, ground and stored the material frozen. At time of use, the plant material was mixed with water and administered by stomach tube to cattle. The amount administered was from 2 to 200 mg $NO_2$/kg body weight with infusions of material daily for several days to irregular administration over 98 days. Although many animals exhibited the characteristic effects of NPA toxicity, the data indicated that the time of onset and severity of responses varied. Some animals had no symptomology at all. The wide range of effects is likely due to variable susceptibility of ruminants to NPA toxicity, which rests on several factors, i.e. on the ability of the ruminant to absorb the intact toxin, followed by subsequent hydrolysis of the NPOH and reduction to NPA, ruminal hydrolysis of the NPOH from the glycoside, followed by absorption of the hydrolyzed NPOH to nitrite. James et al. (1980) noted a difference in pathology between acutely and chronically intoxicated animals. Acute intoxication was associated, in most instances, with severe respiratory distress, pelvic limb weakness, prostration and death. These acutely intoxicated cattle usually had severe lung lesions of lobular alveolar emphysema and collapsed and constricted bronchioles, often with interlobular oedema. Some of the cattle also had widespread small focal haemorrhages in the CNS. Cattle with chronic or delayed intoxication had mainly neurologic signs of knuckling and pelvic limb incoordination. These animals had similar but milder lung lesions than animals with acute intoxication, but with some interlobular fibrosis; in the two animals sampled, there was extensive Wallerian degeneration found in the sciatic nerves. Both acute and chronic cases in cattle had mild Wallerian degeneration of the spinal cord (James et al., 1980). James et al. (1980) noted increases in methaemoglobin, following feedings to the chronically-treated animals. Methaemoglobin returned to baseline within 24 h of feeding. Methaemoglobin was not a major factor in the death of the animals. The increase in methemoglobin was less in acutely intoxicated animals.

James et al. (1980) also examined sheep for nitroaliphatic intoxication. The procedure was the same as for cattle, using various species of *Astragalus*. The amount administered was from 150 to 500 mg $NO_2$/kg from *Astragalus facultatus* in as much as 4 doses over 10 days or 7 doses in 9 days of 38 mg $NO_2$/kg derived from *Astraglus emoryanus*. The sheep exhibited depression, respiratory distress, weakness and other characteristic symptoms. All sheep died or were killed in extremis. Most sheep affected with field or experimentally induced intoxication showed a similar acute symptomology with respiratory distress. The only pulmonary abnormality observed was oedema. Some animals with incoordination showed mild Wallerian degeneration of the spinal cord and some had subcapsular nephrosis (James et al., 1980). Sheep were administered orally with doses ranging from 10 to 30 mg $N(oh)_2$/kg/day (NPA or NPOH) for 1–6 doses administered over as much as 28 days. All animals exhibited one or more signs characteristic of poisoning from the use of these compounds and all died or were killed in extremis (James et al., 1980).

Emory milkvetch, in the pod stage of growth, was collected, dried, ground and stored at 2EC. A 59.6 kg ewe was fed 400 g of the milkvetch (38.9 mg $NO_2$/kg body weight) for 7 days (excluding an intervening weekend). The ewe became weak and unsteady in the hindquarters on the sixth day of treatment and, following feeding on the seventh day, the animal rapidly became weaker, uncoordinated and was euthanized.

Blood was drawn for methaemoglobin 3 h after feeding on the first and third days. The values were 4.6% of total haemoglobin on the first day of feeding and 4.3% on the third (Williams & James, 1976). These results were compared to animals dosed intravenously with NPA and NPOH (20 mg $NO_2$/kg). The NPA-dosed sheep became depressed and uncoordinated in the hindquarters 3 h after treatment. Twenty-four hours later, one sheep became weak, very uncoordinated and fell frequently. When it arose, the animal's fetlocks knuckled over displaying symptoms consistent with NPA toxicity. One sheep was euthanized after one infusion, but the other was infused a second time and died later in the day. Sheep infused with NPOH became weak and uncoordinated in the hindquarters 2 h after treatment. No knuckling of fetlocks was noted. Respiration was laboured and audible. Both animals collapsed and died approximately 4 h after treatment. Methaemoglobin levels were somewhat less in the NPA/NPOH-treated animals at only 2.1–2.5%. The authors concluded that symptoms produced in sheep with Emory milkvetch and NPA were identical. The syndrome produced by NPOH was similar to that produced by NPA, except that the time lapse between the onset of toxic signs and collapse and paralysis was very rapid and the knuckled fetlock stage was not evident (Williams & James, 1976).

