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December 18, 2017

VIA FEDERAL EXPRESS

Susan Carlson, Ph.D.
Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notification for Sodium Thiocyanate for Use in the Lactoperoxidase System

Dear Dr. Carlson:

On behalf of Taradon Laboratory ("Taradon"), we are submitting one paper copy and one electronic copy of this generally recognized as safe ("GRAS") notification for sodium thiocyanate. The electronic copy is provided on a virus-free CD, and is an exact copy of the paper submission. Taradon has determined through scientific procedures that sodium thiocyanate is GRAS for use as part of the lactoperoxidase system, which in conjunction to standard dairy processing procedures such as maintaining appropriate temperatures, pasteurization, or other antimicrobial treatments, may extend the shelf life of the products.

In the US, sodium thiocyanate as part of the lactoperoxidase system is intended to be used as a processing aid to extend the shelf life of a variety of dairy products, specifically fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. The lactoperoxidase system, which is dependent on the use of thiocyanate, is a natural defense system against microbial contamination, and has been reviewed by a number of international organizations, including WHO, because of its use in remote areas for the treatment of milk products. As will be explained, all of the components of the system occur naturally in human and animal liquid secretions, and therefore presents no new exposures to the human body.

If you have any questions regarding this notification, or require any additional information to aid in the review of Taradon's conclusion, please do not hesitate to contact me via email at gary.yingling@morganlewis.com or by telephone, (202)739-5610.

DB1/ 94825429.1

Sincerely,

(b) (6)

Gary L. Ingling

cc: Taradon Laboratory

GRAS NOTIFICATION FOR SODIUM THIOCYANATE FOR USE IN THE LACTOPEROXIDASE SYSTEM

Submitted by:
Taradon Laboratory
Avenue Leon Champagne, 2
B-1480 Tubize
Belgium

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PART 1: SIGNED STATEMENTS AND CERTIFICATIONS

1. This GRAS notice is submitted in accordance with 21 C.F.R. Part 170, Subpart E.

2. Name and Address of Submitting Company:
   Taradon Laboratory
   Avenue Leon Champagne, 2
   B-1480 Tubize
   Belgium
   Tel: +32.495.51.90.64
   Fax: +32.2.390.93.86

3. Name of Notified Substance:
   Sodium thiocyanate

4. Intended Conditions of Use:
   a. List of foods and/or drinking water to be added to:
      Sodium thiocyanate is intended for use in the lactoperoxidase system. The lactoperoxidase system is used in fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

   b. Proposed levels of use:
      Sodium thiocyanate is proposed for use in the lactoperoxidase system. The lactoperoxidase system, is intended for use at a level of 300 mg/L milk used to produce the substances listed above in 1.3.1. The formulation for the lactoperoxidase system is as follows:

      Lactoperoxidase: 1.25%
      Glucose oxidase: 0.75%
      Glucose: 30%
      Sodium Thiocyanate: 5%
      Sucrose: 63%

   c. Purpose of substance in the food product:
      Sodium thiocyanate is intended for use as a component of the lactoperoxidase system to extend the shelf life of fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

      In the US, sodium thiocyanate as part of the lactoperoxidase system is intended to be used as a processing aid to extend the shelf life of a variety of dairy products, specifically fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. The lactoperoxidase system, which is dependent on the use of thiocyanate, is a natural defense system against microbial contamination, and has been reviewed by a number of international organizations, including WHO, because of its use in remote areas for the treatment of milk products. As will be explained, all of the components of the

system occur naturally in human and animal liquid secretions, and therefore presents no new exposures to the human body. The system provides antimicrobial activity against a wide spectrum of spoilage and pathogenic microorganisms. The mode of action of the lactoperoxidase system relies on the production of short-lived intermediary oxidation products of the thiocyanate ion, principally hypothiocyanite (OSCN-), derived from the substance that is the subject of this notification, sodium thiocyanate.

As will be noted, the hypothiocyanite ions react with bacterial membranes, as well as impair the function of bacterial metabolic enzymes; hence their antimicrobial effects (Mickelson, 1977; Reiter & Marshall, 1979). Hypothiocyanite ions are short-lived, surviving only approximately 400 minutes after the initiation of the lactoperoxidase reaction. At the conclusion of treatment with the lactoperoxidase system, only lactoperoxidase, glucose oxidase, glucose, and sucrose remain. Thiocyanate, hydrogen peroxide, and hypothiocyanate are consumed during the process; residual levels are negligible. Due to the short life of the active ingredients, sodium thiocyanate (as part of the lactoperoxidase system) is a processing aid for use in extending the shelf life of variety of dairy products, specifically fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

d. Subpopulation expected to consume product: (if appropriate):
No specific subpopulations are anticipated.

5. Statutory Basis for GRAS Conclusion:
The statutory basis for the GRAS conclusion for sodium thiocyanate is scientific procedures. Taradon Laboratory has assembled the scientific data to conclude that sodium thiocyanate is generally recognized as safe for use as a component of the lactoperoxidase system.

6. It is the view of Taradon Laboratory that the substance is not subject to premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Taradon's conclusion that sodium thiocyanate is GRAS for the intended use as a component of the lactoperoxidase system to extend the shelf life of fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

7. Availability of Information for FDA Review: The data and information that are the basis for Taradon Laboratory's GRAS determination are available for FDA's review, and copies will be sent to FDA upon request, in either electronic format or by paper copy. Requests for copies and arrangements for review of materials cited herein may be directed to:

Gary L. Yingling
Morgan, Lewis and Bockius, LLP
1111 Pennsylvania Ave, NW
Washington, DC 20004
(202) 739-5610

gary.yingling@morganlewis.com

8. Exemptions from FOIA Disclosure:
The information provided in this application does not contain confidential or proprietary information, and therefore no FOIA exemptions are claimed.

9. Authorization to Share Trade Secrets with FSIS:
Should FDA find the need to share the information in this application with FSIS, Taradon has no objections.

10. Certification

On behalf of Taradon Laboratory, I certify that, to the best of my knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and Taradon Laboratory, and pertinent to the evaluation of the safety and GRAS status of the use of sodium thiocyanate for use as a component of the lactoperoxidase system to extend the shelf life of fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

Signed:

(b) (6) [redacted]

Dec 18, 2017

Gary L. Yingling Date
Senior Counsel
Morgan, Lewis, and Bockius LLP

PART 2: IDENTITY OF THE NOTIFIED SUBSTANCE

2.1. Chemical Name

The chemical name of the notified substance is sodium thiocyanate. The thiocyanate is intended for use as part of the lactoperoxidase system, which consists of a lactoperoxidase enzyme, a glucose oxidase enzyme, sodium thiocyanate, sucrose, and glucose.

2.2. Formula

The chemical formula of sodium thiocyanate is NaSCN.

The sodium thiocyanate will be a component of the lactoperoxidase system. The lactoperoxidase system (LPO system) use levels will be 300 ppm of a powder which will be used in 1 liter of the dairy products. The composition of the 300 ppm is:

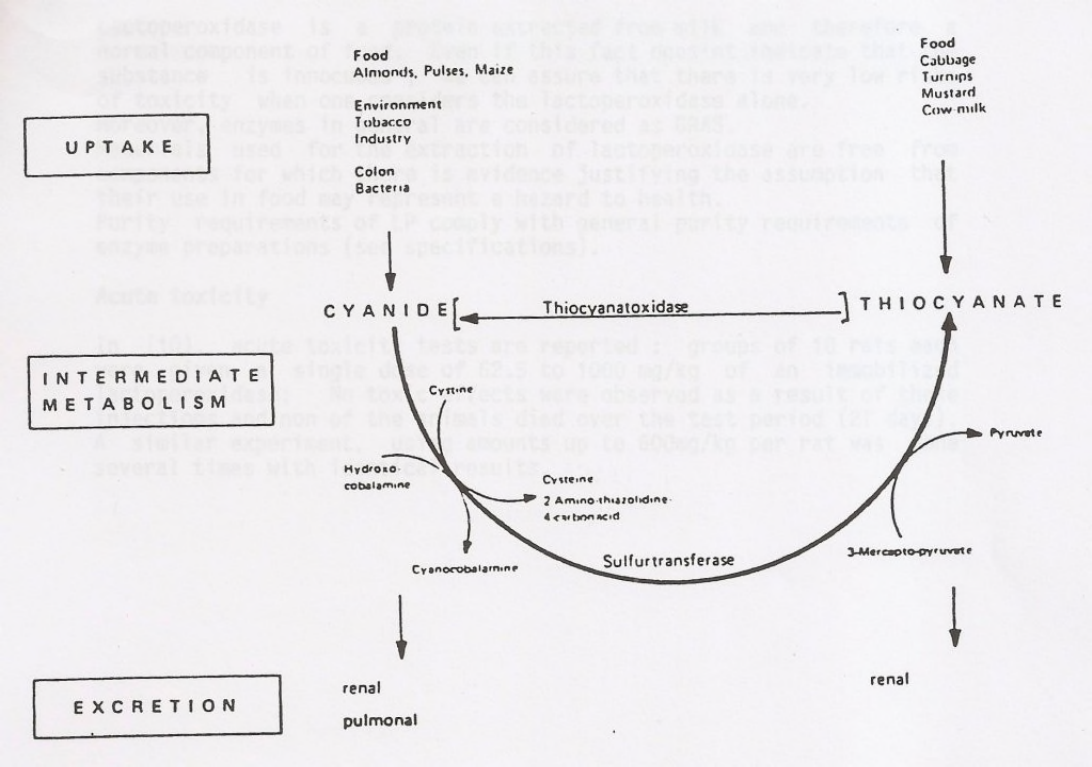
Lactoperoxidase: 1.25%
Glucose oxidase: 0.75%
Glucose: 30%
Sodium Thiocyanate: 5%
Sucrose: 63%

2.3. Composition

A. Thiocyanate

Thiocyanate (SCN^-) occurs ubiquitously in tissues and secretions of mammals. It is present in the mammary, salivary and thyroid glands and their secretions; in organs such as the stomach and kidney; and in fluids such as synovial, cerebral, cervical and spinal fluids, lymph, and plasma. The concentrations depend partly on the feeding regime of the animal, and eating and smoking habits of man. The source is the anion itself, its esters and other precursors such as nitriles, isothiocyanate, and cyanide. It is produced by the metabolism of sulfur amino acids and the detoxification of cyanide (Figure 1), a well-recognized biological function common to man and animal.

Figure 1: Detoxification of cyanide by the sulfurtransferase thiosulfate



The detoxification of the cyanide in the body is catalyzed by rhodanase (sulfurtransferase thiosulfate) occurring in liver and some bacteria. Cyanide reacts with thiosulfate, a product of sulfur amino acid metabolism, to convert cyanide into thiocyanate (SCN^-).

Plants such as clover contain high concentrations of cyanide and are detoxified in ruminants. Plants contain two main groups of SCN^- precursors: glucosinolates and glucosides. Glucosinolate-rich plants belong to Brassicacae, species of Cruciferae (cabbage, kale, SCN-content up to 600 mg/kg, or 10mM). The hydrolysis of glucosinolate is catalysed by thioglucosidase (myrosinase), producing SCN^- and/or isothiocyanate and nitriles. Glucosides are present in potatoes, maize, millet, sugar cane, peas etc. Hydrolysis of the glucosides in the plants directly yields SCN^- (Michajovskij, 1964; Virtanen, 1961 and Virtanen et al., 1960).

In addition to the above, thiocyanate is naturally present in bovine milk; the normal levels depend on the levels of thiocyanate in animal's diet. Concentrations have been reported to vary between 2.3 and 35 mg/l in milk from individual cows.

The high thiocyanate concentrations in saliva has been generally demonstrated, and at one-time saliva was thought to be the only source of SCN^- in human gastric juice. It is now accepted that the parietal cells actively secrete SCN^-. The SCN^- concentration in adult human gastric juice is high, 0.38 mM (22 mg), and even higher than in saliva; up to 2.5 mM SCN^- (145 mg) has been found for the saliva of smokers. Newborn infants have SCN^- anions in their saliva and in their gastric juice, less than that of adults.

The concentration of thiocyanate in the saliva and milk depends partly on the feeding regime of the animal, and eating and smoking habits of man. In case of the smokers, the SCN^- is produced by the metabolism of the sulfur amino acids and the detoxification of cyanide, one of the products of burning tobacco. In cows, it has been demonstrated for a long time that the level of SCN^- is influenced by the fodder. Cows grazing natural pastures with a complex flora of different grasses, weeds and clover were shown to give milk with the highest concentrations of SCN^- as between 0.26 mM (15 mg of SCN^- anion) to 0.35 mM (20 mg of SCN^- anion).

As noted above, thiocyanate is present in man, plants, and animals at variable levels. As to LPO system use in dairy products, the proposed maximum levels of thiocyanate, the estimated intake of SCN^- for the consumers of lactoperoxidase system-treated dairy products is estimated to be between 15 mg to 20 mg of SCN^- ions per liter of milk. Therefore, the intake of SCN^- anions for an average consumer of LPO system-treated dairy products would appear greater than the background from general milk consumption. However, this does not take into account that in the LPO system, the SCN^- is converted to innocuous derivatives such as $OSCN^-$ ions, thus reducing the SCN^- levels or to be eliminated by the kidney and the liver. That is why under the actual use conditions as proposed in this notice, the total content of thiocyanate, once the LPO system is activated in a mixture, does not surpass the natural maximal concentration in any particular cow milk.

B. Sodium Thiocyanate in the Lactoperoxidase System

Thiocyanate is intended for use as part of the lactoperoxidase (LPO) system. Peroxidases, including lactoperoxidase and glucose oxidase, are enzymes (proteins) that are part of the natural, non-immune defense systems in milk and in secretions of exocrine glands such as saliva, tears or intestinal secretions.

These systems of protection, which are less specific than the elements of the immune system, play a defensive role against the invasion by bacteria of the mucous membranes. Peroxidases do not have any antimicrobial activity of their own, but in the presence of specific substrates they constitute a powerful system of defense.

These substrates are hydrogen peroxide H_2O_2, and, depending on the specificity of the enzyme, thiocyanate (SCN^-),chloride (Cl^-), bromide (Br^-) or iodide (I^-). Different peroxidases have different functions, for example, myeloperoxidase, which is present in the leukocytes, catalyzes the oxidation of Cl^-, Br^-, SCN^-, I^- ions, lactoperoxidase catalyzes the same reactions except for Cl^-, whereas horseradish peroxidase catalyzes the oxidation of I^- only.

The oxidation reaction catalyzed by these well recognized enzymes can be summed up as follows:

$$H_2O_2 + X^- \longrightarrow H_2O + OX^-$$

Where $X^- = SCN^-, Cl^-, Br^-$ or I^-

The oxidation product, OX^-, is a short-lived oxidizing agent which will react, for instance, with NH_2 groups or thiols (-SH) of the enzymes essential to the metabolism of the bacteria.

The product that is the subject of this Generally Recognized as Safe (GRAS) notification is sodium thiocyanate, for use in the lactoperoxidase system. The LPO system is not a single enzyme but a system consisting of 5 components: the lactoperoxidase enzyme, the glucose oxidase enzyme, the sodium thiocyanate, sucrose and glucose. The enzyme lactoperoxidase catalyzes the oxidation of thiocyanate using glucose oxidase as a source of H_2O_2 and generates intermediate products with antibacterial properties. These products have a broad spectrum of antimicrobial effects against bacteria, fungi and viruses (de Wit and van Hooydonk, 1996; Naidu, 2000, Wolfson and Sumner, 1993)

Three of the components of the LPO system – namely sucrose, glucose, and glucose oxidase, are GRAS ingredients for use in the foods. As glucose oxidase and glucose are well-defined GRAS substances, a detailed discussion of these components will be reserved. Reference is made to 21 C.F.R. 184.1857 for additional information on glucose, 21 C.F.R. 184.1854 for information on sucrose, and the safety of their use in foods. Reference is also made to that the Codex Alimentarius, where the lactoperoxidase is listed as a processing aid (Annex 1). The Food Chemical Codex also recognizes glucose oxidase as a food processing substance.

2.4. Specifications for food grade material

The specifications for sodium thiocyanate is included in Table 1 below. The sodium thiocyanate used in the production of the lactoperoxidase system is food grade, as are the remaining components of the LPO system. The certificates of analysis for three lots of sodium thiocyanate are included in Annex 2.

All components will meet specifications and standards as required by the Food Chemical Codex, Codex Alimentarius, and regulations as set forth in 21 C.F.R. As sodium thiocyanate is intended for the purposes of this application for the exclusive use as a component of the lactoperoxidase system, the specification for the Taradon Laboratory lactoperoxidase system follows in Tables 2-4. The specification testing results of three batches of the LPO system manufactured by the notifier Taradon Laboratories are available in Annex 3, showing compliance of the production process with the specifications provided. The specifications for the sodium thiocyanate and LPO system follow in the tables below.

Table 1: Thiocyanate Specifications

Characteristic	**Accepted Result**
Description	White free-flowing crystals
Formula	NaSCN
Molecular Weight	81.1
Solubility	Readily soluble in water
Analysis	
Assay minimum purity	98%
Moisture	Maximum 2.0%
pH (1% solution)	6.0-8.0
Chloride	Maximum 0.04%
Sulphate	Maximum 0.04%
Heavy Metals (Pb)	Maximum 0.001%

Iron	<0.0002%
Insolubles	Maximum 0.005%

Table 2: Physical/Chemical Specifications for the Taradon Laboratory Lactoperoxidase System

Property	Average	Minimum	Maximum
Moisture	0.1	0.05	0.2
Fat	0	0	0
Ash	1.5	1.2	1.7
Protein	7.4	6.5	8.5
Density	0.8	0,75	0.85
Refractive Index	NA	NA	NA
Viscosity	NA	NA	NA
Flash Point	NA	NA	NA
Granulation (list pertinent Min. & max. % On/through sieves)	0.1	0.08	0.3

Table 3: Microbial Specifications for the Taradon Lactoperoxidase System

Type	Count	Sample
Aerobic Plate Count	<50	1g
Coliform	Absent	10g
E. coli*	Absent	10g
Yeast	Absent	1g
Mold	Absent	1g
Coagulase Positive Staphylococcus	Absent	10g
Salmonella	Absent	25g
Other: Listeria species	Absent	25g

Table 4: Sensory Specifications for the Taradon Lactoperoxidase System

Property	Standard Description or Target, Minimum, Maximum
Color	Creamy white
Flavor	Sweet
Texture	Dry powder

2.5. Method of Manufacture

As noted above, Taradon Laboratory does not manufacture the sodium thiocyanate, but instead uses the food grade sodium thiocyanate to manufacture the lactoperoxidase system. A schematic of the production of thiocyanate is presented below.