*4.2.3.5. Studies in non-mammals*. Other species are also affected by NPA. A lethal dose of NPA to pigeons is approximately 60–70 mg/kg (Bell, 1974) and the lowest level of crownvetch to chicks (with NPA at 0.05% of the diet) resulting in "mild" symptoms (Shenk et al., 1976). Yin et al. (1992) dosed chicks with irradiated and non-irradiated crownvetch. Mortalities were lowered in chicks fed irradiated crownvetch. Williams (1980) extracted leaves of *Astragalus hamosus* and *Astragalus sesameus* in ethanol, dried the extract, re-dissolved it in water and re-extracted with benzene. The water fraction was then given to chicks via syringe into the crop. Toxic signs were seen and deaths occurred at high doses, but the amount of $NO_2$ was not stated by the authors. Only the chicks dosed with *Astragalus hamosus* showed toxic effects (Williams, 1980).

Brine shrimp (*Artemia salina* L.) were tested for susceptibility to NPA in vivo. A paper disc was inoculated with NPA, the solvent allowed to evaporate and brought into contact with the larvae. The authors found that 90 μg NPA/disc resulted in the deaths of 35% of the brine shrimp so exposed (Panigrahi, 1993). Byers, David, Mayer, and Bierlein (1986) reported that feeding of insects, *Sparganothis fruitworms* and *Sparganothis sulfureana* (Clemens) pinto bean diet, containing low levels of NPA, significantly increased mortality of larvae and reduced pupal weight of both males and females, over four generations.

*4.2.4. Subchronic toxicity studies*

Tan, Brooks, Chu, Nizamuddin, and Schutta (1989) gavaged mice daily (up to 99 days) with water or 0.2, 0.4 and 0.8 of the $LD_{50}$ (221 mg/kg) dose of NPA (approximately 44, 88 and 177 mg/kg). All mice lost weight in the first 10 days of gavage but surviving mice, fed less than 80 mg/kg, gained weight thereafter. Chronic toxicity was associated with decreased open field behaviour, tremulousness and some hind-limb weakness on grasping. Light and electron microscopic neuropathology showed variable adaxonal changes in lateral and ventral spinal cord myelinated axons, but no motor neuron degeneration. The chronic (30 days) oral dose $LD_{50}$ was 49 mg/kg (range, 47–51; Tan et al., 1989).

It has been suggested that dopamine toxicity plays a role in NPA-induced damage. Johnson, Robinson, Ali, and Binienda (2000) studied the dopamine toxicity following long term exposure to low doses of NPA in rats. Adult male SD rats were exposed for 3 months to NPA, weekly, for 24 h periods at 10 and 20 mg/40 ml in drinking water. Dopamine and its metabolites were assessed in the frontal cortex and caudate nucleus. An increase in dopamine turnover was observed in the caudate nucleus of the high dose group following 2 months of dosing. The authors suggested that the production of oxygen radicals associated with dopamine metabolism may contribute to NPA-induced neurotoxicity.

Wullner, Young, Penney, and Beal (1994) studied the systemic effects of NPA, in doses ranging from 12 to 16 mg/kg/day for 30 days, on striatal cytoarchitecture in rats. Administration of NPA, at a dose of 16 mg/kg/day, resulted in large lesions with a central necrotic core that was depleted of both neurons and glia. In animals treated with 12–15 mg/kg/day neither the area nor binding density of the patches were changed.

*4.2.5. Chronic toxicity and carcinogenicity studies*

In 1978, the NCI reported on a carcinogenicity study in rats and mice using NPA (NCI, 1978). Because the investigators had determined that NPA was unstable in food, the NPA was administered via gavage 5 days per week (104 weeks for mice and 110 weeks for rats) to 50 animals per sex per group. Dose levels for the chronic study were established on the basis of the results of the subchronic study. For the chronic study, the low and high doses for the male rats were set at 25 and 50 ppm; for females, 50 and 100 ppm. For both male and female mice, the low and high doses for the chronic studies were set at 75 and 150 ppm. Because it was determined that NPA was unstable in feed, the method of administration used for the chronic study was gavage. Doses were converted from parts per million to milligrams per animal per day (mg/animal/day), based on an estimated food consumption of 17 g/day for male rats, 12 g/day for female rats and 5 g/day for mice (both sexes). The doses in mg/animal/day (Table 8) were used throughout

the study; thus, as the weights of the animals increased, the amounts per unit of body weight decreased (NCI, 1978).