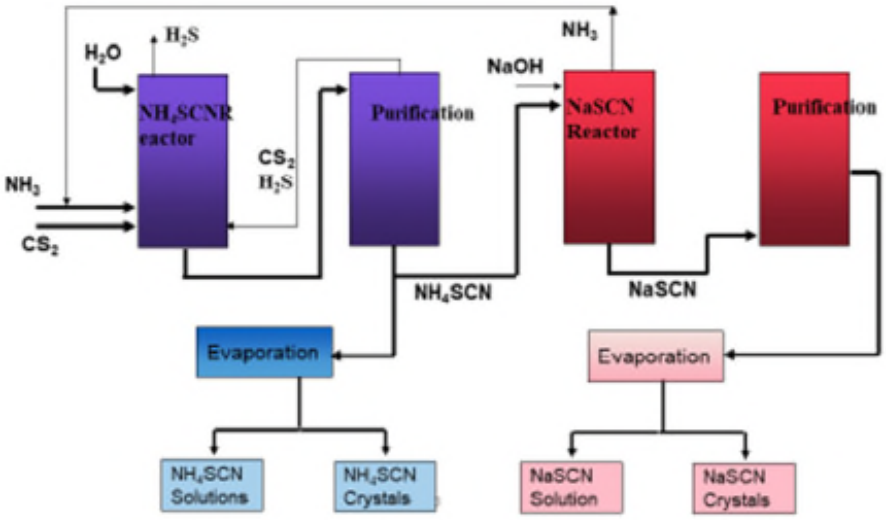
Sodium thiocyanate (NaSCN) production proccess:

Carbon disulfide + ammonia → ammonium thiocyanate + hydrogen sulfide

Ammonium thiocyanate + sodium hydroxide → Sodium thiocyanate + ammonia + water

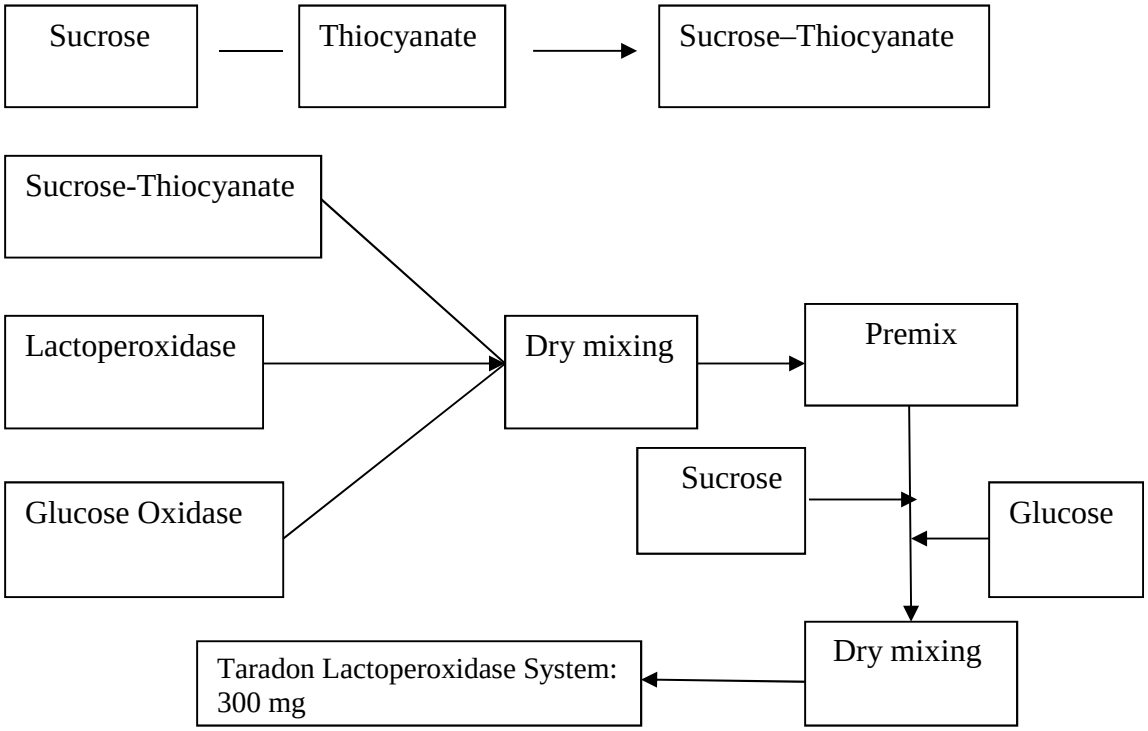
$$CS_2 + 2\,NH_3 \longrightarrow NH_4SCN + H_2S$$

$$NH_4SCN + NaOH \longrightarrow NaSCN + NH_3 + H_2O$$



A schematic of the production of the Taradon lactoperoxidase system is shown below. Taradon's production and commercialization of enzymatic preparations has been certified from the British Retail Consortium.

Figure 1: Lactoperoxidase System Production Scheme



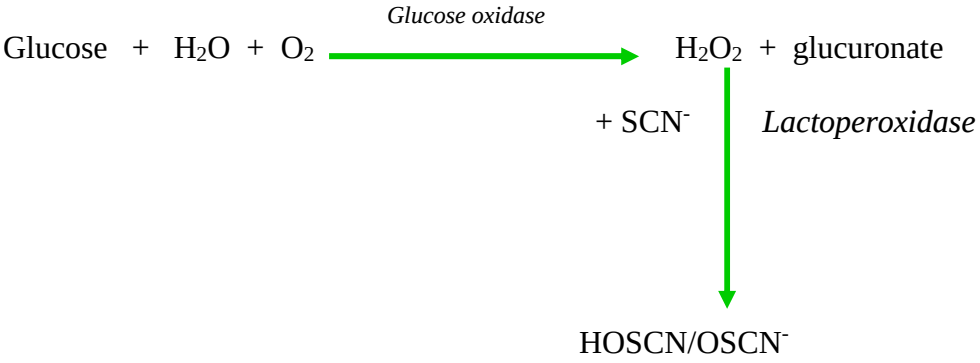
The ingredients of the lactoperoxidase system can be added to the desired product individually during production, or can be mixed to create the lactoperoxidase system prior to the start of the food production and stabilized with an inert support (such as sucrose), and added in the desired volume, during production.

The product must be kept in a cool, dry place. The shelf-life of the lactoperoxidase system mixture is 3 years unopened, and 1 month following opening, assuming it was stored properly. A certificate of analysis accompanies each shipment, documenting compliance with the release specifications.

2.6. Characteristics and Mechanism of Action

Sodium thiocyanate is a critical component of the lactoperoxidase system. The mode of action of the lactoperoxidase system relies on the production of short-lived intermediary oxidation products of the thiocyanate ion, principally the hypothiocyanite ions (OSCN-). The lactoperoxidase system is considered as a natural defense system against microbial infections. All its components occur naturally in human and animal secretions. The system elicits antimicrobial activity against a wide spectrum of spoilage and pathogenic microorganisms.

The overall reaction is as follows:



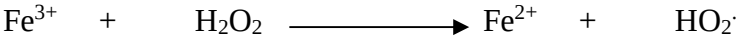
These $OSCN^-$ ions in turn reacts with the bacterial cytoplasmic membranes, as well as impair the function of metabolic enzymes, hence their antimicrobial effects (Mickelson, 1977; Reiter and Marshall 1979)

These $OSCN^-$ ions have a short-lived intermediary (+/- 400 minutes) after the starting of the LPO system reaction. Due to the short-lived of these ions, the LPO system can be considered as processing aids for the production of dairy products.

To understand the reaction mechanism of the lactoperoxidase system, it is important first to determine the structure of the enzyme. Reiter and his collaborators (Oram et al., 1966a; Reiter et al., 1964) showed that an intermediary oxidation product of SCN^- catalyzed by LP and H_2O_2 generated metabolically by the organisms was responsible for the inhibition of some strains of lactic acid streptococci, although some other strains have shown some resistance (Oram et al., 1966b). To understand this mechanism reaction of the lactoperoxidase system, it is important first to understand the structure of the enzyme.

The following four peroxidases, lactoperoxidase (LP), myeloperoxidase, eosinophil peroxidase and thyroid peroxidase constitute the mammalian peroxidases which are distinguished from the peroxidases from plants, fungi and bacteria. Most of the peroxidases, including LP contain ferri-protoporphyrin IX as a prosthetic group (Naidu, 2000; Rae et al., 1998). A characteristic feature of haemoprotein peroxidases is their ability to exist in various oxidation states. There are five known enzyme intermediates for lactoperoxidase. The major intermediates for LP are 1) ferric peroxidase (the native enzyme), 2) Compound I, 3) Compound II, 4) Compound III, and 5) ferrous peroxidase (Pruitt et al., 1991).

The peroxidative reactions are complex and follow different pathways depending upon the concentration of H_2O_2 and whether or not exogenous electron donors are present (de Wit and van Hooydonk, 1996). The first step in the enzymatic mechanism is the initiation reaction of the resting LP (Fe^{3+}) to its ground state, using H_2O_2:



followed by the propagation reactions as illustrated in the Figure 2. The superoxidase radical ($HO_2^{\cdot}$) plays an important role in termination of the catalytic reactions to the resting LP (de Wit et al., 1996).

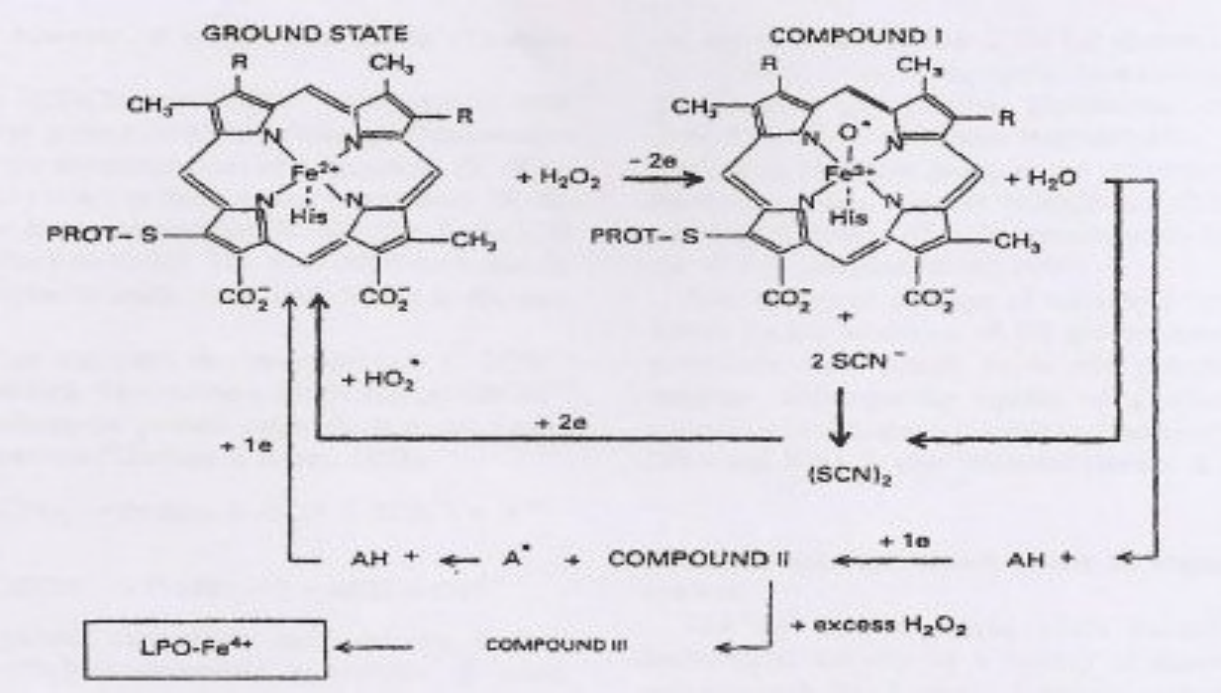


Figure 2: Pathways in the lactoperoxidase-catalyzed reaction mechanism. The normal peroxidalic cycle includes compound I. Insufficient 2-electron donors lead to compound II, and excess of H_2O_2 results in the formation of compound III (de Wit and van Hooydonck, 1996)

The propagation reaction includes the conversion of LP from the ground state into the so-called Compound I state by reaction with H_2O_2. At low SCN^- (<3µM) and halide concentrations. Compound I reacts with H_2O_2 and with any one-electron donor that may be present (such as proteins, peptides, etc.) to form Compound II. Compound II is continuously reduced to the ground state at a low rate. If there is an excess of H_2O_2 (>0.5 mM), Compound II may react with H_2O_2 to form Compound III, leading to a ferrylperoxidase adduct Compound III is involved in metabolic reactions, leading to irreversible inactivation of LP. The oxidant in peroxidase-catalyzed halogenations is not H_2O_2 itself but rather the reaction product of peroxidase with H_2O_2, known as Compound I (de Wit et al., 1996), that is, the thiocyanate ion (SCN^-) is oxidized by Compound I by a direct two-electron transfer of oxidizing equivalent (Pruitt et al., 1991). The next reaction is:

Compound I + X ⟶ Ferric enzyme + XO

Where X represents the halide or the thiocyanate ion and XO is the oxidized product. The products of peroxidation of two-electron donors kill or inhibit the growth and metabolism of many species of microorganisms (Pruitt et al., 1985).

In general peroxidation of H_2O_2 by LP can occur through three different cycles, resulting in divergent antimicrobial activities (de Wit and van Hooydonk., 1996) as follows:

1. In the presence of sufficient oxidizing halide or SCN^- as 2-electron donor for Compound I, giving optimal activation LP.

2. In the presence of insufficient halide or SCN^- of appropriate redox potential,

resulting in dominating I^- electron donors and accumulation of Compound II and reversible inactivation of LP.

3. In the presence of an excess of H_2O_2 resulting in the formation of Compound III, associated with irreversible inactivation of LP.

2.7. Antimicrobial Activity

As noted above, the antimicrobial activity can be broad. Thomas (Thomas et al., 1978) established $OSCN^-$ as an oxidizing agent for bacterial sulfhydryls and proteins to sulfenyl thiocyanate and sulfonic acid derivatives (following the mechanism described here below). This oxidation explains the inhibition of respiration in bacteria and inactivation of SH-depending enzymes in glycolysis. At about the same time, Mickelson (1977) came to the conclusion that a modification by the LPO system of sulhydryl on the inner membrane made *Streptococcus* agalactiae impermeable to glucose and glycolysis.

Marshall and Reiter have also demonstrated (1980), that OSCN- damages the cytoplasmic membrane by the oxidation of SH-groups in E.coli leading to the leakage of potassium ions, amino acids and polypeptides into the medium. Subsequently uptake of glucose, amino acids, purines, pyrimidines in the cell and the synthesis of proteins, DNA and RNA is also inhibited (Reiter and Härnulv, 1984).

The effect on the cytoplasmic membrane of Gram-positive bacteria by the lactoperoxidase system has also been demonstrated by the inhibition of amino transport in *Lactobacillus acidophilus* (Clem et al., 1966 and Slowey et al., 1968) and *Staphylococcus aureus* (Hamon et al., 1973) of glucose transport in *Streptococcus agalactiae* (Michelson, 1977) in *E.coli* (Wray et al., 1987) and of oxygen (Reiter and Pickering, 1964). The lactoperoxidase system inhibits the active transport of glutamic acid, lysine, valine and phentlalanine in *L. acidophilus* (Clem et al., 1966 and Slowey et al., 1968).

Different groups of bacteria show a varying degree of sensitivity to the lactoperoxidase system. Gram negative, catalase positive organisms such as *Pseudomonas, Coliforms, Salmonella* and *Shigellae,* are not only inhibited by the LP-system but also depending on the medium conditions, may be killed. Gram-positive, catalase-negative bacteria, such as *Streptococci* and *Lactobacilli* are generally inhibited but not killed by the lactoperoxidase system (Oram and Reiter, 1966). This difference in sensitivity can be explained by the difference in cell wall structure and their different barrier properties (de Wit and van Hooijdonk, 1996). The inner membrane of Gram-negative bacteria appears to be more extensively damaged by lactoperoxidase treatment than with Gram-positive species (Marshall and Reiter, 1980)

The $OSCN^-$ ions are bactericidal for enteric pathogens including multiple antibiotic resistant *E. coli* strains (Naidu, 2000). The $OSCN^-$ ions damages the inner membrane causing leakage and cessation of uptake of nutrient. The antimicrobial activity of the LP-system against *E. coli seems to be related to the oxidation of bacterial sulfhydryls* (Thomas and Aune, 1978). The oxidation of sulphydryls to sulphenyl derivates inhibit the bacterial respiration, but another groups of

researchers have identified that the inhibitory effect was due to the inhibition of the dehydrogenases in the respiratory chain of *E. coli*.

The issue of whether long-term use of the lactoperoxidase system would result in any microbiological risks, e.g. development of lactoperoxidase system resistant strains, antibiotic resistant or toxin-producing bacteria was considered by the FAO/WHO technical committee (2005). The committee concluded that that the available data indicate that adoption of the LPO system is not likely to stimulate the development of resistance to the lactoperoxidase system itself or antibiotic-resistant microorganisms (Annex 4). This report is discussed in further detail in section 3.2.

2.8. Potential Toxicants

There are no potential toxicants from the sodium thiocyanate added as a processing aid in itself, as it produces unstable intermediates that decompose rapidly before consumption. It is important to consider the LPO system, however, as a reaction product of the system is hydrogen peroxide, or H_2O_2. As noted above, H_2O_2 is a critical component of the system, and rather than adding H_2O_2 directly, it is instead formed in the reaction of glucose, oxygen, and water.

$$H_2O + \text{glucose} + O_2 \longrightarrow \text{gluconic acid} + H_2O_2$$

Although hydrogen peroxide is generated by the oxidation of glucose that occurs naturally during the action of glucose oxidase, it is generally assumed to be not present in milk or dairy products. This is because H_2O_2 is rapidly reduced during the enzymatic oxidation of thiocyanate to produce the hypothiocyanite ion, producing water. In bovine milk, the production of $OSCN^-$ catalyzed by lactoperoxidase depends on the levels of SCN^- and H_2O_2. In the past, International Dairy Federation (IDF) has recommended the use of 300-800 ppm. H_2O_2 for the preservation of milk wherever adequate cooling is difficult, as in developing countries. Since such excessive concentrations affect the clotting of milk inactivate enzymes and denature proteins through the oxidation of amino acids (tryptophan, tyrosine, methionine, histidine and cystine (Methods of Enzymology; vol XI, 3rd Edit. N.Y. Acad Press) the residual H_2O_2 should be eliminated by heat treatment and addition of catalase - a rather complex procedure. Treatment of milk by the lactoperoxidase system requires only very low levels of H_2O_2-10-15 ppm sufficient to oxidize SCN^- in the presence of lactoperoxidase and without affecting the enzyme. Further, these levels are below the levels permitted for use in dairy products for cheese making, as noted in 21 C.F.R. §184.1366

It is interesting to note that at any moment hydrogen peroxide is consumed by the lactoperoxidase/thiocyanate system and that it would never exceed 10 µM: i.e. 3 % of the dose recommended by the International Dairy Federation for the preservation of raw milk by activation of the LPO system (Annex 5). The toxicology of H_2O_2 has been reviewed in the Department of Health and Family services in 1993, has also been evaluated in an IARC monograph in 1985 and by ECETOC (Joint Assessment of Commodity Chemicals N° 22, January, 1993). The US Environmental Protection Agency, after a full toxicological assessment, has established an exemption from the requirement of a tolerance for residues of the biochemical H_2O_2 on all food commodities when used as an algaecide, fungicide and bactericide at the rate of 1% H_2O_2 per application on growing crops and post-harvest crops (vol 64, N° 118, June 1999).

Exogenous H_2O_2 decomposes to oxygen and water on contact with tissues, thus limiting absorption of the intact molecule.

Any H_2O_2 molecule produced is immediately used by the lactoperoxidase so that peroxide cannot accumulate in solution (Reiter et al., 1976). Therefore, there is no concern of any potential toxicity with H_2O_2.

PART 3: DIETARY EXPOSURE

3.1 Estimated Dietary Intake of Sodium Thiocyanate

The focus of the exposure assessment was the exposure to thiocyanate. As noted previously, thiocyanate will be used as a component of the LPO system, along with glucose, sucrose, and glucose oxidase. It is important to point out that a significant portion of the thiocyanate is converted into unstable intermediates that decompose spontaneously before consumption. In this estimated daily intake study, this phenomenon is not taken into account, and as a consequence, the exposure study of thiocyanate can be considered as the worst case scenario.

Thiocyanate is proposed for use in the following five milk-based food and beverage categories: fresh cheeses (including mozzarella and cottage cheese), frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. Table 5 lists the proposed food use categories and their corresponding thiocyanate concentration that is naturally occurring, proposed for use in food, and the total maximum thiocyanate levels in proposed foods which accounts for both the naturally occurring thiocyanate levels in food plus the proposed use levels.

Table 5: Proposed Uses

	Thiocyanate (mg/kg)		
Food Category	Naturally Occurring	Proposed Use	Total (Natural + Proposed Use)
Fresh Cheese			
Mozzarella	15	0*	15
Cottage Cheese	15	15	30
Frozen Dairy Desserts	3	1.5	4.5
Fermented Milk	15	15	30
Flavored Milk Drinks	15	15	30
Yogurt	30	15	45

*The proposed use for mozzarella is in the water the cheese is stored in, not the actual cheese itself.

Using the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES) 2009-2012. consumption data, Exponent estimated the 2-day average daily intake on a *per capita* and *per user* basis. In the analysis, the 2-day average intake of thiocyanate was estimated by multiplying the reported intake of foods from the 24-hr recall with the proposed corresponding thiocyanate use level (see Table 5) and the cumulative sum over the two 24-hr recalls was divided by two. This was then repeated using the maximum levels of thiocyanate (i.e., naturally occurring level plus proposed use level). Intake estimates of thiocyanate were derived from all proposed uses combined for the total U.S. population and expressed in units of milligram per day (mg/day), and are presented below in Table 6. The results are presented in the table below, and the full Exponent report is available in Annex 6. The total use for the mean and 90th percentile users of thiocyanate are well below those values established in the toxicology studies.

Table 6: Estimated Exposure

	Total U.S. Population					
			Per Capita (mg/day)		Per User (mg/day)	
EDI based on:	Unweighted N	% User	Mean	90th Percentile	Mean	90th Percentile
Proposed Use	7,576	49	0.59	2	1.2	3.33
Maximum Use (Natural + Proposed)	10,208	67	1.63	5.52	2.44	7.24

The levels from the proposed uses are below the naturally occurring levels found in milk, which range from 2.3 and 35 mg/l of thiocyanate in milk from individual cows.

PART 4: SELF-LIMITING LEVELS OF USE

The thiocyanate level of 5% of the 300 mg of LPO system is the maximum recommended dose. At concentrations exceeding this level, the taste and texture of the product may be impacted. The concern at higher levels is the taste and texture alterations; there is no issue of safety.

PART 5: EXPERIENCE BASED ON COMMON EXPERIENCE IN FOOD BEFORE 1958

This section is not applicable to this application.

PART 6: NARRATIVE

6.1. History of Safe Use of Sodium Thiocyanate

One of the most critical elements of the safety of sodium thiocyanate as proposed in this notification is the fact that the antibacterial effect of thiocyanate via the LPO system is mediated by short-lived oxidation products of thiocyanate. These intermediates are very unstable and those not reacting with bacteria decompose spontaneously. Products treated with LPO system would not have any active agents when they reach the consumer. In fact, kinetic studies, as described below, have shown that the entirety of the thiocyanate in the LPO system is consumed in the reaction, and will not reach the consumer.

Another critical element supporting the safety of thiocyanate is its existence in nature, and the regular consumption of thiocyanate by consumers. The presence of thiocyanate in vegetables and plants is well documented (Felker et al., 2016, Serrano et al., 1988, Chandler et al., 2014). These vegetables include cabbages, radishes, Brussels sprouts, kale, and broccoli, cassava, yam, sugar cane, among other popular vegetables and plants. Plants contain two main groups of thiocyanate precursors: glucosinolates and glucosides. Hydrolysis of glucosides directly yields thiocyanate. Glucosinolate-rich plants belong to Brassicacae as well, as documented by a USDA paper that examined the levels of glucosinolates in vegetables (Carlson, et al., 1987). The hydrolysis of glucosinolate is catalyzed by thioglucosidase (myrosinase), producing SCN^- and/or isothiocyanate and nitriles. Glucosides are present in potatoes, maize, millet, sugar cane, peas etc. The following table is taken from Serrano, et al, which lists the thiocyanate concentrations in parts per million, (as obtained from two separate methods of hydrolysis in a dessicated sample).

Table II. Quantities of SCN^- in Vegetables

	SCN^- in dry sample (ppm)	
Sample	**Chemical hydrolysis**	**Enzymatic hydrolysis**
Chicorium endivia, var. *latifolia*	0.2734	1.1941
Allium sativum	5.5801	12.6849
Brassica oleracea var.*botrytis*	41.1221	89.1432
Daucus carota	0.3825	1.6312
Lycopersicon esculentum	0.6655	0.8219
Apium graveolens	0.2871	0.4692
Cucumis sativus	1.7197	4.6999
Brassica oleracea	9.8576	16.4011
Spinacea oleracea	0.4419	2.5488
Allium cepa	0.4395	0.7843
Lactuca sativa	0.1811	3.9206
Brassica oleracea var. *gemmifera*	39.9501	97.7351

As the figure points out, the amount of thiocyanate encountered in the diet on a regular basis is not inconsequential, and can easily exceed the amounts that will be used in the proposed system. This consumption data is even more striking when it is considered against the fact that the thiocyanate in the LPO system, as proposed in this GRAS notification, is entirely consumed in the reaction. Consumers eating the vegetables in the table above would instead consume the entirety of the thiocyanate concentration in the portion of the plant consumed.

Thiocyanate is also naturally present in human saliva, tears, gastric fluids, nasal lining fluid, epithelial lining fluid, and plasma. (Chandler, et al., 2014). Tenovuo and Larjava evaluated the safety of one of the thiocyanate intermediates, hypothiocyanate (or OSCN-) in dental studies, as

the salivary system in humans utilizes thiocyanate (Tenovuo and Larjava, 1984). They used both *in vitro* and *in vivo* methods to evaluate the increased concentration of hypothiocyanate generated by supplementation with hydrogen peroxide, and found no toxic effects were exerted on human cells following exposure. The presence of lactoperoxidase and thiocyanate has been well documented in infant saliva. As shown in a paper published in 1975, the levels of lactoperoxidase and thiocyanate present in infant saliva, though one third of the level present in adults, is still sufficient to exhibit antimicrobial activity (Gothefors and Marklund, 1975). Interestingly, the levels of lactoperoxidase vary, with some levels higher than those seen in adults observed. The authors also conclude that the presence of thiocyanate and the lactoperoxidase activity is present in both humans and cows, underscoring its biological significance, as well as its prevalence. The use of thiocyanate under the conditions proposed in this notification would not be an introduction to a new substance in the human population.

Human use of thiocyanate for clinical purposes is also well established. Thiocyanate was used for years to treat high blood pressure, and in ways its use continues, though now as a metabolite. Interestingly, FDA has evaluated the toxicity of thiocyanate in its approvals of Nitropress® (sodium nitroprusside), a drug used for hypertension. Sodium nitroprusside is metabolized to cyanide and subsequently thiocyanate in the human body. Recently, this drug was evaluated for use in the pediatric population. As noted in the briefing materials for the March 6, 2017 Pediatric Advisory Committee meeting,[1] the Pediatric Postmarketing Pharmacovigilance and Drug Utilization Review states that thiocyanate is only mildly toxic at serum concentrations of 60 mg/L, and is not life threatening until concentrations of 200 mg/L are encountered. The concentrations for use as a drug are far above that which would be encountered in the proposed use of thiocyanate included in this GRAS notification, especially when it is considered that in the proposed use of this GRAS notification, the thiocyanate is consumed in the reaction, and little if any will be consumed in the food products in which it is used.

This information is also supplemented by the book chapter included in Annex 7. This document is a chapter from a book, *Medical and Biological Significance of Thiocyanate* (Weuffen, 1982) Though originally published in German, an English translation is provided. This chapter outlines the extensive testing conducted on thiocyanate, including toxicology as well as pharmacokinetics. This chapter also describes the extensive human exposures to thiocyanate.

6.1.1 International and Regulatory Approvals of Thiocyanate and the Lactoperoxidase System

Thiocyanate, as part of the lactoperoxidase system, has been approved as a processing aid to extend the shelf life of dairy products by various international regulatory and scientific advisory bodies including: Codex, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Food Standards Australia New Zealand (FSANZ), the French Agency for Food Safety (AFSSA), and others.

[1] Available at: https://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/PediatricAdvisoryCommittee/UCM543823.pdf

In a well written and concise document, the Codex document Codex Code of Practice, Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase System, CAC/GL 13-1991 sets forth the Codex-approved specifications and practices for use of the thiocyanate-containing lactoperoxidase system for the stabilization of milk (Codex Committee on Milk and Milk Products, 2012). Codex notes that refrigeration remains the method of choice for safe milk transport. The Codex-approved LPO system utilizes the lactoperoxidase already present in milk and the system is initiated by sodium percarbonate (rather than glucose and glucose oxidase) to generate the hydrogen peroxide necessary to convert thiocyanate to hypothiocyanite.

In Sweden, the National Food Administration has evaluated the efficiency of the lactoperoxidase system and existing toxicological data and has decided to allow the use of LP-activation in milk where raw milk cannot be properly cooled (The National Food Administration, 1980) (Sweden, 1980; Swedish Waterhouse, 2012).

The lactoperoxidase system was approved by the National Expert Committee on Food Additives in the People's Republic of China as "an acceptable preservative used for milk preservation."

In France, the Ministry of the Economy of Finance and Industry gave a permit for the addition of the lactoperoxidase system to the brine "destined for the production of smoked salmon" in April 1998. In 2003, the AFSSA (French Food Safety Agency) authorized the use of the $OSCN^-$ ions (oxidation product of the SCN^-) without the presence of the LPO system, as a processing aid for the treatment of fresh-cut, ready-to-eat salads (Agence Française de Sécurité Sanitaire des Aliments (AFSSA), 2012). In 2002, the Finnish Ministry approved the system for similar uses.

In Australia and New Zealand (2002), the FSANZ approved the use of the lactoperoxidase system containing 40 mg/liter of SCN- in the agro-food industry as a processing aid functioning as an antibacterial agent for meat and meat products.

In 1990, JECFA concluded that the LPO system was acceptable for use in milk preservation and does not present a toxicological hazard (FAO/WHO, 2005; JECFA, 1990; JECFA, 2005). In 2005, an FAO/WHO technical meeting concluded that the LPO system is "a safe method of preventing milk losses due to microbial spoilage when used according to the Codex guidelines either alone or in combination with other approved procedures."

These uses demonstrate the safe use of sodium thiocyanate as part of the lactoperoxidase system in dairy products.

6.2. Summary of Literature Discussing Sodium Thiocyanate

6.2.1. FAO/WHO Technical Report

In 2005, the FAO/WHO Technical Meeting was held to evaluate the use of thiocyanate as a component of the lactoperoxidase system for preservation of raw milk. The resultant report of that meeting discusses in detail the lactoperoxidase system, as well as the potential risks and benefits of its use. This report is included in Annex 4.

The report discusses the efficacy of the lactoperoxidase system, and acknowledges its broad antimicrobial activity against bacteria, viruses, mold, yeasts, protozoa, and other milk spoilage microorganisms. The mechanism of action is considered primarily bacteriostatic, and also points out that the lactoperoxidase system does not promote microbial growth or encourage resistance. Further, the report also clearly states that use of the lactoperoxidase system cannot be used to disguise or hide spoiled milk.

FAO/WHO Technical Group devoted a significant portion of the report to the safety of the LPO system, and the report includes an extensive review of the literature pertaining to the use of thiocyanate. The authors affirm that hypothiocyanate is found in saliva, and has a short half-life in milk, making the residual levels of no concern of safety. The report also discusses the extensive list of studies performed in iodine deficient populations and those with thyroid disorders, given the potential concern for interference with iodine metabolism at very high plasma levels of thiocyanate. While there is some evidence of mild alterations after consumptions of 45 milligrams of thiocyanate, levels which are much higher than intended for the used proposed in this GRAS notice, other studies found no alternation in thyroid function, even in iodine deficient populations. They also evaluated a study conducted over a 10-year period in the American tropics, with no adverse effects of thiocyanate in the LPO system treated milk found. Reference is made to a 2-year rat carcinogenicity study of sodium thiocyanate, which found no evidence of carcinogenicity. The Technical Group concluded that there was no significant toxicological risk to the general public from consumption of thiocyanate in the LPO system.

The report concluded that the LPO system is a safe and effective method for preservation of raw milk. The FAO/WHO Technical Group believed that the system had numerous advantages, and no significant risk that would prevent its application to the global community.

6.2.2. Other Relevant Scientific Articles Discussing Sodium Thiocyanate

While the FAO/WHO report provides a comprehensive review of the relevant literature, we also wished to highlight several studies which also demonstrate both the safety and effectiveness of the thiocyanate and the LPO system in dairy products. The referenced scientific articles are provided in Annex 8.

Another paper published in 1975 explores the efficacy of thiocyanate and the LPO system against milk spoilage organisms (Bjorck, et al., 1975). The authors determined that the LPO system was antimicrobial against several gram-negative bacteria including certain strains of *E.coli* and *Pseudomonas*. The importance of glucose and glucose oxidase was elucidated, and was found to be a key component of the system, supporting the production of hydrogen peroxide. The paper also notes that the system is removable, and has no lasting impact on the milk once the LPO system components have been removed.

Two studies have also evaluated the efficacy of thiocyanate in the LPO system against *Listeria monocytogenes*. The first used a model broth culture system, and found that against the strain Scott A, LPO system exerted a bacteriostatic effect, rather than a bactericidal effect (Siragusa and Johnson, 1989). However a second study which evaluated multiple strains in raw milk

found the LPO system has a bactericidal effect, but this effect is strain and temperature dependent (Gaya, Medina, and Nunez, 1991). The LPO system in this study against *Listeria monocytogenes* was most effective at refrigeration temperatures. Given that the intended use of the product in this notification is the prolongation of shelf-life, the ability to prevent the growth of bacteria also important. Further, as has been stated numerous times, the use of the thiocyanate system does not negate the need for pasteurization, and also assumes users will use the appropriate manufacturing and processing techniques to ensure a safe end product.

A review article published in 2005 contains an extensive discussion on the mechanism of action of the thiocyanate in the LPO system, as well as an overview of the efficacy studies performed with the LPO system (Seifu, Buys, and Donkin, 2005). These studies have found a bactericidal effect on numerous gram-negative bacteria, including *H. pylori*, *Actinobacillys actinomycetemcomitans*, and *Fusobacterium nucleatum*, and a gram positive bacteria *Streptococcus sanguis*. Growth and or enzyme inhibition were also noted for a variety of bacterial strains, including *Streptococcus mutans*, and *Yersinia enterocolitica*, as well as the HIV-1 virus. The LPO system was also bactericidal and bacteristatic against *Staph aureus*, a major cause of bovine infections as well as human infections. The LPO system was also found to be bactericidal against the human pathogens *Salmonella typhimurium* and *Campylobacter jejuni*, as referenced by the authors. The specific mechanism of action is dependent on the type of pathogen the LPO system faces, and multiple mechanisms are reviewed. Another review, published by Taradon Laboratories in conjunction with Liege University Plant Pathology Laboratory, provides an extensive review of the chemical actions of the LPO system, specifically focusing on the antimicrobial activity (Bafort, et al., 2014). Both of these review articles support the efficacy of thiocyanate (as the active component of the LPO system) for the intended use, as a processing aid to extend the shelf-life of certain dairy products.

Finally, a study directly relating to the proposed use of this notification, an extension of shelf life, was conducted in 2015 (Pokhrel and Das, 2012). This study evaluated the ability of thiocyanate in the LPO system to extend the shelf life of raw milk. The LPO system provided a significant increase in shelf life compared to control at temperatures of 25°C and 5°C. In the 5°C group, shelf life of milk was extended by 2 days. The paper underscores the efficacy of thiocyanate for the intended use.

6.3. Toxicology Studies

6.3.1. Toxicity Studies on Sodium Thiocyanate Alone

The National Cancer Institute did a two-year chronic toxicity/carcinogenicity bioassay of sodium thiocyanate (alone or in combination with sodium nitrite) in F344 rats at the National Cancer Institute. (Lijinsky and Kovatch, 1989). The animals received sodium thiocyanate at a level of 3.2 grams/liter in drinking water. The authors noted that the "none of the treatment appeared to have a noticeable adverse effect on the rats, even though treatment with sodium thiocyanate lasted 112 weeks." The authors evaluated all major organs as part of the study, and did not appear to limit the evaluation to carcinogenicity alone. The results of this study led to the clear conclusion that sodium thiocyanate is not carcinogenic to rats, and while not a toxicology study, the fact that the rats had no adverse effects over such a long exposure, suggests the thiocyanate has a high level of safety.

This type of chronic exposure, with no observed adverse effects and no difference over control is relevant to general human safety, and the doses were delivered via water consumption, which is relevant to the addition of sodium thiocyanate to food. As mentioned above, it is important to recall that thiocyanate is naturally present in foods, and has been added to milk for many years (Fernandez et al. (2005).

Thiocyanate has been evaluated for various health purposes for nearly a century. It is also known to reduce blood pressure in essential hypertension, and intravenous injection of the thiosulfate ion is an antidote for cyanide poisoning (Chen, Rose, and Clowes, 1934). It was later shown that pretreatment of animals with sodium nitrite and thiosulfate resulted in the conversion of cyanide to thiocyanate (Frankenberg and Sorbo, 1974). In fact, the primary goal of one of the current cyanide antidotes, Nithiodote (sodium nitrate and thiosulfate), is to serve as a sulfur donor to convert cyanide to the "relatively non-toxic" thiocyanate for ready elimination.[2]

If we go back 70 years, we see that Wood, et al**.** (1947) did an experiment on the conversion of thiocyanate sulfur to sulfate. They noted that the origin and fate of thiocyanate ion in the body has been of interest for more than a century, and that because the thiocyanate ion does not permeate the normal cell, it can be used as a method for determination of extracellular fluid volume. The study looked at the excretion of sulfate and thiocyanate ion in the urine after the injection of radioactive labeled potassium thiocyanate. In the rates, they found that 1 to 4.5 percent of the injected thiocyanate appeared as the sulfate, but the major part was excreted unchanged. In fact, it was concluded that very little thiocyanate is metabolized by the organism.

Anderson and Chen (1940) investigated the absorption and toxicity of sodium and potassium thiocyanate. They noted that small amounts of ingested cyanide could be converted to thiocyanate and excreted from the body. They dosed a dog, rats, and rabbits with sodium and potassium thiocyanate. The NOAEL for sodium and potassium thiocyanate in the test animals was 100-200 mg/kg (rats) and 20.8-24.4 mg/kg (dogs), far above the concentrations anticipated for use in the current application. This study supported earlier studies indicating that safety of thiocyanate was dose dependent, and that lower doses caused little to no adverse effects when treating high blood pressure in humans (Barker, 1936).