This methodology left much to be desired, as it was difficult to back-calculate the exact amount of NPA received by the rats, especially since no body weight data (other than graphs) were presented in the report. Therefore, in order to determine how much NPA was consumed, it was necessary to consult standard testing industry data (Covance Laboratories, Vienna, VA), which assume that a rat consumes food in an amount equal to 10% of its body weight per day. Using these data, rats administered 50 ppm (0.85 mg/animal/day) consumed 2.5 mg NPA/kg body weight/day and this number was used as the no observed effect level (NOEL; see later). The study design is presented in Table 8.

In the rat study, general appearance, body weights and mortalities of treated groups did not differ from controls. An adequate number of animals remained on study to detect any late-appearing tumours. A variety of neoplasms occurred in both control and dosed groups. Each type of neoplasm had been encountered previously as a spontaneous neoplasm in rats (NCI, 1978). In male rats, there was a slight increase in hepatic neoplastic nodules, but the increase was not significant and was within the normal range for the Fischer 344 strain (Haseman, Arnold, & Eustis, 1990). Treated males also had a higher, but non-statistically significant increase in incidence of pancreatic islet cell adenomas, although no such changes were observed in females or treated mice of either sex. It was concluded that NPA was not carcinogenic in rats or mice.

Focal myocardial fibrosis was observed in dosed vs. control rats. This may well be due to the stress of gavaging because it was only seen in gavaged rats (i.e. and not in non-gavaged controls). Clinically silent degenerative myocardial disease of unknown etiology is common in F344 rats and may be produced by stress, diet or environment (MacKenzie & Alison, 1990). Further, this condition does not appear dose-related because, although there was a doubling of dose between the low and high dose levels, there was not a doubling of this effect in animals of either sex. The lack of dose-response would indicate that the fibrosis was not a specific biochemical lesion, as would an enzyme inhibitor such as NPA.

In the mouse study, the mean body weights of both the low- and high-dose males and females were lower than those of controls during the greater part of the bioassay. Mortalities were not excessive, nor was there a difference between treated and control groups. A sufficient number of mice of each sex were at risk for the development of late-appearing tumours. A variety of neoplasms occurred in both the control and dosed groups. Each of the types of neoplasms have been encountered previously as a spontaneous lesion in the mouse. Proliferative hepatocellular lesions occurred in both sexes, but there was no indication that they were related to administration of NPA. Several chronic inflammatory, degenerative and other proliferative conditions were observed in all groups and were not related to administration of NPA.

Therefore, because an effect could not be directly attributed to the administration of NPA, the no observed adverse effect level (NOAEL) for NPA in a rodent chronic bioassay was 50 ppm (2.5 mg/kg/day). The NOAEL was based on the male rat study since this was the highest level tested which could be assumed to be safe in the most sensitive animal model.

Palfi et al. (1996) studied the cognitive performances of chronically NPA-treated and control baboons, using the object retrieval detour task (ORDT). Fourteen *Papio anubis* baboons were included in the study; four received intramuscular doses of NPA and 10 controls received saline for 20 weeks. The dose regimen for the administration of NPA was designed to yield a linear

Table 8
NPA bioassay study design

| Species | Sex | Dose[a] (mg/animal/day) | Duration of dosing (weeks)[b] |
|---|---|---|---|
| Rats (Fischer 344) | Males | 0 (Control) | |
| | | 0.425 | 110 |
| | | 0.85 | 110 |
| | Females | 0 (Control) | |
| | | 0.6 | 110 |
| | | 1.2 | 110 |
| Mice (B6C3F1) | Males | 0 (Control) | |
| | | 0.375 | 104 |
| | | 0.75 | 104 |
| | Females | 0 (Control) | |
| | | 0.375 | 104 |
| | | 0.75 | 104 |

[a] Doses are equivalent to 25 and 50 ppm for male rats, 50 and 100 ppm for female rats, and 75 and 150 ppm for both sexes of mice.
[b] Controls were not gavaged.