There is also little concern that the thiocyanate will be converted to cyanide. Smith (1973) investigated this theory after noticing that the toxic doses of thiocyanate and cyanate produced similar symptoms. Following the administration of high doses of thiocyanate to mice (6.3 mmole/kg), the author concluded that high doses of thiocyanate had inconsistent symptoms of toxicity, and this cannot be accounted for by conversion of the thiocyanate to the cyanide, as the rates of conversion required would exceed the capabilities of the biologic system.

Boxer and Rickards (1952) studied of the metabolism of the carbon of cyanide and the carbon of thiocyanate in rats and a dog. In the dog, they were able to show that a portion of the cyanide was converted to thiocyanate in the urine. In the rat, they were able to demonstrate that the cyanide and

[2] Prescribing Information, Nithiodote. Available at: https://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=ff4941b3-9901-4aab-adcf-c5327bede34e

thiocyanate reached a dynamic equilibrium by the specific activity of hydrogen cyanide being exhaled by the rats after an injection of radioactive cyanide. The authors also conclude that the carbon in thiocyanate and cyanide are converted to carbon dioxide, and exhaled. The authors also found that, consistent with previous studies discussed in this notification, thiocyanate is largely excreted in the urine, unchanged.

The references listed above demonstrate that thiocyanate alone has been evaluated and the data from the studies show that the thiocyanate is safe in the low levels proposed for use in this GRAS notification. These reference further support long use of thiocyanate in humans for medicinal purposes, in addition to the long history of exposure through natural sources such as vegetables.

6.3.2. Corroborative Studies

Since this GRAS Notice is concerned with the use of the thiocyanate in the lactoperoxidase (LPO) system, we now wish to discuss the corroborative data that demonstrates the safety of sodium thiocyanate in the system.

The evaluation of thiocyanate via the LPO system is important, as the sodium thiocyanate is intended for use as a part of the system, and is consumed as a part of the reaction. As explained previously, the antibacterial effect of the LPO system is mediated by short-lived oxidation products of thiocyanate. These intermediates are very unstable and those not reacting with bacteria decompose spontaneously. Products treated with thiocyanate in the LPO system would not have any active agents when they reach the consumer.

Toxicological risks associated with the addition of thiocyanate to foodstuffs at the proposed levels of use would be very low, because we can assume that all the SCN- is consumed by the system, and the toxicology and kinetic studies conducted to date support this conclusion (Annex 9).

Below are the summaries of the safety studies performed to date. The unpublished corroborative studies have been included as Annexes to this notification.

6.3.2.1. Acute Toxicity Studies of Sodium Thiocyanate

a. Acute Toxicity Studies in the Scientific Literature

In the literature, another study was found which evaluated the effects of thiocyanate in cows. Cannulated calves (Reiter et al., 1980) were fed 200 ml of raw milk containing *E.coli* , followed by 2000 ml of raw milk containing lactoperoxidase, thiocyanate, and one of various sources of hydrogen peroxide (either glucose oxidase/glucose or magnesium peroxide or a hydrogen peroxide producing strain of *Lactobacillus casei*). In abomasal samples taken immediately after feeding and periodically thereafter initial inoculums were reduced by at least 99.9%. No adverse effects were reported.

b. Corroborative, Unpublished Kinetic Studies Performed by Taradon

Taradon has conducted extensive unpublished, corroborative studies on the kinetics of thiocyanate, particularly in the lactoperoxidase system. The studies are included in Annex 9, in a report that is a compilation of data generated by Taradon. If from a theoretical point of view, thiocyanate can be regenerated from the reduction of hypothiocyanite (i.e. when $OSCN^-$ reacts with bacteria), Taradon has failed to show this effect *in vitro*, as shown on page 5 of the report included in Annex 9 (relevant data is for the second system). These data from these investigation supports the conclusion that the thiocyanate is not regenerated from the reduction of hypothiocyanate.

c. Corroborative, Unpublished Acute Toxicity Studies Performed at Pasteur Institute

While the Pasteur studies are not published and, therefore, merely collaborative, we believe that they are of value because they are focusing on the Lactoperoxidase System. The following studies were done at Pasteur.

Acute toxicity of thiocyanate in the LPO system was tested by the Pasteur Institute under OECD and GLP principals, in mice and rats at two dosage levels, one optimized to produce the highest levels of hypothiocyanite and one which delivered all four ingredients up to their solubility limit. The latter formulation produces no hypothiocyanite because of the excess hydrogen peroxide present. The study report for both the mouse and the rat studies are included as Annex 10.

Mouse Study

In the evaluation of thiocyanate in the LPO system in mice performed by the Pasteur Institute, the LPO system was administered orally in water (25mL/kg) to "Souris" OF1 mice, 10M and 10F/group) after an 18-hr fast. The mice were observed for 15 days. There was no control group. The LPO system Formula A (maximal hypothiocyanite) contained 4,000 mg/L glucose, 18.72 mg/L lactoperoxidase, 2 mg/L glucose oxidase, and 68.9 mg/L sodium thiocyanate; the total dose was 102 mg/kg bw). The LPO system Formula B (maximum dose) contained 625 g/L glucose, 2.9 g/L lactoperoxidase, 0.32 g/L glucose oxidase, and 10.7 g/L sodium thiocyanate; the total dose was 16 g/kg bw. Formula B is approximately 165 times higher than that delivered in Formula A.

There were no deaths, signs or toxicity or abnormal weight gain in the mice receiving Formula A. Necropsy revealed no lesions other than desquamation of the stomach mucosa (10/10 males and 1/10 females) and red spots on the mucosa of one male.

Four of ten male mice died and no female mice died after receiving Formula B. No toxic symptoms were observed in the female mice. Sedation was observed in two of the surviving males and return reflex was inhibited in one of the surviving males. Weight gain was transiently lower at day 5, but returned to normal by day 10. Necropsy revealed bleeding (1/10 M) and desquamation of stomach mucosa (7/10 F). No other signs of toxicity were observed. The authors conclude the LD0 for males was greater than 102 mg/kg bw but less than 16 g/kg bw and for females the LD0 was greater than 16 g/kg bw. It is important to note that Formula B is far

beyond any dose that would be administered in the proposed levels of use for thiocyanate in this notice, which proposes a 90th percentile use of 3.33 mg/day.

Rat Study

In the rat study conducted by the Pasteur Institute to evaluate the acute toxicity of thiocyanate and the LPO system, the LPO system was administered orally in water (10 mL/kg) to Sprague Dawley OFA rats (10M and 10F/group) after an 18-hr fast. The rats were observed for 15 days. The Lp-system Formula A (maximal hypothiocyanite) contained 4,000 mg/L glucose, 18.72 mg/L lactoperoxidase, 2 mg/L glucose oxidase, and 68.9 mg/L sodium thiocyanate; the total dose was 40.9 mg/kg bw). The Lp-system Formula B (maximum dose) contained 833 g/L glucose, 3.9 g/L lactoperoxidase, 0.42 g/L glucose oxidase, and 14.3 gm/L sodium thiocyanate; the total dose was 8.5 g/kg bw. No control group was included. No deaths, signs of toxicity, or abnormal weight gains were observed for either the Formula A or Formula B groups. The authors conclude that the LD0 is greater than 8.5 g/kg bw, which is far above the proposed use of thiocyanate in this GRAS notice, which proposes a 90th percentile use of 3.33 mg/day.

6.3.2.2. Subacute Toxicity Studies of Sodium Thiocyanate

Subacute toxicity has been evaluated for thiocyanate, as well as the LPO system, in both *in vivo* and *in vitro* experiments. One such study, which found benefit to chronic exposure to thiocyanate, was conducted in human lung epithelial cells (Xu, Szep, and Lu, 2009). Patients with cystic fibrosis have a gene mutation that prevents thiocyanate release in the respiratory epithelium. Thiocyanate and lactoperoxidase were found to prevent injury to lung epithelium due to hydrogen peroxide and hypochlorite, and the authors suggest that inadequate exposure to thiocyanate through diet could worsen the injury, and state that the general human population has plasma thiocyanate concentrations of 1-140 uM at baseline. This study suggests that thiocyanate is not only safe for respiratory epithelium, but is protective.

Reiter et al. (1981) fed neonatal calves (> 200 animals) with either whole milk or milk substitute, both containing thiocyanate in the LPO system (whole milk + 20 ml of a solution containing 1.6 g KSCN/L, 300 g glucose/L, and 20 ml of a solution containing 0.5 g glucose oxidase/L) for 5 weeks or until weaning. Weight gain was increased compared to controls by 3 weeks and sustained until the conclusion of the study. No adverse effects were reported.

Similar results were reported by Still et al. (1990) using young calves fed with a formulation containing thiocyanate in the LPO system, which is a whey-based feed complement containing lactoferrin and the lactoperoxidase system (20 mg/L lactoperoxidase, 1 mg/L glucose oxidase, 25 mg/L thiocyanate, and 1 g/L glucose). The results showed that LPO system significantly increased the weight gain of calves that received this formulation.

Wang, et al. (1987) also found that prolonged exposures to doses of thiocyanate (in milk with hydrogen peroxide, simulating the LPO system) up to 7.966 mg/day in mice and up to 5.112 mg/day also found no adverse effects in the animals. All animals were normal in health and weight gain at the conclusion of the study, which used a staggered dose schedule. Histopathology was performed and no relevant toxicologic findings were noted. A copy of this article, and a translation, are included in Annex 8.

These results demonstrate that the lactoperoxidase system can be activated *in vivo* without any adverse effect.

In addition to the literature sources presented above, Taradon is providing the full study report of a unpublished, corroborative 12-week rat study, conducted by the Pasteur Institute, following OECD and GLP guidelines (Annex 11). This study evaluated thiocyanate as part of the lactoperoxidase system, in male and female OFA Sprague-Dawley rats. The rats received treatment with one of four doses of thiocyanate (0, 0.05, 0.15, and 0.25 mg/kg) as part of the lactoperoxidase daily for 12 weeks. Doses were set based on the thiocyanate concentration. No treatment related lesions were found at necropsy, and no toxicologically significant differences were observed in organ weight or histopathology. The study conclusions were that there was no increased mortality and no treatment related toxic clinical signs following 12 weeks of thiocyanate exposure.

6.3.2.3. Chronic Exposures to Sodium Thiocyanate

Chronic thiocyanate exposures are also known to result from cigarette smoke exposure, resulting from the metabolism of cyanide to thiocyanate. The Agency for Toxic Substances and Disease Registry ("ATSDR") reports that smokers had 24-hour levels of urinary thiocyanate of 3.2 ug/mL in smokers, compared to 2.15 ug/mL in non-smokers.[3] Given that even non-smokers are found to have a chronic exposure to thiocyanate, likely though exposure in the diet as previously stated, as well as through the exposure to passive smoking, the use of thiocyanate as part of the lactoperoxidase system will be consistent with already existing dietary exposures. As stated in the WHO guidelines[4] for use of the lactoperoxidase system in milk products, "The levels of thiocyanate resulting from this treatment are within the physiological levels reported to occur in milk under certain circumstances and feeding regimes. They are also far below the thiocyanate levels known to exist in human saliva and certain vegetables, e.g. cabbage and cauliflower." Therefore, humans are chronically exposed to thiocyanate both through the saliva present in the oral cavity and the vegetables consumed daily. The presence of thiocyanate in physiologic fluids such as saliva at higher than those that would reach the consumer in food products that use thiocyanate as proposed for use in this GRAS notification again underscores the safety of thiocyanate.

6.3.2.4. Mutagenicity/Genotoxicity Studies of Sodium Thiocyanate

Hypothiocyanite produced by the LPO system using hydrogen peroxide, lactoperoxidase, and potassium thiocyanate was found to be cytogenic, but not mutagenic, in the Ames assay using *Salmonella typhimurium* indicator strains TA 1535, TA 1537, TA 1538 and hisG-46 (White Jr., et al., 1983). Hypothiocyanite generated enzymatically at an estimated initial concentration of 970 μM and by direct addition of hypothiocyanite at concentrations of 0, 0.11, 0.33, 1.1, 3.3, 11, 33, and 90 μM. Cell toxicity was noted at concentrations of 33 and 90 μM in all four strains. Hypothiocyanite was not toxic for *Saccharomyces cerevisiae* D-7 at concentrations up to 860

[3] Agency for Toxic Substances and Disease Registry. Toxicological Profile for Cyanide. 2006. Available at: https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=72&tid=19

[4] World Health Organization. Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase System. CAC/Gl 13-1991.

μM and did not oxidize calf thymus DNA after *in vitro* incubation for 30 min at room temperature (White Jr., et al., 1983).

6.3.2.5. Cytotoxicity Studies of Sodium Thiocyanate

The cytotoxic effects of various components of the LPO system, including thiocyanate, have been studied alone or in combination for cytotoxic effects. Lactoperoxidase was reported to lyse erythrocytes *in vitro* in the presence of hydrogen peroxide and iodine (McFaul et al., 1986). The cytolysis required the presence of iodine ions and was not observed when iodine was replaced by bromide, thiocyanate, or fluoride.

Moreover, Everse and collaborators (1985) have shown that the peroxidase system has a no toxicity level for normal tissues, but a specific antitumoral action by studying the effect of injection of a mixture of glucose oxidase and horseradish peroxidase immobilized onto small solid beads.

Tenovuo et al. (1984) reported that lactoperoxidase alone (5 ppm), thiocyanate alone (10 mM), or the combination of the two has no apparent effect on ^{3}H-thymidine incorporation, nor did they cause visual damage to the cells in human fibroblasts *in vitro*. Hydrogen peroxide at concentrations of 100 μM caused over 80% reduction in ^{3}H-thymidine incorporation compared to the controls. 200 μM of H_2O_2 was totally inhibitory. Peroxide-treated cells were partially or totally lysed when examined under microscope. Hypothiocyanite generated before addition to the cells at concentrations up to 300 μM had no effect on ^{3}H-thymidine incorporation in this study. Hypothiocyanite generated in presence of the cells by adding varying concentrations of hydrogen peroxide to the medium already containing cells, lactoperoxidase, and thiocyanate had no apparent effect on ^{3}H-thymidine incorporation, as long as there was no unreacted hydrogen peroxide left in the medium.

This study indicates that elevated concentrations of hypothiocyanite at levels that inhibit bacterial metabolism did not damage human cells.

6.4. GRAS Conclusion

The information submitted as part of this GRAS notice demonstrates that sodium thiocyanate is GRAS for the proposed intended use as a component of the lactoperoxidase system. Three of the ingredients of the system are present in the human and animal body, including thiocyanate, the subject of this notice, which is present in human saliva and gastric juice.

The chemical reaction of thiocyanate in the LPO system lasts approximately 400 minutes, which would be completed prior to the consumption of the product. Because the sodium thiocyanate is almost completely consumed in the reaction that drives the antimicrobial protection of the LPO system, it is not available in significant amounts at the time of consumption (Baufort et al., 2014). The exposure assessment provided in this GRAS notification ignores these kinetics of the reaction, and assumes that there is no elimination of the sodium thiocyanate from the reaction, and is a worst-case scenario. Furthermore, the levels of use proposed for the sodium thiocyanate are below those values which can be found naturally in vegetables.

Beyond the presence of thiocyanate in human biologic fluids and in many of the green vegetables that are consumed daily, the safety of thiocyanate is further supported by toxicologic studies of both thiocyanate and the LPO system. As noted in the acute toxicity studies included in the notice, the LD0 ranged from 8.5 g/kg/bw to greater than 16 g/kg/bw. As noted by the exposure assessment included in Part 3, these doses are magnitudes higher than the total consumption anticipated per day in the worst-case scenario exposure assessment, which found a product-related use level of 3.33 mg/day in the 90th percentile user. Even if the natural uses are included as well, the highest dose would be 7.24 mg/day. Again, these values do not account for the fact that nearly all of the thiocyanate is eliminated in the LPO system reaction, and is not consumed by the user. Similarly, subchronic studies found that even after 12 weeks of thiocyanate administration, no adverse effects were found. A 2-year chronic toxicity study fed rats 3.2 g thiocyanate/L water, and found no evidence of carcinogenicity. These studies reveal safe use levels of grams, which are 1000 times larger than the proposed milligram/kg uses in the application.

Thiocyanate has been studied in humans for over a century. It is known as an antidote to cyanide poisoning, and the current standard of care for cyanide poisoning is in fact a conversion of the ingested cyanide to thiocyanate. Thiocyanate is excreted largely unchanged in the urine, suggesting it is not metabolized extensively. As discussed above, when it is metabolized, it is converted to carbon dioxide. Numerous studies have been performed in various animal models using thiocyanate alone, with a NOAEL for sodium and potassium thiocyanate in the test animals was 100-200 mg/kg (rats) and 20.8-24.4 mg/kg (dogs), concentrations far exceeding those proposed in this notice (Chen and Anderson, 1940).

Thiocyanate has been suggested through animal studies to have a potential link to hypothyroidism, as it can act as a competitive inhibitor of iodine uptake. However, as noted by the World Health Organization in its review of the use of thiocyanate as part of the LPO system to preserve milk, the serum concentrations of thiocyanate required to initiate these effects are far beyond anything that would be reached using the LPO system.[5] This conclusion is supported by a clinical study by Dahlberg, who found that even in iodine-deficient people, daily intake of 4.75 mg of thiocyanate did not have a negative impact on thyroid function (Dahlberg et al., 1985). Another review evaluated the toxicity of thiocyanate and found that no reports of toxicity were found in humans with less than 500 uM plasma concentrations of thiocyanate, a level beyond that which would be achieved through the use of thiocyanate in the LPO system (Chandler et al., 2012). Given that thiocyanate is a naturally occurring substance, endogenous to human saliva and serum, this lack of thyroid toxicity except at high levels is not surprising, and speaks to the safety of the use of thiocyanate as proposed in this GRAS notification.

Taradon Laboratories has concluded that the information submitted and referenced allows them to state that the sodium thiocyanate is generally recognized as safe as a processing aid as part of the lactoperoxidase system for use in fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt.

[5] World Health Organization. (2005). Benefits and potential risks of the lactoperoxidase system of raw milk preservation. Report of an FAO/WHO Technical Meeting.

PART 7: LIST OF REFERENCES

Pursuant to 21 C.F.R. 170.255, the list of supporting data and information referenced in the GRAS notice is contained below.

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7. LIST OF ANNEXES

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Annex 1

GUIDELINES FOR THE PRESERVATION OF RAW MILK BY USE OF THE LACTOPEROXIDASE SYSTEM

CAC/GL 13-1991

INTRODUCTION

Milk is an easily perishable raw material. Contaminating bacteria may multiply rapidly and render it unsuitable for processing and/or unfit for human consumption. Bacterial growth can be retarded by refrigeration, thereby slowing down the rate of deterioration. Under certain conditions refrigeration may not be feasible due to economical and/or technical reasons. Difficulties in applying refrigeration are specially a problem for certain areas in countries setting up or expanding their milk production. In these situations, it would be beneficial to have access to a method, other than refrigeration, for retarding bacterial growth in raw milk during collection and transportation to the dairy processing plant.

In 1967 the FAO/WHO Expert Panel on Milk Quality concluded that the use of hydrogen peroxide might be an acceptable alternative in the early stages of development of an organized dairy industry, provided that certain conditions were complied with. However, this method has not achieved any general acceptance as it has several drawbacks, most important of which is the difficulty of controlling its use: it may be misused to disguise milk of basic hygienic quality produced under poor hygienic conditions. The toxicological aspects of the use of relatively high concentrations of hydrogen peroxide in milk have also been questioned.

A chemical method for preserving milk would still be of great advantage in certain situations. The search for such a method has therefore continued. Interest has recently been focused on the indigenous antibacterial systems in milk to determine if these could be applied practically to preserve raw milk. During the last decade, basic and applied research has demonstrated that one of these systems, the lactoperoxidase/thiocyanate/hydrogen peroxide system (LP-system) can be used successfully for this purpose.

1. SCOPE

1.1 This Code of Practice describes the use of the lactoperoxidase system for preventing bacterial spoilage of raw milk (bovine and buffalo) during collection and transportation to a dairy processing plant. It describes the principles of the method, in what situations it can be used, its practical application and control of the method. It should be stressed that this method should be utilized when refrigeration of the raw milk is not feasible.

2. PRINCIPLES OF THE METHOD

2.1 The lactoperoxidase/thiocyanate/hydrogen peroxide system is an indigenous antibacterial system in milk and human saliva. The enzyme lactoperoxidase is present in bovine and buffalo milk in relatively high concentrations. It can oxidise thiocyanate

ions in the presence of hydrogen peroxide. By this reaction, thiocyanate is converted into hypothiocyanous acid (HOSCN). At the pH of milk HOSCN is dissociated and exists mainly in the form of hypothiocyanate ions ($OSCN^-$). This agent reacts specifically with free sulphydryl groups, thereby inactivating several vital metabolic bacterial enzymes, consequently blocking their metabolism and ability to multiply. As milk proteins contain very few sulphydryl groups and those that are present are relatively inaccessible to $OSCN^-$ (masked), the reaction of this compound is in milk quite specific and is directed against the bacteria present in the milk.

2.2 The effect against bacteria is both species and strain dependent. Against a mixed raw milk flora, dominated by mesophilic bacteria, the effect is bacteriostatic (predominantly inhibitory). Against some gram-negative bacteria, i.e. pseudomonads, *Escherichia coli,* the effect is bactericidal. Due to the mainly bacteriostatic effect of the system it is not possible to disguise poor quality milk, which originally contained a high bacterial population, by applying this method.

2.3 The antibacterial oxidation products of thiocyanate are not stable at neutral pH. Any surplus of these decomposes spontaneously to thiocyanate. The velocity of this reaction is temperature dependent, i.e. more rapid at higher temperatures. Pasteurisation of the milk will ensure a complete removal of any residual concentrations of the active oxidation products.

2.4 Oxidation of thiocyanate does not occur to any great extent in milk when it has left the udder. It can, however, be initiated through addition of small concentrations of hydrogen peroxide (see Section 4). The high concentrations of hydrogen peroxide used to preserve milk (300–800 ppm), destroy the enzyme lactoperoxidase and thereby preclude the oxidation of thiocyanate. With this method the antibacterial effect is thus an effect of hydrogen peroxide itself.

2.5 The antibacterial effect of the LP-system is, within certain limits, proportional to the thiocyanate concentration in the milk (provided that an equimolar amount of hydrogen peroxide is provided). The level thiocyanate in milk is related to the feeding of the animals and can thus vary. The practical use of the method consequently requires addition of some thiocyanate to ensure that a level necessary to achieve the desired effect, is present in the milk.

2.6 The levels of thiocyanate resulting from this treatment are within the physiological levels reported to occur in milk under certain circumstances and feeding regimes. They are also far below the thiocyanate levels known to exist in human saliva and certain common vegetables, e.g. cabbage and cauliflower. In addition, results from clinical experiments have clearly demonstrated that milk treated according to this method will not cause any interference of the iodine uptake of the thyroid gland, neither in persons with a normal iodine status nor in cases of iodine deficiency.

3. INTENDED UTILIZATION OF METHOD

3.1 This method should only be used in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities for maintaining the quality of raw milk. Use of the LP-system in areas which currently lack an adequate infrastructure for collection of liquid milk, would ensure the production of milk as a safe and wholesome food, which otherwise would be virtually impossible.

3.2 The method should not be used by the individual farmers but at a suitable collecting point/centre. These centres must be equipped with proper facilities for cleaning and sanitising the vessels used to hold and transport milk.

3.3 The personnel responsible for the collection of milk should be in charge for the treatment of the milk. They should be given appropriate training, including training in general milk hygiene, to enable them fulfil this in a correct way.

3.4 The dairy processing the milk collected by use of the lactoperoxidase system should be made responsible for ensuring that the method is used as intended. This dairy should set up appropriate control methods (see Section 5) to monitor usage of the method, raw milk quality and quality of the milk prior to processing.

3.5 The method should primarily be used to prevent undue bacterial multiplication in raw milk during collection and transportation to the dairy processing plant under conditions stated in 3.1. The inhibitory effect of the treatment is dependent on the temperature of the stored milk and has been found to act for the following periods of time in laboratory and field-experiments carried out in different countries with raw milk of an initial good hygienic standard:

Temperature, C	Time, h
30	7–8
25	11–12
20	16–17
15	24–26

3.6 The use of the lactoperoxidase method does not exclude the necessity of pasteurization of the milk before human consumption. Neither does it exclude the normal precautions and handling routines applied to ensure a high hygienic standard of the raw milk.

4. PRACTICAL APPLICATION OF THE METHOD

4.1 The lactoperoxidase system can be activated in raw milk to give the above stated antibacterial effect by an addition of thiocyanate as sodium thiocyanate and hydrogen peroxide in the form of sodium percarbonate by the following procedure:

- 14 mg of NaSCN is added per litre of milk. The milk should then be mixed to ensure an even distribution of the SCN-. Plunging for about 1 minute with a clean plunger is normally satisfactory.
- Secondly, 30 mg of sodium percarbonate is added per litre of milk. The milk is then stirred for another 2–3 minutes to ensure that the sodium percarbonate is completely dissolved and the hydrogen peroxide is evenly distributed in the milk.

4.2 It is essential that the sodium thiocyanate and sodium percarbonate are added in the order stated above. The enzymatic reaction is started in the milk when the hydrogen peroxide (sodium percarbonate) is added. It is completed within about 5 minutes from the addition of H_2O_2; thereafter, no hydrogen peroxide is present in the milk.

4.3 The activation of the lactoperoxidase system should be carried out within 2–3 hours from the time of milking.

4.4 Quantities of sodium thiocyanate and sodium percarbonate needed for the treatment of a certain volume of milk, for example 40 or 50 litre milk churns, should be distributed to the collecting centre/point in prepacked amounts lasting for a few weeks at a time. The technical specifications of the thiocyanate and sodium percarbonate which should be used are stated in Appendices I and II.

5. CONTROL OF USAGE

5.1 The use of the lactoperoxidase system for preserving raw milk must be controlled by the dairy processing plant receiving the milk. This should be a combination of currently used acceptance tests, e.g. titratable acidity, methylene blue, resazurin, total viable count and analyses of the thiocyanate concentration in the milk. Since the thiocyanate is not consumed in the reaction, treated milk arriving at the dairy plant would contain approximately 10 mg above the natural amount of thiocyanate (the latter can be determined by analysing untreated milk from the same area) per litre of milk. The analytical method for SCN^- is described in Appendix III Testing should be undertaken at random. If the concentration of thiocyanate is too high (or too low), investigation must be carried out to determine why the concentration is outside specification. The dairy processing plant should also be responsible for the control of the chemicals to be used at the collection centre for the activation of the lactoperoxidase system.

5.2 Analysis of the bacteriological quality of the milk (methylene blue, resazurin, total plate count) should also be carried out to ensure that good hygienic standards are not neglected. Since the effects of the system are predominantly bacteriostatic, an initial high bacterial population in the milk can still be revealed by such tests.

APPENDIX I

TECHNICAL SPECIFICATION OF SODIUM THIOCYANATE

Definition

Chemical name	Sodium thiocyanate
Chemical formula	NaSCN
Molecular weight	81.1
Assay content	98–99%
Humidity	1–2%

Purity (according to JECFA* specification)

Heavy metals (as Pb)	< 2 ppm
Sulfates (as SO_4)	< 50 ppm
Sulfide (S)	< 10 ppm

* Joint FAO/WHO Expert Committee on Food Additives.

APPENDIX II

TECHNICAL SPECIFICATION OF SODIUM PERCARBONATE

Definition

Chemical name	Sodium percarbonate (*)
Chemical formula	$2Na_2CO_3 \cdot 3H_2O_2$
Molecular weight	314.0
Assay content	85%

Commercial available sodium percarbonate recommended to be used has the following specification:

Sodium carbonate peroxyhydrate	> 85%
Heavy metals (as Pb)	< 10 ppm
Arsenic (as As)	< 3 ppm

(*) For information where sodium percarbonate could be obtained commercially, please apply to IDF General Secretariat, Silver Building, Blvd. A. Reyers 70/B, B-1030 Brussels, Belgium.

APPENDIX III

ANALYSIS OF THIOCYANATE IN MILK

Principle

Thiocyanate can be determined in milk, after deproteinisation, with trichloroacetic acid (TCA), as the ferric complex by measuring the absorbance at 460 nm. The minimum level of detection by this method is 1 to 2 ppm of SCN^- .

Reagent solutions

1. 20% (w/v) trichloroacetic acid: 20 g TCA is dissolved in 100 ml of distilled water and filtered.
2. Ferric nitrate reagent: 16.0 g $Fe(NO_3)_3.9H_2O$ is dissolved in 50 ml 2 M HNO_3* and then diluted with distilled water to 100 ml. The solution should be stored dark and cold.

   * 2M HNO_3 is obtained by diluting 138.5 ml 65% HNO_3 to 1 000 ml with distilled water.

Determination

4.0 ml of milk is mixed with 2.0 ml of 20% TCA solution. The mixture is blended well and then allowed to stand for at least 30 minutes. It is thereafter filtered through a suitable filter paper (Whatman No. 40). 1.5 ml of the clear filtrate is then mixed with 1.5 ml of the ferric nitrate reagent and the absorbance measured at 460 nm. As a blank, a mixture of 1.5 ml of ferric nitrate solution and 1.5 ml of water is used. The measurement must be carried out within 10 minutes from the addition of the ferric nitrate solution as the coloured complex is not stable for any length of time. The concentration of thiocyanate is then determined by comparison with standard solutions of known thiocyanate concentration, e.g. 10, 15, 20 and 30 µg/ml of thiocyanate.

Annex 2

SODIUM THIOCYANATE

Certificate of Analysis

Delivery Address

Order item	5100455684 000020
Delivery item	6100587611 000020
Material number	5404306
Customer ref.	

Analysis

Batch number	1403070719
Quantity	

Characteristic	Unit	Values	Spec Limits min.	Spec Limits max.	Method of Analysis
Appearance	-	White crystals			
Assay (on dried basis)	%	99,5	98,0	-	AG/89.1
pH (5%-Solution)	-	6,3	5,0	9,0	F/89.2
Iron	mg/kg	0,1	-	3,0	KOL 92/02

Our certificates of analysis are based on analyses carried out on random samples taken from the production batches from which you have been supplied. Thi certificate of analysis does not exempt you from testing the suitability of the delivered product for your applications.

Date:18.07.2014

Approved by:

Thomas Schauzu
(QHSSE Manager)

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D-50735 Köln
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Phone +49 221 7496-0
Fax +49 221 7496-190

This Certificate of Analysis has been produced electronically and is effective without a signature.

CARBOSULF

SODIUM THIOCYANATE

Delivery Address

Certificate of Analysis

Order item	5100562161 000020
Delivery item	6100724415 000020
Material number	5404306
Customer ref.	

Analysis

Batch number 1501071319

Quantity

Characteristic	Unit	Values	Spec Limits min.	Spec Limits max.	Method of Analysis
Appearance	-	White crystals	-	-	
Assay (on dried basis)	%	99,9	98,0	-	AG/89.1
pH (5%-Solution)	-	6,1	5,0	9,0	F/89.2
Iron	mg/kg	0,1	-	3,0	KOL 92/02

Our certificates of analysis are based on analyses carried out on random samples taken from the production batches from which you have been supplied. This certificate of analysis does not exempt you from testing the suitability of the delivered product for your applications.

Date:05.02.2015

Approved by:

Thomas Schauzu
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CARBOSULF

SODIUM THIOCYANATE

Certificate of Analysis

Delivery Address

Order item 5100976166 000020
Delivery item 6101263638 000020
Material number 5404306

Customer ref.

Analysis
Batch number 1606070619
Quantity

Characteristic	Unit		Spec Limits min.	max.	Method of Analysis
Appearance	-	White crystals			
Assay (on dried basis)	%	99,9	99,0	-	AG/89.1
pH (5%-Solution)	-	5,7	5,0	9,0	F/89.2
Iron	mg\kg	0,1	-	3,0	KOL 92/02

Our certificates of analysis are based on analyses carried out on random samples taken from the production batches from which you have been supplied. This certificate of analysis does not exempt you from testing the suitability of the delivered product for your applications.

Date:06.07.2016

Approved by:

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AkzoNobel

Product Specification

Sodium Thiocyanate crystalline

Chemical Name:	Sodium thiocyanate
Molecular Formula:	NaSCN
Molecular Mass:	81,1 g/mol
CAS-No.:	540-72-7
EC-No.:	208-754-4

Properties

Bulk density:	approx. 750 kg/m^3
Solubility in water (20°C):	approx. 1250 g/l
Melting point:	approx. 310 °C

Specification

Appearance:	white crystals
Content (on dried basis):	min. 99,0 %
Moisture:	max. 3,0 %
Iron:	max. 3 mg/kg
pH (5% aqueous solution):	5,0 – 9,0
Ammonia (as NH_3)	max. 200 mg/kg

Typical Characteristics

Chloride:	< 200 mg/kg
Sulphate:	< 300 mg/kg
Heavy metals:	< 10 mg/kg

Analytical methods are available on request.

Major Applications

In the fiber industry in spinning baths for acrylic fibers.
In the water treatment industry as corrosion inhibitor.
In agriculture as an intermediate in the manufacture of herbicides.
In the photographic industry as sensitizer and stabilizer.
In concrete industry as hardening accelerator.

Storage

Store in a cool and dry place and avoid any contact to a strong acid.
Use resistant equipment like polymer materials and high grade alloys. Iron corrosion can result in red coloration of product when exposed to UV-light. Although the product is stable when stored under ambient conditions without exposure to other chemicals, it is advised to re-analyze before use after 3 years of storage. Thiocyanates are hygroscopic and will attract humidity from air. This might result in higher moisture content in the product after some time.

Packing and Transport

Sodium thiocyanate is delivered in:	25 kg net in paper bags
Hazard Identification No.:	none
UN-No.:	none

Safety advice

For transport, handling and first aid instructions we refer to our Material Safety Data Sheet (MSDS).

Issue June 2015/T. Morris

Annex 3

TARADON LPO-SYSTEM

CERTIFICATE OF ANALYSIS

Lot: CS-DON 20161024

Per gr of powder

Lactoperoxidase activity:	12,500 ABTS units
Glucose Oxydase activity:	325 ABTS units
Na-Thiocyanate :	50 mg
Glucose:	300 mg
$OSCN^-$ production:	> 100 µM

Bacterial count:

Total plate count	< 10 CFU / g
E. coli	Absent /g
Yeast and Molds	< 10 CFU / g
Staphylococcus aureus	Absent / 2gr
Salmonella	Absent / 5gr

Manufaturing date : 24/10/2016
Expired date : 24/10/2017

Jean-Paul Perraudin
Quality Control Coordinator

TARADON LPO SYSTEM

CERTIFICATE OF ANALYSIS

Lot: CS-DON 20160818

Per gr of powder

Lactoperoxidase activity:	12,500 ABTS units
Glucose Oxydase activity:	310 ABTS units
Na-Thiocyanate :	50 mg
Glucose:	305 mg
$OSCN^-$ production:	> 100 µM

Bacterial count:

Total plate count	< 10 CFU / g
E. coli	Absent /g
Yeast and Molds	< 10 CFU / g
Staphylococcus aureus	Absent / 2gr
Salmonella	Absent / 5gr

Manufaturing date : 18/08/2016
Expired date : 18/08/2017

Jean-Paul Perraudin
Quality Control Coordinator

TARADON LPO-SYSTEM

CERTIFICATE OF ANALYSIS

Lot: CS-DON 20151028

Per gr of powder

Lactoperoxidase activity:	12,625 ABTS units
Glucose Oxydase activity:	315 ABTS units
Na-Thiocyanate :	50 mg
Glucose:	300 mg
$OSCN^-$ production:	> 100 µM

Bacterial count:

Total plate count	< 10 CFU / g
E. coli	Absent /g
Yeast and Molds	< 10 CFU / g
Staphylococcus aureus	Absent / 2gr
Salmonella	Absent / 5gr

Manufaturing date : 28/10/2015
Expired date : 28/10/2016

Jean-Paul Perraudin
Quality Control Coordinator

Annex 4

Benefits and Potential Risks of the Lactoperoxidase system of Raw Milk Preservation

Report of an FAO/WHO technical meeting

O Headquarters, Rome, Italy, 28 November - 2 December, 2005

World Health Orga i i

FAO FIAT PANIS

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Web sites:
http://www.fao.org/ag/agn/
http://www.fao.org/ag/dairy.html

Benefits and Potential Risks of the Lactoperoxidase System of Raw Milk Preservation

Report of an FAO/WHO technical meeting
FAO Headquarters, Rome, Italy
28 November - 2 December 2005

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Contents

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Appreciation is also extended to all those who responded to the call for data that was issued by FAO and WHO and brought to our attention information that was not readily available in the mainstream literature and official documentation.

The preparatory work and technical meeting convened to prepare this report was coordinated by the Animal Production and Food Quality and Standards Services of FAO. This included Anthony Bennett, Sarah Cahill, Ruth Charrondiere, Maria de Lourdes Costarrica, Frederic Lhoste and Simon Mack in FAO and Hae Jung Joon and Jørgen Schlundt in WHO. Publication of the report was co-ordinated by Anthony Bennett, Sarah Cahill and Frederic Lhoste. Art direction by Nicoletta Forlano and design by James Morgan. James Edge edited the report.

The work was supported and funded by the Animal Production and the Food Quality and Standards Services of FAO and the WHO Department of Food Safety, Zoonoses and Foodborne Disease.

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Declarations of interest

Ing. Ponce: As a researcher at the National Centre for Animal and Plant Health (CENSA), he is the author of a patent on a product based on the activation of the Lactoperoxidase system. He does not have rights for its commercial exploitation nor profits derived from it as established by Cuban Laws of Intellectual Property.