increase in doses until obvious spontaneous abnormal movements could be observed (mean starting dose, 14 mg/kg/day; mean final dose, 33 mg/kg/day). A significant impairment in the ORDT was observed in the NPA animals after 3–6 weeks of treatment, occurring in the absence of spontaneous abnormal movements but in the presence of apomorphine-inducible dyskenesias. Prolonged NPA treatment resulted in the progression appearance of spontaneous abnormal movements. Histological evaluation showed selective bilateral caudate-putamen lesions with sparing of the cerebral cortex, notably the prefrontal cortex. The authors concluded that, chronic NPA treatment replicates, in primates, the basic pathophysiological triad of HD, including spontaneous abnormal movements, progressive striatal degeneration and a frontostriatal syndrome of cognitive impairment. Earlier, authors from this study have shown that chronic systemic administration of NPA to baboons can produce various dyskinetic movements and dystonic postures associated with selective striatal lesions (Brouillet et al., 1993b; 1995; Ferrante, Hantraye, Brouillet, Kowall, & Beal, 1993).

*4.2.6. Reproductive toxicity*

In the range-finding study for the bioassay, NPA was added to rat feed in concentrations ranging from 100 to 900 ppm (NCI, 1978). Five males and five females were tested at different doses and controls were used. All animals were dosed for 6 weeks, then observed for 2 weeks. On histologic examination, testicular atrophy with spermatogenic arrest was reported in male rats at 150 ppm, although no incidence data or statistical analyses were presented (NCI, 1978). This finding should not be regarded as dispositive for the following reasons: (1) this finding occurred with nearly equal incidence between controls and treated groups in the bioassay, wherein the animals were exposed to NPA for 110 weeks, a term 18 times greater than the range-finding study; (2) NPA was found to be unstable in the diet (the method of administration in the range finding study) and therefore, the changes may have been elicited by a degradant; (3) a similar occurrence was not significant in mice in the range finding or bioassay studies; (4) the effect seen in the range-finding study may well have been the one of the pathologic sequelae to arrested development in the range-finding animals; and (5) this finding is not corroborated by any other investigators.

*4.2.7. Mutagenicity studies*

Controversy exists in the scientific literature concerning the mutagenicity of NPA. Hansen (1984) considered NPA a non-carcinogenic mutagen and, as such, it is a rarity. This information, together with the knowledge that NPA used in the NCI bioassay contained impurities, led Hansen to investigate the findings of the previous researchers. Hansen first obtained a new lot of NPA from the supplier (Aldrich Chemical Co., Milwaukee, Wisconsin), consisting of white crystals. A sample of the NPA used in the carcinogenicity bioassay (NCI, 1978) and subsequent positive mutagenicity assays (Dunkel & Simmon, 1980) was obtained from the chemical repository of the National Toxicology Program. This earlier lot had been purchased from Aldrich by NCI in 1973 and was yellow–orange, but upon recrystallization, yielded white crystals.

Hansen (1984) then took a sample from the NCI lot of NPA, which had been reported mutagenic, in strain TA100 (1500 μg/plate) without metabolic activation (Dunkel & Simmon, 1980), but mutagenic activity was almost totally lost after recrystallization. This sample was not mutagenic in strain TA98, before or after recrystallization. A new, purer commercial sample was non-mutagenic in strains TA98, TA100 and TA1538, with or without metabolic activation. The NPA was tested up to the level of bacterial toxicity which, for all three strains was greater than 1500 μg/plate without liver enzymes and greater than 500 μg/plate with liver enzymes. Hansen (1984) concluded that the mutagenicity attributed to NPA was due to the presence of impurities. Impurities may have included β-propiolactone, from which NPA is synthesized and which also produces base-pair mutagenicity. The mutagenic impurity would not be present in the natural state, since natural synthesis occurs by a different route (Hansen, 1984). With this knowledge, it is possible to divide studies into two pools, those which used the NCI sample and those which did not. It is probable that Caspary (1988a) used the NCI sample, since other work published that year by Caspary used the NCI sample.

In addition to Hansen (1984), other investigators, not using the contaminated NCI–NPA, demonstrated that NPA was not mutagenic in the experimental system utilized (Oshiro, Myhr, Bowers, & Caspary, 1988; Piper, Balwierz, & Soelter, 1991). Further, β-propiolactone acts as a positive mutagen in these test systems (RTECS, 1994). Therefore, data presented indicate that NPA is not mutagenic. A summary of mutagenicity assays is presented in Table 9.