Abbreviations

CAC	Codex Alimentarius Commission
CCFH	Codex Committee on Food Hygiene
COMESA	Common Market for Eastern and Southern Africa
FAO	Food and Agriculture Organization of the United Nations
GEMS	Global Environment Monitoring System
GLP	The FAO Global Lactoperoxidase Experts Group
JECFA	Joint FAO/WHO Expert Committee on Food Additives
HTST	High Temperature Short Time
IDD	Iodine Deficiency Disease
IGAD	Inter-Governmental Authority on Development
LP-s	Lactoperoxidase system
ppm	Parts per million
SADC	Southern African Development Community
UHT	Ultra-high temperature (sterilization) / Ultra heat treated (milk)
WHO	World Health Organization

Executive Summary

This technical meeting was jointly organised by the Animal Production and the Food Quality and Standards Services of the Food and Agriculture Organization of the United Nations (FAO), in cooperation with the Department of Food Safety, Zoonoses and Foodborne Disease of the World Health Organization (WHO) to obtain the best available scientific advice on issues related to the use of the lactoperoxidase system (LP-s) in raw milk preservation.

After reviewing the available scientific information (References, Appendix A and B), the technical meeting concluded that the LP-s is a safe method of preventing milk losses due to microbial spoilage when used according to the Codex guidelines either alone or in combination with other approved procedures. The LP-s is particularly suitable for application in situations where technical, economical and/or practical reasons do not allow the use of cooling facilities for maintaining the quality of raw milk. Use of the LP-s does not preclude or replace the need for the pasteurization of raw milk to improve safety for human consumption.

Post harvest losses are a major issue in dairying in developing countries. Smallholder dairy farmers could increase their participation in worldwide milk production, processing and marketing if they could reduce their losses using any approved milk preservation method. Refrigeration is the preferred means of milk preservation but does require high capital investment and can incur high running and maintenance costs. The LP-s provides a cost effective method to increase the availability of milk that contributes to income generation, household food security and nutrition in developing countries.

The LP-s elicits antimicrobial activity against a wide variety of milk spoilage and pathogenic microorganisms including bacteria, HIV-1 virus, moulds, yeasts, mycoplasma and protozoa. Furthermore, the LP-s does not promote the growth of pathogenic microorganisms after completion of the bacteriostatic effect[1]. The activated LP-s is effective in raw milk of different species, the overall activity being primarily bacteriostatic[2], depending on the initial total bacterial load, species and strains of contaminating bacteria and the temperature of milk.

[1]Under laboratory conditions.

[2]The LP-s is classified as a 'microbiostatic' in the Codex Code of Hygienic Practice for Milk and Milk Products (CAC/RCP/57 – 2004) (CAC, 2004b).

Observations from laboratory and field studies indicate that the LP-s does not induce any significant adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products. Under practical conditions the activated LP-s cannot be used to disguise milk of poor microbiological quality.

None of the components of the LP-s presents a significant toxicological risk to public health at the levels proposed. Where iodine deficiency is common, public health measures to rectify the iodine deficiency are needed whether or not the LP-s is used.

In adopting the "Guidelines for the preservation of raw milk by use of the lactoperoxidase system" in 1991, the Codex Alimentarius Commission agreed to emphasise that the LP-s should not be used for products intended for international trade. This provision is considered a major obstacle to the adoption of the system, limiting both regional and international trade in LP-s treated milk and dairy products.

Based on the available data and an assessment thereof, the technical meeting considered the LP-s to be a safe method of raw milk preservation when implemented according to established Codex guidelines. The meeting concluded that this report provides a scientific basis for Codex to reconsider the provision related to the limitation on the international trade of LP-s treated milk and dairy products.

Recommendations

In making its recommendations, the meeting reiterated the safety of the Lactoperoxidase system of raw milk preservation when used according to the existing guidelines (CAC, 1991b), recommending its use in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities. Based on its deliberations the following specific recommendations were made.

TO CODEX

Consider expanding the guideline for the use of this system with regard to temperature of application of the LP-s to also include the temperature range from 31°C to 35°C for 4–7 hours and down to 4°C for 5–6 days.

Develop milk and dairy product standards that can be easily adopted at regional or national level through the encouragement and support of active participation of a representative range of country members in the development of standards.

Remove the current provision regarding the restriction on the use of LP-s in milk or dairy products intended for international trade as the meeting found no scientific or technical basis or economic justification for the provision.

TO MEMBER COUNTRIES, FAO, WHO, CODEX, NGO'S AND THE DAIRY INDUSTRY

Acknowledge the LP-s as an effective and feasible method of raw milk preservation that does not display a negative impact on the further processing of milk.

Owing to its bacteriostatic effect, give consideration to the application of the LP-s as part of a programme to improve milk hygiene and safety along the milk chain.

Consider the application of the LP-s to complement cooling in order to extend the keeping quality of raw milk and halt proliferation of milk spoilage and pathogenic microorganisms.

Use the LP-s to improve the quality of processed products based on its proven bacteriostatic effect from milk collection to final processing and in particular to extend milk collection distances in developing countries, thereby increasing the amount of milk available for marketing. This can have significant direct benefits for both milk producers and consumers.

Recognise that the use of the LP-s is an economically viable option (either standalone or in combination with refrigeration) to significantly reduce milk losses and increase milk availability.

In addition to recommendations specific to the use of the LP-s a number of other related issues were discussed, based on which the technical meeting made the following recommendations.

Promote the consumption of milk as a valuable source of human nutrition contributing to healthy development and growth.

Promote the contribution of small-scale dairying to household nutrition, food security, and poverty alleviation.

Implement measures to rectify iodine deficiency in recognised IDD areas accompanied by appropriate monitoring of its prevalence. Milk can also be a valuable source of iodine, providing there is adequate iodine in the diet of the milk-producing animals.

1. Introduction

This technical meeting was jointly organised by the Animal Production and the Food Standards and Quality Services of the Food and Agriculture Organization of the United Nations (FAO), in cooperation with the Department of Food Safety, Zoonoses and Foodborne Disease of the World Health Organization (WHO) to get the best available scientific advice on issues related to the Lactoperoxidase system (LP-s). The LP-s consists of the addition of sodium thiocyanate and hydrogen peroxide to reactivate the existing lactoperoxidase enzyme in milk that maintains the initial quality of the milk without refrigeration until the milk can be processed or pasteurized.

FAO and WHO recognise the important role smallholder dairy producers play in supplying milk and dairy products to markets in developing countries. Their continued participation in these markets is encouraged. Milk is an important commodity that contributes to household nutrition and health, and can also provide an income. Therefore, approaches for enhancing the availability of safe milk and dairy products are important for the continued improvement of household nutrition and health.

This meeting was part of the FAO/WHO activities on the provision of scientific advice to Codex and to their member countries. The Codex guidelines (CAC /GL 13 – 1991(CAC, 1991b)) for the preservation of raw milk by use of the LP-s were adopted in 1991 at which time the Codex Alimentarius Commission (CAC) also "agreed to emphasise that the lactoperoxidase system not be used for products intended for international trade" (CAC, 1991a). Since then many member countries have raised concerns over this provision. In this regard, FAO and WHO have been asked to provide scientific advice based on comprehensive and relevant information in order to support appropriate decision-making within the Codex system on the use of the LP-s (CAC, 2004a).

Experts from five regions – Africa, Asia, Europe, North and Latin America, and the Caribbean – participated in the meeting in their independent professional capacities and not as representatives of their governments, employers, or institutions. The meeting was supported by a number of submitted papers following an open call for information and data from member countries on issues relating to the LP-s. In particular, issues related to microbiological effects and performance, human health and nutrition, processing and technology, and economic value and trade were addressed. These documents, as listed in Appendix A, were distributed to the experts prior to the meeting. Additional materials consulted and provided by participants during the meeting are included in the Reference section and Appendix B of this report.

1.1 BACKGROUND

This meeting was part of the FAO/WHO activities on the provision of scientific advice to Codex and to their member countries. The Codex guidelines (CAC /GL 13 – 1991(CAC, 1991b)) for the preservation of raw milk by use of the LP-s were adopted in 1991 at which time the Codex Alimentarius Commission (CAC) also "agreed to emphasise that the lactoperoxidase system not be used for products intended for international trade" (CAC, 1991a). Since then many member countries have raised concerns over this provision. In this regard, FAO and WHO have been asked to provide scientific advice based on comprehensive and relevant information in order to support appropriate decision-making within the Codex system on the use of the LP-s (CAC, 2004a).

Lactoperoxidase is an enzyme that is naturally present in milk. One of its unique biological functions is a bacteriostatic effect in the presence of hydrogen peroxide and thiocyanate. Both of these substances are naturally present in milk in varying concentrations. The method of activating the LP-s in milk is to add about 10 ppm (parts per million) of thiocyanate (preferably in powder form) to the raw milk to increase the overall level to 15 ppm (around 5 ppm is naturally present). The solution is thoroughly mixed for 30 seconds and then an equimolar amount (8.5 ppm) of hydrogen peroxide is added (generally in the form of a granulated sodium carbonate peroxyhydrate). The activation of the lactoperoxidase has a bacteriostatic effect on the raw milk and effectively extends the shelf life of raw milk for 7–8 hours under ambient temperatures of around 30°C or longer at lower temperatures. This allows adequate time for the milk to be transported from the collection point to a processing centre without refrigeration.

There are several ways in which the spoilage of milk may be controlled, including refrigeration, heat treatment (pasteurization in bulk or in pouch), microfiltration (with or without pasteurization), bactofugation, high-pressure treatment and use of chemical preservatives (including salting at levels of 3–12%). Some of these procedures require expensive equipment and are not widely applicable, particularly in small-scale dairy production and processing systems in developing countries where up to 80% of the milk produced may enter the informal market.

The FAO Global Lactoperoxidase Experts Group (GLP) was set up in July 1998. The main objective of this group was to promote the LP-s and carry out demonstrations in specific regions in the world where refrigeration is difficult. The partners involved in this group were the Lund University of Sweden, WHO, the International Dairy Federation, and FAO, with support from the Governments of Sweden, France, Hungary and Ireland. The strategy of the GLP was to inform countries and assess their interest in these issues, to identify regional partner institutions, national institutions and experts, conduct national training and demonstrations in collaboration with the relevant ministries and follow-up through national experts and governments. The outputs from the GLP included posters and manuals on the use of the LP-s in English, French and Spanish, the printing and distribution of Field Manuals, the implementation of training and demonstrations in 35 countries, annual meetings and the Bushmilk *(Lait de brousse)* programme in West Africa.

Codex adopted the "Guidelines for the preservation of raw milk by use of the lactoperoxidase system" in 1991 (CAC, 1991a, b). Issues concerning the LP-s of raw milk preservation have been raised in numerous Codex meetings, most recently during the meeting of the Codex Alimentarius Commission in Geneva in 2004 (CAC, 2004a). Issues related to the guidelines have also been raised as a concern by numerous FAO member countries.

In 2002, the GLP requested that the Codex Committee on Milk and Milk Products (CCMMP) consider amendments to the guidelines (CAC, 2002a). Highlighting the need for a scientific basis for any amendments, the committee referred the issue to the Codex Executive Committee later in 2002, which agreed that this might be of particular interest to developing countries and invited Regional Committees to consider the issues (CAC, 2002b). It was recognised that all relevant health aspects of this complex issue should be considered to ensure that any revision of the guidelines would be based on sound science and risk analysis.

In 2002, the Codex Coordinating Committee for Africa supported these decisions and maintained that until uncertainties related to the process were resolved the provisions on the use of this system should be maintained (CAC, 2002c). The Codex Coordinating Committee for North America and South West Pacific (CCNASWP) in 2002 also recommended that further reviews by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) of the chemical and microbiological safety of the LP-s should be undertaken before revising the guidelines (CAC, 2002d). In 2003, the Codex Committee on Food Hygiene (CCFH) concluded that the current provision excluding the use of the lactoperoxidase system for products intended for international trade should continue to be applied and that there was no need for the revision of the existing Guidelines in the framework of Codex or a JECFA review (CAC, 2003).

The issue was raised again in the CCFH in 2004 when the committee was informed that new data were being generated. It was also discussed at the 27th session of the Codex Alimentarius in 2004 in the course of the adoption of the Draft Code of Practice for Milk and Milk Products, during which the following text was added to the code, "The use of the lactoperoxidase system for milk and milk products in international trade will be re-examined by the Committee on Food Hygiene (CCFH) after completion of an expert review by FAO and WHO of available data and considering the FAO Lactoperoxidase Expert Group report about potential risks and benefits of lactoperoxidase system. CCFH will then review the issue in 2006" (CAC, 2004a).

1.2 SCOPE AND PURPOSE OF THE TECHNICAL MEETING

The current meeting was implemented to respond to member country concerns and to provide scientific advice to the next session of the CCFH in 2006 on the benefits and possible risks associated with the LP-s for raw milk preservation and any dairy products derived from treated milk.

The objective of the technical meeting was to determine the benefits (economic and nutritional) and the level of health risks, if any, posed by the application of the LP-s, advise on the safety of the LP-s treated milk and derived milk products, and to address the issue of the limitation on the use of LP-s treated milk or derived products intended for international trade.

The group agreed to discuss these issues under the following four headings and a chairperson and rapporteur was assigned for each of the four subject areas.

1. The microbiological effects and performance of the LP-s
Chairperson: C. Michiels
Rapporteur: H. Korhonen

2. The effects of LP-s treated milk and dairy products on human health and nutrition
Chairperson: J. Vanderveen
Rapporteur: R. Walker

3. Milk processing, technology and preservation
Chairperson: J. P. Ramet
Rapporteur: A. Grandison

4. Economic value and trade of LP-s treated milk and dairy products
Chairperson: H. G. Muriuki
Rapporteur: O. C. Emata

This report summarises the deliberations, findings and conclusions of the meeting.

2. Microbiological Effects and Performance of the Lactoperoxidase System

The effectiveness of the LP-s in maintaining the hygienic quality of raw milk for a limited period of time has been established in many experimental and field studies conducted in different geographical regions. The method can be applied to preserve raw milk from different species. The effectiveness depends on the initial amount and type of microbiological contamination and the temperature of milk during the treatment period. The LP-s exerts primarily a bacteriostatic effect in raw milk. Experimental data and experience from practice indicate that the LP-s can be applied beyond the temperature limits (15–30°C) referred to in the 1991 Codex guidelines (CAC, 1991b). At the lower end of the temperature scale, several studies indicate that activation of the LP-s can delay growth of psychrotrophic milk bacteria and thus delay milk spoilage for several days compared to what can be achieved with refrigeration alone.

It is important to emphasise that the purpose of the use of the LP-s is not to render milk safer for consumption but to preserve its initial quality. Good hygienic practices in milk production are critical to the efficacy of the LP-s and to the microbiological quality of the milk. The safety of milk is only achieved through a combination of good hygienic practices and heat treatment of milk, independent of the LP-s. This effectiveness of the LP-s under various conditions and against a range of microorganisms is addressed below.

2.1 EFFECTIVENESS OF THE LACTOPEROXIDASE SYSTEM FOR PREVENTING SPOILAGE OF RAW MILK

a) Effectiveness under conditions as specified in the Codex guidelines

The Codex guidelines focus on the application of the LP-s for preventing spoilage of raw milk (bovine and buffalo) during collection and transportation to a dairy processing plant, under conditions where adequate refrigeration is not feasible. The guideline is based on a number of scientific papers from the late 1970s elucidating the working principles of the method and providing proof of concept (Björck, Claesson and Schulthess, 1979; Reiter et al., 1976; Björck, 1978).

Since the adoption of the Codex guidelines, a substantial amount of data on the effectiveness of the LP-s has accumulated, not only from laboratory and field studies, but also from experience with the large-scale adoption of the system in commercial milk production in some countries. During the meeting, summary reports showing results from many

countries, for example Cuba, Colombia, Peru, Venezuela, Cameroon, Kenya, Uganda and Pakistan, covering a wide range of different production conditions, were presented and have been reviewed (Björck, Claesson and Schulthess, 1979; Bibi and Bachmann, 1990; Ponce et al., 2005; Albujar, Ludena and Castillo, 2004; Siirtola, 2005; Fonteh, Grandison and Lewis, 2005). Overall, these data confirm the effectiveness of the LP-s for preventing spoilage of non-refrigerated raw milk within the framework defined in the Codex guidelines, i.e.:

- The principles of good hygienic practice in milk production must be respected in order to guarantee a good initial microbiological quality of the raw milk (see below)

- The inhibitory effect of the treatment is dependent on the storage temperature of LP-s treated milk as follows (Table 1):

Table 1: Extension of milk keeping quality by the LP-s at different temperatures

Temperature (°C)	Time (hours)	Reference
31-35	4-7	Ponce et al., 2005
30	7-8	CAC,1991b
25	11-12	CAC,1991b
20	16-17	CAC,1991b
15	24-26	CAC,1991b
4	5-6 days	Zapico et al., 1995; Lin and Chow, 2000

It should be emphasised that these spoilage delay times should be considered indicative, because they are affected to a great extent by the initial bacterial load (see below).

b) Effectiveness under different ambient conditions

The temperature dependence of the effectiveness of the LP-s as shown above, and as already specified in the original CAC guidelines (CAC, 1991b), illustrates that with respect to prevention of spoilage of raw milk, the LP-s can be complementary to refrigeration. In other words, it can compensate for a lack of refrigeration whenever the latter cannot be supplied. However, the efficacy of the LP-s persists for a limited period of time, which decreases as the ambient temperature increases. This temperature dependence of the effectiveness of the LP-s was defined only in a range between 15 and 30°C in the original Codex guidelines. However, milk storage temperatures may exceed 30°C during daytime, and may fall below 15°C during night-time in some regions without refrigeration facilities. Therefore, the effectiveness of the LP-s at temperatures outside this range is a relevant issue.

Temperature is one of the most important factors influencing microbial growth. The role of refrigeration and the cold chain in maintaining the quality and safety of both raw and pasteurized milk is well recognised. Many bacteria are mesophilic, growing best at

temperatures of 30°C to 40°C. However, psychrotrophic and psychrophilic bacteria can grow at low temperatures, with some strains capable of surviving and growing at temperatures down to 0°C. *Listeria monocytogenes* is an example of a pathogenic bacterium that can grow at very low temperatures. However, in products such as milk that have a diverse microflora, it would normally be outgrown by the psychrotrophic spoilage bacteria, such as members of the genera, *Pseudomonas, Bacillus* and *Micrococcus.*

Some recent field studies that have been carried out with raw milk treated by the LP-s and stored at 30–35°C showed a consistent inhibition of microbial growth for 4–7 hours (Ponce *et al.*, 2005).

Effectiveness of the LP-s may also be relevant to microbial quality and safety issues in relation to extended storage of raw milk under refrigerated conditions. Current issues of concern with regard to low temperature storage include the formation of heat stable proteases by psychrotrophic *Pseudomonas* spp. and the outgrowth of psychrotrophic pathogens such as *Listeria monocytogenes* and some *Bacillus cereus* spp.. At this end of the temperature scale, several studies indicate that activation of the LP-s can delay growth of psychrotrophic milk bacteria and thus delay milk spoilage for several days compared to what can be achieved with refrigeration alone. For example, studies in Taiwan indicated a six-day extension of the spoilage-free storage period of raw milk at 4°C upon activation of the LP-s (Lin and Chow, 2000). Another study showed that the LP-s prevented the growth of psychrotrophic *Pseudomonas fluorescens* for five days at 4°C and for three days at 8°C (Zapico *et al.*, 1995). A summary table comparing LP-s, refrigeration and the combination of LP-s with refrigeration is included as Appendix C.

c) Effectiveness in milk of different species (bovine, buffalo, sheep, goat, camel)

The lactoperoxidase enzyme is present in the milk of all mammals. Although there are variations at the species and even at the individual animal level (Fonteh, Grandison and Lewis, 2002), the enzyme levels in the milks that are used for human consumption are not believed to be a limiting factor for the effectiveness of the LP-s. In general, the available studies show that the time/temperature combination as outlined for cow and buffalo milk are also applicable to goat and sheep milk. In camel milk, the activation of the LP-s may induce a longer-lasting bacteriostatic effect than in cow's milk due to the presence of higher levels of other indigenous antimicrobial components (Ramet, 2001). Less information is available for milk from other species.

d) Effectiveness in relation to principles of hygienic milk production

The Codex guidelines state that, *"Due to the mainly bacteriostatic effect of the system it is not possible to disguise poor quality milk, which originally contained a high bacterial population, by applying this method", and, "The use of the LP-s does not exclude the necessity of pasteurization of milk before human consumption. Neither does it exclude*

the normal precautions and handling routines applied to ensure a high hygienic standard of the raw milk" (CAC, 1991b).

Microbiological studies conducted over the last 10 to 15 years support this view. Invariably, the antibacterial efficacy of the LP-s is found to be inversely correlated to bacterial cell density. The antibacterial efficacy of the LP-s is low at high bacterial concentrations, primarily bacteriostatic at intermediate concentrations and primarily bactericidal at low concentrations. This follows from both laboratory observations with pure cultures of pathogenic or spoilage bacteria suspended in buffer or broth (El-Shenawy, Garcia and Marth, 1990; Garcia-Graells *et al.*, 2003), and from field studies in milk with its natural mixed microflora (Ponce, 2005; Albujar, Ludena and Castillo, 2005). Consequently, safeguarding a high bacteriological milk quality before application of the system by adopting good hygienic practices is critical to its efficacy. In this respect, the use of the LP-s for preserving the quality of the milk before pasteurization does not differ from use of refrigeration for the same purpose. It is important to emphasise that the purpose of both methods is to prevent (microbiological) deterioration of the milk after milking and before pasteurization, not to render the milk safer for consumption, which is achieved by subsequent pasteurization of milk.

2.2 EFFECTIVENESS OF THE LACTOPEROXIDASE SYSTEM AGAINST PATHOGENIC MICROORGANISMS

The antimicrobial activity of the LP-s in milk, whey and synthetic media has been demonstrated against a wide range of microorganisms, including bacteria, HIV-1 virus, moulds, yeasts, mycoplasma and protozoa (for reviews see Korhonen, 1980; Reiter and Härnulv, 1984; IDF, 1991; Wolfson and Sumner, 1993; Stadhouders and Beumer, 1994; de Wit and van Hooijdonk, 1996; van Hooijdonk, Kussendrager and Steijns, 2000; Seifu, Buys and Donkin, 2005). These microorganisms cover non-pathogenic starter cultures and spoilage bacteria as well as pathogenic organisms that cause gastrointestinal infections in humans and udder infections in cows. However, considerable differences have been found in the sensitivity of different bacteria to the LP-s. Depending on the bacterial species or even the strain of the organism, the effect can be either bactericidal or bacteriostatic even under identical conditions. The LP-s has been found to be less effective against some non-pathogenic streptococci and lactococci.

The variations in sensitivity between strains may be explained by different cell wall structures and inhibitory compounds generated by the organisms concerned. Lactic acid bacteria, for example, are deficient in the catalase enzyme, and many species metabolically produce H_2O_2, which is accumulated in the growth medium. This H_2O_2 can activate the LP-s and lead to the self-inhibition of bacterial growth. Many dairy cultures are sensitive to the LP-s, and while some reports indicate interference with the fermentation processes (Wright and Tramer, 1958; De Valdez, Bibi and Bachmann, 1988; Seifu, Buys and Donkin, 2003), the impact is not consistent. This issue is also addressed in section 4.4. Most Gram-negative bacteria possess the catalase enzyme, which decomposes any

generated H_2O_2. These bacteria, therefore, are not self-inhibited in milk through the LP-s and, to activate the system, H_2O_2 has to be supplied from an exogenous source, e.g. by the addition of sodium percarbonate. Under such conditions Gram-negative pathogenic and spoilage bacteria can be killed or their growth arrested for a certain period of time (Reiter *et al.,* 1976; Sandholm *et al.*, 1988; Dionysius, Grieve and Vos, 1992).

A number of studies on the impact of the LP-s on some of the most common milk-borne pathogens and other microorganisms causing infections in humans and domestic animals have been undertaken. Some of those on common milk-borne pathogens, namely *Escherichia coli, Salmonella spp., Campylobacter spp., Staphylococcus aureus, Listeria monocytogenes, Yersinia enterocolitica* and *Brucella melitensis* are summarised in Table 2. In various experimental studies, the bacteriostatic or bactericidal effect of the LP-s has been demonstrated against several other human pathogenic microorganisms, such as *Streptococcus mutans* (Carlsson, Iwami and Yamada, 1983), *Aeromonas hydrophila* (Santos *et al.*, 1995), *Candida albicans* (Lenander-Lumikari, 1992) and *Helicobacter pylori* (Shin *et al.,* 2002). Also, the LP-s has been shown to inhibit the reverse transcriptase enzymatic activity of HIV-1 virus (Wang, Ye and Ng, 2000). Furthermore, a recent study by Armenteros *et al.*, (2005) has shown that the activation of the LP-s in raw milk does not exacerbate the presence of human pathogens including *E. coli* O157: H7, *L. monocytogenes, S. aureus and* S. Typhimurium when introduced into raw milk under laboratory conditions.

The LP-s is considered as one of the body's natural defence mechanisms against microbial infections. Increased concentrations of lactoperoxidase and thiocyanate ions are found in milk from mastitic cows as compared to milk from healthy animals. In general, the same applies to other major antimicrobial factors occurring in milk, e.g. immunoglobulins, lactoferrin, lysozyme and phagocytic cells (Korhonen *et al.*, 1977; Reiter, 1978; Reiter, 1985; Reiter and Perraudin, 1991; Korhonen, 2002). The LP-s has been shown to be bactericidal or bacteriostatic in vitro against many microorganisms that cause udder infections, e.g. *E. coli, S. aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis and Pseudomonas aeruginosa* (Mickelson, 1966; Reiter *et al.,* 1976; Marshall, Cole and Bramley, 1986; Sandholm *et al.*, 1988). Many of these bacteria also pose a potential risk to human health. There is some experimental data to show that the LP-s in mastitic milk is not as effective as in milk from healthy cows because of a higher concentration of reductive agents and higher catalase enzyme activity present in mastitic milk (Sandholm *et al.,* 1988). No studies have so far been reported on the antibacterial activity of the LP-s against antibiotic-resistant mastitis organisms or coagulase-negative staphylococci. These organisms are frequently isolated from mastitic udders.

Table 2: Summary of studies on the impact of the LP-s on some common milk-borne pathogens

Pathogen	Effect of LP-s	Demonstrated in	Reference
Escherichia coli, including *E. coli* O157:H7	Bactericidal	Raw cow milk, buffer solution and synthetic medium Infected calves and piglets	Reiter *et al.*, 1976; Reiter, Marshall and Philips, 1980; Earnshaw *et al.*, 1990; Farrag, El-Gazzar and Marth, 1992a; Grieve, Dionysius and Vos, 1992; Zapico *et al.*, 1995; Kangumba, Venter and Coetzer, 1997; Heuvelink *et al.*, 1998; Bosch, van Doormen and De Vries, 2000; Seifu, Dunkin and Buys, 2004
	Reduced gastrointestinal colonization rate of coliform bacteria Bacteriostatic	Raw cow, goat and camel milk, culture medium and infant formula	
Salmonella Typhimurium	Bactericidal and bacteriostatic (dependent on number of organisms)	Raw milk	Reiter *et al.*, 1976; Purdy *et al.*, 1983; Earnshaw *et al.*, 1990; Pitt, Harden and Hull, 2000
Salmonella typhi, other *Salmonella* spp.	Bactericidal	Culture medium, infant formula and fresh cheese	
Campylobacter jejuni (various strains)	Bactericidal	Cow Milk	Borch *et al.*, 1989; Beumer *et al.*, 1985
Staphylococcus aureus (several strains)	Bactericidal and bacteriostatic	Cow, goat and camel milk	Kamau, Doores and Pruitt, 1990; El-Agamy *et al.*, 1992; Kangumba, Venter and Coetzer, 1997; Pitt, Harden and Hull, 2000; Seifu, Donkin and Buys, 2004
Listeria monocytogenes (several strains)	Bactericidal and bacteriostatic (activity depending on temperature, length of incubation and strain)	Raw cow and goat milk, UHT milk, soft cheese and in synthetic medium	Dennis and Ramet, 1989; Siragusa and Johnson, 1989; Bibi and Bachmann, 1990; El-Shenawy, Garcia and Marth, 1990; Gaya, Medina and Nuñez, 1991; Zapico *et al.*, 1993; Pitt, Harden and Hull, 1999; Seifu, Donkin and Buys, 2004; Gay and Amgar, 2005
Yersinia enterocolitica	Bactericidal	Cow milk	Beumer *et al.*, 1985; Farrag, El-Gazzar and Marth, 1992b
Brucella melitensis	Bactericidal	Goat milk	Seifu, Donkin and Buys, 2004;

2.3 POSSIBLE CONSEQUENCES OF THE LONG-TERM USE OF THE LACTOPEROXIDASE SYSTEM ON ITS ANTIMICROBIAL EFFICACY

The issue of whether long-term use of the LP-s would result in any microbiological risks, e.g. development of LP-s resistant, antibiotic-resistant or toxin-producing bacteria was considered.

Some studies show that the efficacy of the LP-s could be interfered with by residues in milk of certain antibiotics used in the treatment of mastitis (Ali-Vehmas, Vikerpuur and Sandholm, 1994). Mutants of *Escherichia coli* with increased tolerance to the LP-s have recently been isolated in the laboratory and characterised (De Spiegeleer *et al.,* 2005). For one category of such mutants (*waaQ* and *waaO*), LP-s tolerance was linked to a deficiency in the outer core polysaccharide of the lipopolysaccharides, which causes a reduced permeability of the outer membrane for the hypothiocyanate anion (OSCN-) due to a reduced porin content in the outer membrane. This type of mutation also causes a slightly elevated resistance to some penicillins (Nikaido, 2003). However, LP-s tolerant mutants have never been isolated from LP-s treated milk, which may be due to a reduced fitness under these conditions. For example, the *waaQ* mutation mentioned above causes a so-called rough phenotype, which is also associated with enhanced sensitivity to lactoferrin and lysozyme, two other important antimicrobial factors in milk. Thus, the available data indicate that adoption of the LP-s is not likely to stimulate the development of resistance to the LP-s itself or antibiotic-resistant microorganisms. However, as with all antimicrobial systems and due the ability of microorganisms to adapt the meeting considered that ongoing monitoring and research in this area is warranted.

2.4 CONCLUSIONS AND RECOMMENDATIONS

The LP-s elicits antimicrobial activity against a wide variety of milk spoilage and pathogenic microorganisms including bacteria, viruses, moulds, yeasts, mycoplasma and protozoa. The overall activity is primarily bacteriostatic[3], depending on the initial total bacterial load, species and strains of contaminating bacteria and the temperature of milk. While its effectiveness against well-known milk spoilage and pathogenic microorganisms is well established, further studies would be useful on the efficacy of the LP-s against milk-borne viruses and emerging pathogenic microorganisms.

The activated LP-s is effective in raw milk of different species and available studies also indicate that the same time-temperature as outlined in the Codex guidelines (CAC, 1991b) can be applied to goat and sheep milk.

The LP-s does not promote the growth of pathogenic microorganisms after completion

[3] The LP-s is classified as a 'microbiostatic' in the Codex Code of Hygienic Practice for Milk and Milk Products(CAC/RCP/57 – 2004) (CAC, 2004b).

of the bacteriostatic effect and there is no evidence to show that the long-term use of the LP-s would lead to any such microbiological risks, e.g. development or accumulation of toxin-producing bacteria.

Under practical conditions the activated LP-s cannot be used to disguise poor microbiological quality of milk. Good hygienic practices in milk production are critical to the efficacy of the LP-s.

LP-s is effective in refrigerated raw milk. Experimental and field studies have demonstrated that the activated LP-s is effective in prolonging the keeping quality of raw milk both for up to 5–6 days in refrigerated milk (+4°C) and up to 4–7 hours at high ambient temperatures (from 31 to 35°C).

The application of the LP-s is not likely to stimulate the development of resistance to the LP-s itself or other antimicrobial agents but due to the dynamic nature of microorganisms ongoing monitoring of the situation would be reasonable.

Based on the above the meeting recommended that:

- When refrigeration is not technically feasible or economically viable the LP-s be applied to raw milk to halt proliferation of milk spoilage and pathogenic microorganisms.

- The application of the LP-s should be considered as part of a programme to improve milk hygiene and safety along the milk chain, owing to its bacteriostatic effect.

- Consideration be given to the application of the LP-s to complement cooling in order to extend the keeping quality of raw milk.

- Codex consider expanding the guideline for the application of the LP-s with regard to temperature of application to also include the temperature range from 31 to 35°C for 4–7 hours and down to 4°C for 5–6 days.

- Monitoring for the development of resistance be undertaken to detect the development of any resistant microorganisms.

3. Human Health and Nutrition

Milk has an important nutritional role in the diet, particularly for growing children, throughout the world and not just in developing countries. It represents a major source of protein, calcium, phosphorus, magnesium, and fat-soluble vitamins and may make a significant contribution to dietary intakes of some other vitamins and minerals including iodine. Milk can also be a useful vehicle for supplementation of nutrients such as vitamins A and D (WHO, in press). Lactose in milk is involved in regulating osmotic pressure but an additional role in facilitating calcium absorption in infants has been suggested (Abrams, Griffin and Davila, 2002; Garrow, James and Ralph, 2000).

There is a negative correlation between milk consumption and morbidity and mortality from childhood diseases and in this respect the provision of school milk programmes has been effective in improving childhood health and nutritional status (Scrimshaw and San Giovanni, 1997).

While the condition of lactose intolerance may limit the amounts of milk that can be consumed without adverse effects by some individuals/populations, up to one cup of milk (approx. 200ml) is generally tolerated. Furthermore, lactose serves as a substrate in lactic fermented milk products, leading to a reduction in the levels in such products and yeast fermentation results in hydrolysis of lactose by microbial Đ-galactosidase. Considering the important role of milk in human nutrition and health this section addresses the impact of the application of the LP-s for raw milk preservation from a public health and nutrition perspective.

3.1 THE LACTOPEROXIDASE SYSTEM IN CONTEXT

The LP-s differs uniquely from other preservation systems in that it is a natural biological protective system in the biology of animals. It functions as a protective antimicrobial mechanism in mucosal tissue, including in the oral cavity and lung (Tenovuo, 2002; Geiszt *et al.,* 2003). In this regard, the LP-s does not introduce substances into milk that are not normal human metabolites.

The LP-s can be applied to reduce spoilage of milk where refrigeration is not immediately available. However, the use of LP-s is not exclusive and may be combined with other procedures (e.g. refrigeration) to reduce losses of milk both in the formal and informal markets. The safety evaluation of the use of the LP-s in milk by JECFA at its 35th meeting (see below) was restricted by the terms of reference to the application of the system "when refrigeration is virtually impossible", and by the Guidelines drafted by the Joint FAO/WHO Committee of Government Experts on the Code of Principles

concerning Milk and Milk Products. It is recognised that the safety issues concerning the broader application of the LP-s in conjunction with other methods for controlling spoilage, including refrigeration, were not addressed at that time.

3.2 POTENTIAL HEALTH ISSUES ASSOCIATED WITH THE USE OF THE LACTOPEROXIDASE SYSTEM: TOXICOLOGICAL ASPECTS

As noted above, the components or metabolites of the LP-s, namely lactoperoxidase, the thiocyanate ion and hypothiocyanate have been detected in animal and human tissues and secretions, including milk. The levels of hydrogen peroxide introduced into the milk via sodium percarbonate are lower than those previously considered acceptable by the 24th meeting of the JECFA (WHO, 1980) and are, therefore, not of concern.

The use of the LP-s does not require the addition of further lactoperoxidase above the levels of the enzyme occurring in raw milk. As there is no change to the enzyme concentrations naturally present in milk, this component is not considered of toxicological significance.

Hypothiocyanate has been detected in human saliva (Thomas, Bates and Jefferson, 1980) and has a very short half-life in milk, so that residual levels in milk treated with the LP-s do not pose a toxicological risk. The breakdown products are considered innocuous.

In the earlier evaluation, at its 35th meeting in 1990 the JECFA concluded that *"when used according to the draft guidelines, the lactoperoxidase system does not present a toxicological hazard and, furthermore, that the system should be used in preference to hydrogen peroxide alone for the preservation of raw milk, though only where absolutely necessary i.e. in the absence of adequate refrigeration facilities"*. Very few new data on the toxicology of thiocyanate have become available since the previous JECFA evaluation.

The present group examined the potential toxic effect of thiocyanate, which was considered to interfere with iodine metabolism and uptake by the thyroid (WHO, 1990). The mode of action of the goitrogenic effect is via competitive inhibition of iodine and tyrosine oxidation leading to lower levels of thyroxine (T4) and inhibition of uptake by the thyroid. However, this effect occurs at relatively high plasma thiocyanate concentrations (60–80 micromolars or 4.8-6.4 milligram/litre) whereas at lower levels (0.5–1.0 µmolar) there is a stimulatory effect by interacting with thyroid peroxidase (Green, 1978).

At high plasma thiocyanate concentrations there is an increased excretion of iodine and a reduced iodine uptake by the thyroid gland, resulting in a low thiocyanate/iodine (SCN/I) excretion ratio. The value of the threshold level for this ratio seems to be three (Delange and Ahluwalia, 1983) after which endemic goitre appears. This phenomenon can occur only when the iodine intake is below about 100 micrograms per day. At SCN/I ratios of lower than two there is a risk to cognitive function and development (Erman *et*

al., 1983). A low ratio leads to abnormal levels of the thyroid stimulating hormone (TSH) and low thyroxine (T4). Ayangade, Oyelola and Oke (1982) found that in pregnant women the thiocyanate level of the cord blood was proportional to the maternal serum thiocyanate level, indicating that thiocyanate can cross the placental barrier and affect the foetus. However, there is very little thiocyanate in breast milk indicating that the mammary gland does not concentrate thiocyanate and so breast-fed infants are not affected.

In this context, in clinical studies on sodium thiocyanate in milk, negative effects on iodine metabolism were only observed at concentrations of 200–400 milligrams/litre (Vilkki and Piironen, 1962). Furthermore, in studies in normal euthyroid individuals no significant effects on thyroid function (T4, T3, TSH) resulted from consumption of 8 milligrams of thiocyanate in milk daily for 12 weeks (Dahlberg *et al.*, 1984) although serum and urinary levels increased. Conversely, the group with a (presumed) daily consumption of milk containing about 45 milligrams/litre had higher serum levels of T4 and lower T3 and TSH levels than a control group (Banerjee *et al.*, 1997). It should be noted that this last study was published only as a short communication and the level of reporting did not allow the group to conduct a critical evaluation.