*4.2.8. Genotoxicity*

Genotoxicity testing of NPA, in three *Drosophila* short-term somatic assays, was conducted by Batiste-Alenton, Xamena, Creus, and Marcos (1995). The somatic mutation and/or recombination tests in *Drosophila melanogaster* were evaluated by *zeste-white, white ivory* and wing spot assay. In *zeste-white* assay, frequency of eye colour mosaicism was investigated in adult *Drosophila* males of the UZ strain after larval treatment with 0, 5 and 10 mM NPA. For *white ivory* test, similar experiments were conducted with the (wi)4 strain of *Drosophila*. In *wing spot* assay, induction of mosaicism was studied in *Drosophila* after larval treat-

ment with NPA at 0, 5, 10 and 20 mM concentration. NPA produced positive results in both the *zeste-white* and the *wing spot* tests, although there was no dose-response. Because of lack of dose-response, the authors classified NPA as weakly genotoxic.

*4.2.9. Cytotoxicity*

Kinosita et al. (1968) found that NPA was cytotoxic to hamster cells in vitro at a concentration of $10^{-2}$M, but exhibited stimulatory effects at low concentrations ($10^{-6}$).

In vitro studies have shown that the presence of a nitro group on an aliphatic raises questions as to the ability of substances such as NPA to affect smooth muscle. Castillo, Reyesm, Rosas-Lezama, Valencia, and Hong (1994) reported a concentration-dependent decrease of contractile force and heart rate in isolated guinea pig atria by NPA. The authors speculated that the cardiodepressor activity may be explained by interference with the availability of cytosolic free $Ca^{2+}$. Hong et al. (1990) and Castillo et al. (1993) examined the relaxation effect of NPA on rabbit aortic rings in vitro and found that NPA did not produce relaxation via endothelium and it is not mediated by muscarinic, β-adrenergic, or histaminergic ($H_1$) receptors. Aortic rings, precontracted with KCl, were less sensitive to relaxation by NPA, suggesting that at least part of the mechanism of vasodilation is not related to an inhibition of calcium influx through the voltage-dependent $Ca^{2+}$ channel. The profile provided strong evidence that, like nitrovasodilators, NPA exerts relaxant effect through guanylate cyclase stimulation and consequent increase in cyclic GMP levels into the vascular smooth muscle cells. The anti-hypertensive effect of NPA to renal hypertensive dogs could be attributed mainly to its vasodilating action (Hong et al., 1990).

Lopez et al. (1998) studied the NPA-induced bradycardia in isolated atria. In isolated spontaneously beating atria, NPA ($10^{-4}$ M) decreased heart rate by 62%. NPA did not decrease the amplitude or duration of action potentials. However, the duration of intervals between two action potentials was prolonged from 530 to 1400 ms after exposure to $10^{-2}$ M NPA. NPA inhibited oxygen consumption by heart mitochondria when either malate/glutamate or succinate were used as metabolism substrates. NPA did not affect cytochrome c oxidase activity but decreased atrial ATP content by 65%. Results of this study show that NPA decreases atrial rate by increasing the action potential phase four, probably by inhibition of mitochondrial respiration, thereby decreasing cardiac ATP content, suggesting that NPA-induced bradycardia might be related to intracellular ATP depletion.

Using in vitro preparations of brain homogenate, Erecinska and Nelson (1994) found that NPA inhibited synaptosomal respiration in a dose-dependent manner; the degree of inhibition by the same concentration of the compound was greater, however, when respiration was stimulated by concomitant increase in ATP usage. The most rapid event, after addition of NPA, was a

Table 9
Mutagenicity assays with NPA

| Assay | Results | Sample origin | Reference |
|---|---|---|---|
| TA1535 | Positive | NCI | Dunkel and Simmon, 1980 |
| TA100 | Positive | NCI | |
| TA1535 | Positive | NCI | Zeiger, 1987 |
| TA100 | Positive | NCI | |
| TA1537 | Negative | NCI | |
| TA98 | Negative | NCI | |
| TA100 | Positive | NCI | Hansen, 1984 |
| TA98 | Negative | NCI | |
| TA100 | Negative | Commercial | Hansen, 1984 |
| TA98 | Negative | Commercial | |
| TA1538 | Negative | Commercial | |
| CHO cells HGPRT assay | Negative | Commercial | Myhr et al., 1988 |
| CHO cells HGPRT assay | Negative | Commercial | Oshiro et al., 1991 |
| CHO micronucleus assay | Negative | Commercial | Oshiro et al., 1991 |
| Survival assay (Rauscher leukaemia virus-infected rat embryo cells) | Positive | NCI | Traul, Takayama, Kachevsky, Hink, and Wolff, 1981 |
| Mouse lymphoma | Positive | NCI | Caspary, Langenback, Penman, Crespi, Myhr, and Mitchell, 1988b |
| Human lymphoblast | Positive | NCI | |
| CHO SCE | Positive | NCI | |
| CHO Chromosomal aberrations | Positive | NCI | |
| Mouse lymphoma | Positive | NCI (?) | Caspary, Daston, Myher, Mitchell, Rubb, and Less, 1988a[a] |
| Mouse lymphoma | Positive | NCI(?) | |
| Rat hepatocyte DNA repair | Positive | NCI | Williams, Mori, and McQueen, 1989 |

[a] A summary of two studies performed at different laboratories.

decrease in [creatine phosphate]/[creatine] and an increase in [lactate]/[pyruvate]. A fall in [ATP]/[ADP] and [GTP]/[GDP] was initially less pronounced but closely followed that in [CrP]/[Cr]. In the absence of glutamine, NPA caused a pronounced decrease in internal aspartate level and a small reduction in glutamate concentration, whereas [GABA] rose; the sum of these three amino acids inside synaptosomes fell, but there were no increases in their external levels. With glutamine in the medium, the reduction in intra-synaptosomal aspartate was accompanied by increases in intra-synaptosomal glutamate and GABA. The external concentration of glutamate rose substantially in the presence of the inhibitor. NPA had no effect on basal release of either glutamate (and GABA) or biogenic amines but increased efflux occurring upon addition of non-saturating concentrations of the depolarizing agents, veratridine and KCl.

Deshpande and Nishino (1998) studied the in vitro protection of NPA-induced toxicity of astrocytes by basic fibroblast growth factor (bFGF) and thrombin. NPA (0.017–1.7 mM) produced concentration- and time-dependent astrocyte loss as indicated by decrease in the number of glial fibrillary acidic protein (GFAP) positive cells and increase in LDH levels of the culture medium. The NPA-induced loss was apparent within 12 h and was maximal at 24 h. The presence of bFGF (10 ng/ml) attenuated the NPA-induced cell loss. Thrombin, at 0.01 nM, protected against the toxicity of NPA, while higher concentrations (10–100 nM) produced greater cell loss. The results indicated that NPA produced acute astrocyte toxicity and was attenuated by bFGF and low concentrations of thrombin.

Olsen, Rustad, Tonnum, Paulsen and Hassel (1999) investigated NPA toxicity in primary cultures of cerebellar granule cells and astrocytes. NPA inhibited SDH and tricarboxylic acid cycle activity to the same degree in neurons and astrocytes. Even so, NPA was 16 times more toxic to neurons than to astrocytes. The reported $LC_{50}$ for neurons and astrocytes was 0.7 and 11 mM, respectively. The authors suggested that the relative resistance of astrocytes to NPA may be related to their low tricarboxylic acid cycle activity (5–10% of that in neurons) and to the inability of NPA to cause astrocytic calcium overload. The authors concluded NPA is predominantly an astrocyte-sparing neurotoxin.

Riepe et al. (1992) studied the effects of measured amounts of NPA in the organ bath on the brains of Sprague-Dawley rats. NPA (1 mM) produced a hyperpolarization for variable lengths of time before evoking an irreversible depolarization in the pyramidal cell layer of the hippocampal region CA1. The hyperpolarization was caused by an increased potassium conductance that is attenuated by glibenclamide (1–10 μM), a selective antagonist of ATP-sensitive potassium channels; in contrast, diazoxide (0.5 mM), an agonist at this channel, induces a hyperpolarization in CA1 neurons of rat hippocampal slices. The transient hyperpolarization after prolonged (approximate 1 h) application of NPA, is followed by a depolarization that is incompletely reversed by brief application of glutamate antagonists [d-2-amino-5-phosphonopentanoic acid (APV), 6,7-dichloroquinoxaline-2,3-dione (CNQX), 3-()-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) and 7-chloro-kynurenic acid (7CI-KYN)]. Early application of glibenclamide (within the initial 5 min) blocked or reduced hyperpolarization and accelerated depolarization. These data suggest that metabolic inhibition by NPA initially activates ATP-sensitive potassium channels. Events other than activation of glutamate receptors participate in the final depolarization resulting from uncoupling of oxidative phosphorylation (Riepe et al., 1992).

Siedel, Jiang, and Wolf (2000) treated neuroblastoma cells with different doses of NPA and analyzed the changes in gene expression by means of mRNA differential display (DDRT–PCR). Using 18 primers combination, authors identified a set of 33 candidate cDNA, deriving from 29 excised DDRT bands whose expression appeared to be changed in response to the NPA insult (mostly elevated). The differential mRNA expression of the ribosomal protein S6 and L40, of the protein kinase A catalytic beta subunit, and of the intercellular adhesion molecule could be verified using Northern hybridization and RT–PCR, respectively. The authors concluded that the results may prove useful in elucidating the multiple processes causing neurodegeneration subsequent to lesions by NPA.

### 4.3. Reports of accidental poisonings

Accidental poisonings with NPA were first widespread in the Western literature with the reports of Liu et al. (1989) who referred to "deteriorated sugarcane poisoning" (DSP) and later as "mouldy sugarcane poisoning" (Liu, 1993; Liu et al., 1992; MSP; Peraica, Radic, Lucic, & Pavlovic, 1999). DSP/MSP was at first known as an acute food poisoning of unknown etiology, occurring in 13 provinces in China. It was reported to primarily affect the CNS and occurred seasonally with most cases occurring during February–April. From 1972 to 1988, 847 cases were reported, 84 of whom died and many were left "lifelong disabled" (Liu et al., 1989). By 1989, there were 884 cases with 88 deaths. The main epidemiological feature was the small number of persons in one outbreak (one in five persons), with the victims being mostly children and young people (Liu et al., 1992). Generally, incubation period was from 10 min to 8 h, but in most cases was 2–3 h (Liu et al., 1989) and less than 5 h (Ming, 1995). Clinical signs included sudden-onset nausea, vomiting, abdominal pain and diarrhoea. Some patients also developed double vision,

somnolence, nystagmus, convulsions, decerebrate rigidity and coma. The patients that regained consciousness were mute and incontinent. Some developed delayed dystonia 7–40 days later. Clinical signs included grimacing, sustained athetosis of hands and fingers, torsion spasm, spasmodic torticollis, hemiballismus and painful spasms of the extremities. Computerized tomography (CT) of these patients revealed bilateral hypodensity of the putamen and, to a lesser extent, the globus pallidum. The caudate and claustrum were occasionally involved. Like the spastic parapareses of lathyrism and cassavism, the clinical features of toxic dystonia were permanent (Spencer et al., 1993). Abnormal electroencephalographic results were seen, but body temperature, heart, liver, lung, cerebrospinal fluid, blood, urine and faeces were all normal (Liu et al., 1992). Liu et al. (1992) stated that the characteristic injuries in rats described by Hamiton and Gould (1987b) were very similar to the lesions in bilateral lenticular nuclei shown in the CT scans of the patients with MSP. The development of delayed symptoms was predicted by abnormalities identified in the basal ganglia on cranial CT scans (Ming, 1995). In adults, NPA caused gastrointestinal symptoms, whereas signs of severe encephalopathy were not common (Ludolph et al., 1991).

Curiously, most of the reported cases were children, which contradicts the animal studies of Brouillet et al. (1993b) and others, who noted that older animals were more susceptible than young ones. However, the difference in age-dependent susceptibility was revealed not to be a biological factor in the Chinese poisonings, but a cultural one. As explained by Liu et al. (1992) and Spencer et al. (1993), sugarcane is supplied to children as a fresh fruit substitute in spring, at a time when fruit prices are high and especially during the time of the Chinese New Year as a seasonal or holiday confectionery.

Upon examination of the sugarcane samples that caused the poisoning, an undisclosed number did not appear normal, with the bark having lost its normal luster, the colour of the pulp having changed to light brown and several supplies smelled of mouldy food and alcohol (Liu et al., 1992). Strains of *Arthrinium* were found in the sugarcane samples and in deteriorated sugarcane sampled from food markets in the areas of reported poisoning. No strains of *Arthrinium* were isolated from the normal sugarcane samples. NPA was identified as the toxin in the deteriorated samples (Liu et al., 1989). Levels of NPA in the sugarcane samples ranged from 285 to 6660 ppm (Liu et al., 1992), but the amount consumed by the patients was not estimated. The highest concentration of NPA detected in cultures from the sugarcane was 4000 ppm (Liu et al., 1992).

NPA provides an animal model of HD which closely resembles the human aspects of the disease, in terms of both pathology and symptomatology (Table 10; Alexi et al., 1998). While NPA-like mechanisms of metabolic dysfunction may not be the specific culprit in HD, animal models employing metabolic lesioning provide deeper insight into neurodegenerative disorders. Animal models also open different avenues for therapeutic intervention in neuronal damage resulting from metabolic compromise.

Table 10
NPA as an animal model of Huntington's disease (Modified from Alexi et al., 1998)

| Neuropathology | NPA | HD |
|---|---|---|
| GABA | ↓ | ↓ |
| NADPHd/NOS/SST/NPY | ↓= | ↓= |
| ChAT | ↓=[a] | ↓= |
| Calbindin | ↓ | ↓ |
| Substance P | ↓ | ↓ |
| Fibres of passage | = | = |
| Dendritic alterations | Yes | Yes |
| Movement dysfunction | | |
| Chorea | Yes | Yes |
| Dystonia | Yes | Yes |
| Dyskinesia | Yes | Yes |

HD is from published post mortem results. Arrows indicate a decrease in phenotype marker, equal sign indicate no change, and arrows combined with equal sign indicates that the change was relatively less severe than those of other markers. ChAT, choline acetyltransferase; GABA, γ-aminobutyric acid; HD, Huntington's disease; NADPHd, NADPH diaphorase; NOS, nitric acid synthase; NPY, neuropeptide Y; SST, somatostatin.

[a] Results were not quantified.

## 5. Conclusions

NPA is produced by the nearly ubiquitous moulds *Aspergillus, Penicillium* and to a lesser extent, *Arthirnium*. The use of *Aspergillus* as an economic mould in the production of foods and the accidental contamination of foods provides for an historically lengthy and widespread exposure of humans to NPA. Based on the data of Kinosita et al. (1968), exposure of Japanese populations to NPA through *miso* and soy sauce alone may be as much as 5.5 mg/day (79 μg/kg/day for a 70 kg person) and may be considerably more if *sake* and other sources are taken into consideration. Despite this widespread consumption of foods containing NPA and the ubiquity of the presence of moulds capable of its synthesis, human poisonings of NPA are confined to a specific set of circumstances. That is, the accidental poisonings in China were the result of gross mishandling of sugarcane and sale of discoloured and off-odour products, circumstances which would not occur under current Good Manufacturing Practices.

NPA is absorbed in the gastrointestinal tract, enters the circulation and is metabolized to nitrite, although some may bind succinate dehydrogenase upon oxidation. It is likely that most orally ingested NPA is metabolized in the liver. There are no data available on excretion of NPA. The pathologic sequelae attendant to NPA toxicity are consistent among all species. Once the threshold dose for the particular species is achieved and succinate dehydrogenase bound, the cell is rendered energetically impoverished and ischemia of the organ ensues. In acute poisonings, striatal damage occurs and neuromuscular sequelae are manifested. In recent years, NPA has been exploited as a chemical for the investigation of various neurodegenerative disorders in humans, such as HD.

The $LD_{50}$ for mice and rats is between 60 and 120 mg/kg, although that for rabbits and other mammals may be slightly higher. The 30 day $LD_{50}$ in mice is approximately 50 mg/kg. A bioassay study was conducted in rats and mice and NPA did not exhibit carcinogenicity or chronic toxicity. The NOAEL in the bioassay for rats was 50 ppm for males, and 75 ppm for females (2.5 and 3.75 mg/kg/day, respectively). Mutagenicity data are mixed, but all positive assays can be traced back to the use of a single impure sample of NPA. Once this sample was purified, it was no longer mutagenic; nor was the newly manufactured NPA. Further, naturally occurring NPA is generated through a pathway that does not produce the mutagenic contaminant.

Therefore, from the data presented earlier, an ADI can be determined on the basis of the NOAEL in the chronic rodent study and the customary (in the case of a chronic study) safety factor of 100. This would yield an ADI of 25 μg/kg/day or 1.750 mg/day for a 70 kg human.

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