From the foregoing it can be concluded that the groups likely to be at highest risk from thiocyanate exposure are iodine-deficient subjects. However, in one study in which iodine-deficient adults were given milk containing 19 milligrams thiocyanate/litre (controls 3.6 milligrams/litre) leading to an additional daily intake of 4.75 milligrams, there was no apparent effect on thyroid function (Dahlberg *et al.*, 1985). The milk used in this study contained iodine at a concentration of 100 micrograms/litre.

There were no experimental data available on the effects of dietary thiocyanate on reproductive function or on the genotoxicity of thiocyanate. Plasma thiocyanate concentrations can reach 100 milligrams/litre during sodium nitroprusside therapy, but toxicity often occurs at concentrations above 120 milligrams/litre. Plasma concentrations in the order of 200 milligrams/litre have been reported in fatalities.

A two-year chronic toxicity/carcinogenicity bioassay of sodium thiocyanate (alone or in combination with sodium nitrite) has been conducted in F344 rats. The animals received sodium thiocyanate at a level of 3.2 grams/litre in drinking water. The results of this study led to the conclusion that sodium thiocyanate is not carcinogenic to rats (Lijinsky and Kovatch, 1989).

The clinical symptoms of overt iodine deficiency during pregnancy as manifested in foetal development and growth of children have been known for more than eighty years. These include stillbirth, abortion and congenital anomalies (Hetzel, 1983; Mastovinovic, 1983). In recent years, research has revealed that iodine deficiencies during pregnancy, even in which overt maternal symptoms are lacking, can have an effect on the growing child, such as hearing deficits (Wang and Yang, 1985).

The normal levels of thiocyanate in milk depend on the levels of thiocyanate and its precursors in the animals' diet, including thioglycosides (glucosinolates) and cyanogenic glycosides. Concentrations have been reported to vary between 2.3 and 35 milligrams/litre in milk from individual cows and to be around 8 milligrams/litre in bulked milk (Ponce *et al.*, 2005). Higher levels occur in colostrum and in mastitis milk. Similar results were obtained for cow milk (6–12 milligrams/litre; mean 8.5 milligrams/litre) and goat milk (6.6–8 milligrams/litre; mean 7 milligrams/litre) (Fonteh, Grandison and Lewis, 2002). When used according to the Codex guidelines, the level of supplementation of sodium thiocyanate in activating the LP-s is 10–15 milligrams/litre so that overall levels in activated bulk milk would be in the order of 20 milligrams/litre, a factor of 10–20 lower than those reported to lead to detected effects on iodine metabolism. A study of the thiocyanate concentrations in milk mixtures under practical conditions of the American tropics indicates that they oscillate between 5.8 and 8.12 milligrams/litre, although the levels in milk of individual cows vary widely, ranging from 2.9 to 34.8 milligrams/litre. That is why the total content of thiocyanate, once the LP-s is activated in a milk mixture, does not surpass the natural maximal concentration in any particular cow milk (Ponce *et al.*, 2005). Evidence of undesirable effects were not observed in the populations consuming milk activated with the LP-s for more than 10 years (Fernandez, Marrero and Capdevila, 2005).

Thiocyanate is found in animal and human tissue and fluids where it is part of the defensive system (e.g. high in colostrums and in milk of cows with mastitis) and is a metabolite of the detoxication process of cyanogenic glycosides. Thiocyanate is also present in foods of plant origin and it is formed in the human or animal body from substances in plants such as glucosinolates (in brassica an average 100 milligrams/kilogram) or cyanogenic glycosides. Thiocyanate is present in raw lima beans (100–3100 milligrams/kilogram), raw cassava tubers (10–462 milligrams/kilogram), raw cassava leaves (68–468 milligrams/kilogram), dried cassava root cortex (2450 milligrams/kilogram), almonds (6.2 milligrams HCN/bitter almond), bamboo shoots tips (8000 milligrams/kilogram), stone fruits and sorghum (2500 milligrams/kilogram) (FAO, 1990). Cyanides readily decompose upon heating, and cooked foods contain little or no cyanide, e.g. cooked cassava tubers had 1-10 milligram/kilogram depending on the cooking method and the initial content. Glucosinolates and glucosinolate breakdown products are hydrophilic, and as much as 63% of the glucosinolate content of a vegetable may leach into the cooking water during boiling (WHO, 1993).

The additional intake of sodium thiocyanate from one cup (200 ml) of LP-s treated milk would correspond to 3 milligrams of sodium thiocyanate which is also present in 30 grams of raw cabbage, 1 gram of raw lima beans or 8 grams of raw cassava tuber. When applying the food supply of the 13 GEMS/Foods regional diets (See Appendix D), exposure to sodium thiocyanate is estimated to be in the range of 2.8 to 9.5 milligrams/day. If all milk were treated with the LP-s the exposure would increase to 5.9 to 21.2 milligrams/day.

The highest potential risk from thiocyanate would arise with infants because of the high need for energy per kilogram bodyweight and the unitary diet. As an example, in a 10 kilogram infant, 500 millilitres of LP-s treated milk would result in 1 milligrams/kilogram body weight of sodium thiocyanate compared to 0.3 milligrams/kilogram body weight from untreated milk. The LD_{50} dose of orally administered sodium thiocyanate in rats, a measure of acute toxicity, is reported to be 764 milligrams/kilogram body weight (FAO/WHO, 1965). Clearly, acute toxicity is not a relevant aspect of exposure through the LP-s treated milk.

In non-smokers, plasma thiocyanate concentrations range from 0.1 to 0.4 milligrams/litre, while in heavy smokers concentrations typically range from 5 to 20 milligrams/litre (WHO, 1995). Thiocyanate is concentrated in other human body fluids, notably saliva and gastric juice, where levels typically range from 10 to 300 milligrams/litre (Björck, Claesson and Schulthess, 1979; Korhonen, 1980; Reiter and Härnulv, 1984; Farrag and Marth, 1992, Food Standards Australia and New Zealand, 2002).

3.3 NUTRITIONAL EFFECTS

The LP-s reduces losses of milk through microbial spoilage and can thus increase the volume of milk available as an important nutritional component of the diet. Although a reduction in folate levels in milk may occur as a result of LP-s treatment, milk is not considered to be a significant dietary source of folate and the overall dietary impact is not considered important.

3.4 EFFECTS ON MILK-BORNE PATHOGENS

Although LP-s may be effective to a limited degree against some pathogens, it should not be considered as an alternative to pasteurization in this regard. The effects on a number of pathogens are dealt with in more detail in section 2. There are no available data on the effects of the LP-s on milk-borne viruses, although some research has been undertaken on the impact of the LP-s on HIV-1 (Wang, Ye and Ng, 2000).

3.5 CONCLUSIONS AND RECOMMENDATIONS

Overall, the meeting considered the LP-s to be a safe method of preventing losses of milk owing to microbial spoilage when used according to the guidelines (and with an extended temperature range as recommended under 2.5) either alone or in combination with other approved procedures.

It was concluded that the advantages of the LP-s mainly result from significantly reduced spoilage losses of milk and thus improved availability of milk as a good nutrient source in the diet and benefiting both milk producers and consumers.

Milk improves health and reduces morbidity and mortality from childhood disease. Therefore, the application of the LP-s could be considered as part of a system to improve public health by increasing the availability and safety of milk.

Based on the available scientific information the meeting concluded that none of the components of the LP-s presents a significant toxicological risk to public health at the levels proposed. Nevertheless, where iodine deficiency is common, public health measures to rectify the iodine deficiency are needed whether or not the LP-s is used.

Based on the assessment, the LP-s is a safe method of raw milk preservation when implemented according to established guidelines (with an extended temperature range as recommended under 2.5); it can reduce milk losses which is a major benefit for both milk producers and consumers.

Based on the above the meeting recommended that:

- The LP-s be considered safe, when used according to the Codex guidelines, for use in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities and that it be applied as part of an integrated programme to improve milk production and quality.

- Milk consumption be promoted because of its value in human nutrition for healthy development and growth.

- Measures to rectify iodine deficiency be implemented in recognised IDD areas accompanied by appropriate monitoring of its prevalence. It was noted that milk could also be a valuable source of iodine, providing there is adequate iodine in the diet of the milk-producing animals.

4. Processing and Technology

Milk is recognised as a highly nutritious food and valuable source of vitamins and minerals. It is, however, highly perishable and has, in its raw state, a relatively short shelf-life. There are numerous processes for prolonging the shelf-life of milk and dairy products and an increasing array of technologies that can be applied to improve the safety and quality of milk.

While refrigeration and heat treatment of raw milk are also highly effective in and widely used for extending shelf-life, more advanced physical treatments are also evolving and being applied such as microfiltration and high pressure processing. The cost of these processes and associated technologies is relatively high as compared to the combination of heating and cooling such as in pasteurization processes (high temperature short time or low temperature long time). Also in many rural areas even the cost of cooling remains prohibitively high. The use of LP-s is not designed to replace adequate heat treatment, which kills harmful bacteria, but has the potential to increase the quality and quantity of raw milk available for further processing into dairy products.

The LP-s is one of the growing families of biostatics that can have beneficial effects in the processing of milk by extending the shelf life and improving the quality of milk collected or preserved. This section reviews the LP-s activation/inactivation and examines potential risks and benefits of the system.

4.1 METHODS OF ACTIVATING THE LACTOPEROXIDASE SYSTEM

Addition of thiocyanate/peroxide

Thiocyanate ions (in the form of sodium or potassium salt) are the substrate for lactoperoxidase and are normally added to milk at a level of approximately 14 milligrams/litre, although this could be adjusted in relation to variation in levels in milk. This is followed by addition of peroxide, either in the form of hydrogen peroxide or sodium percarbonate.

Hydrogen peroxide would be added at a level of 1-10 milligram/litre. This dose is difficult to achieve accurately and could lead to detrimental overdosing. Hydrogen peroxide is unstable and also reacts with proteins, although the latter is unlikely to cause processing problems at this concentration. Therefore sodium percarbonate (30 milligrams/litre) is recommended by Codex as the source of peroxide ions, as it leads to slower release of the active agents.

Activation kits consisting of sachets of thiocyanate and percarbonate can be obtained from a range of companies at a cost of treatment of US$0.0025–0.01 per litre of milk, and are recommended for administration by trained personnel only. It should be noted that the majority of the cost arises from packaging that limits the range of package sizes, especially for small volumes of milk. Most kits are designed for use with 50 litre batches of milk, although kits for treatment of 500 to 10,000 litres are commercially available. The major problems associated with these materials are as follows:

i) thiocyanate is hygroscopic and may deteriorate with time, although this problem may be obviated by the use of coatings or hermetically sealed containers;
ii) some sources of thiocyanate do not comply with accepted quality standards;[4]
iii) percarbonate may produce oxygen leading to 'blown' packets of activator.

Addition of glucose oxidase (1–2 milligrams/litre) to milk, following thiocyanate ions, has been demonstrated on a laboratory scale to activate the LP-s by conversion of glucose to gluconic acid and peroxide. There is usually sufficient glucose present in raw milk as a result of β-galactosidase action, particularly derived from yeasts, although addition of 2–3 grams/litre exogenous glucose is a further possibility. It is an expensive method and dose control at such low levels of addition would be very difficult.

Addition of lactic starter bacteria (catalase negative) could be used in milk for cheese-making in cases where chemical additions were unacceptable. Use of 10^4–10^5 cells/millilitre is effective, for instance in combating psychrotrophic organisms.

Addition of microorganisms (introduced deliberately or inadvertently) such as yeasts or Corynebacteria can activate the LP-s. The use of the latter following surface rinsing with thiocyanate has been shown to be effective in controlling *Listeria* on the surface of soft cheese. Autoinhibition by contaminating microorganisms may contribute to shelf-life extension in pasteurized milk and milk products where significant levels of activity remain following heat treatment (see section 4.2 next page).

Leucocytes may activate LP-s through production of hydrogen peroxide, although their presence is obviously undesirable, reflecting mastitic infection.

Hydrogen peroxide residues from disinfectant solutions following cleaning of milk containers may also activate the system.

[4] Purity criteria of thiocyanate has been specified by the Joint FAO/WHO Expert Committee on Food Additives (available from the JECFA database for food additives HYPERLINK "http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en" http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en)

4.2 THERMAL INACTIVATION OF THE LACTOPEROXIDASE SYSTEM

The kinetics of thermal inactivation of the lactoperoxidase enzyme are well established (e.g. Ramet, 2004; Barrett, Grandison and Lewis, 1999). In practical terms, batch pasteurization (e.g. 65°C/30 minutes) has little effect on enzyme activity, HTST pasteurization (72°C/15 seconds) results in retention of approximately 70% lactoperoxidase while treatment at 80°C or more (including conventional or UHT sterilisation) leads to complete destruction of the enzyme. It has been suggested (Marks, Grandison and Lewis, 2001) that this residual activity explains the fact that milk pasteurized at 72°C has a longer shelf life than milk subjected to 80°C, which has implications in cases where the milk industry may contemplate increasing severity of pasteurization conditions. In fact, it is possible that residual lactoperoxidase plays a role in the keeping quality of pasteurized milk and dairy products generally.

Lactoperoxidase activity could be used as a marker enzyme for effectiveness of HTST heat treatment because of its similarity to phosphatase in terms of thermal inactivation. The official method involves estimation of phosphatase, although this is not useful in camel milk (Ramet, Abeideirrahmane and Ould Mohammed, 2004) where phosphatase remains active following heat treatment at 82–86°C for two minutes. A lactoperoxidase assay would clearly be more appropriate as a marker in the latter case.

4.3 OTHER APPROVED METHODS OF MILK PRESERVATION

The major approved methods of milk preservation are refrigeration and/or heat treatment, although both methods have limitations with respect to processing.

REFRIGERATION

While refrigeration[5] is clearly very effective in inhibiting growth of bacteria, limited negative physical and chemical effects occur which could have small effects on processing parameters. The most important are solubilisation of β-casein, solubilisation of minerals, changes to fat crystallisation and alteration of the balance of bacteria in milk, with an increase in psychrotrophic organisms. Residual proteolytic and lipolytic enzyme activity coming from psychrotrophs following processing gives rise to problems including rancid or bitter off-flavours in products (especially cheese), gelation in UHT milk and gelation in reconstituted calf-feeding powders.

In some countries refrigeration is not feasible at some production sites because of the prohibitive cost (in terms of both initial investment and running costs), but also because of technical problems, such as the absence or unreliability of an electricity supply. The LP-s could be used as a complementary treatment where a power supply is unreliable.

[5] According to the Codex guidelines, milk for further processing should be cooled within two hours to or below 6°C when collected on a daily basis, or to or below 4 °C when not collected every day (CAC, 2004b).

Heat treatment

Obviously heating is the most effective way of destroying microorganisms and is applied to milk in treatments of varying severity (thermisation, pasteurization, sterilisation). Several negative chemical effects occur in products depending on severity of treatment. Whey protein denaturation leads to changes in functionality which can lead to problems owing to reduced syneresis of cheese curd, although high heat treatments are necessary to produce satisfactory yoghurt texture, where syneresis is undesirable. Attachment of β-lactoglobulin to β-casein on the casein micelle surface at high temperatures results in milk with reduced ability to coagulate with clotting enzymes. Hence rennet cheese-making from sterilised milk is not possible. Heating of milk leads to the Maillard reaction (between proteins and reducing sugars) giving rise to browning reactions as a result of melanoidin formation, and also to 'cooked' off-flavours. Heating of milk gives rise to insolubilisation of calcium phosphate (and complexes with proteins) which leads to fouling of processing surfaces, and may require the heated milk to be supplemented with calcium salts before cheesemaking.

It should be noted that heat treatment is more effective if the initial cell counts are minimised before processing, hence application of the LP-s prior to heating provides a complementary, possibly synergistic, combination.

4.4 EFFECTS OF THE LACTOPEROXIDASE SYSTEM ON ORGANOLEPTIC QUALITY OF MILK AND THE MANUFACTURE OF PRODUCTS

It can be surmised that use of the lactoperoxidase system might lead to limited chemical changes to the milk – e.g. through oxidation of fat and proteins. Subsequent physical effects, combined with microbiological changes could lead to negative effects on organoleptic quality of milk and milk products, and the manufacture and texture of some products. However, a report from Ponce *et al.* (2005) indicates that such effects have not been observed in practice.

It has been found that enrichment of raw milk with reagents used for lactoperoxidase activation does not modify sensory properties of the treated milk compared to control milk (Ramet, 2004). The flavour of fermented goats' milk and cheese may actually be improved as a result of the action of the lactoperoxidase changing the balance of microflora (Seifu, Buys and Donkin, 2005).

There is a clear potential for inhibition of lactic starters due to lactoperoxidase activity, resulting in reduced acid production and coagulation problems with acid-gelated products. In addition, interaction of lactoperoxidase with sulphydryl groups of proteins could alter texture of gelled products – e.g. reduction in β-lactoglobulin/β-casein interaction in yoghurt. Evidence for these phenomena is mixed. Evidence from Latin American studies suggests that the lactoperoxidase system has no negative effects on the quality of cheese and fermented products when milk has been subjected to adequate heat

treatment following the use of the LP-s (Ponce *et al*., 2005). Ozer *et al*., (2003) reported some limited effects of LP-s activation on yoghurt gel texture, while Revol-Junelles and Milliere (2005) and Seifu, Buys and Donkin (2005) reviewed the topic and found some evidence of slower rennet clotting and weaker gels in cheese, and lower acid production in yoghurt. However, the effects were generally very limited and reports are not consistent.

The sensitivity of the lactic acid starter bacteria to LP-s action mainly depends on the susceptibility of the specific strains. Susceptibility can be categorised into three groups as follows (Seifu, Buys and Donkin, 2005; Guirguis and Hickey, 1987):

- The most sensitive group of organisms which generate hydrogen peroxide, e.g. *Lactobacillus acidophilus, Lactobacillus bulgaricus;*

- Organisms that are sensitive but do not have the ability to generate hydrogen peroxide and thus require an exogenous source of hydrogen peroxide e.g. *Lactobacillus helveticus, S. thermophilus*;

- Organisms resistant to inhibition e.g. *Lactococcus lactis.*

In summary, it is concluded that any effects of LP-s activation on processing of milk are quite limited. There is no evidence that LP-s activation results in any serious negative effects.

It should be emphasised that using the LP-s to maintain the microbiological status of milk for processing should lead to superior product quality and this has been borne out by some of the FAO field trials on different fermented products (FAO, 2004a).

4.5 OTHER METHODS OF MICROBIOLOGICAL CONTROL

Microfiltration is used in some countries to reduce bacterial populations prior to pasteurization. It is feasible that it could be used as a "stand-alone" technique in the future. The process has the benefit that it is a purely physical treatment based on membrane filtration, which could circumvent many of the disadvantages of heat treatment. A disadvantage is that the diameters of fat globules and microorganisms are similar such that microfiltration is limited to skimmed milk, which can subsequently be remixed with heat treated fat-rich streams, if required. Microfiltration has also been proposed as an alternative solution to the health risks in manufacture of cheese from raw milk. However, it is unlikely that microfiltration will be adopted at present in countries where refrigeration is not routinely carried out because of technical complexities and higher costs.

High-speed centrifugation has been applied to reduce bacterial cell and spore counts in milk prior to hard and semi-hard cheesemaking. Again this is a physical process, but is unlikely to be adopted at present in developing countries because of technical complexities.

High pressure processing (400–800 MPa) has the potential to inactivate microorganisms in milk and alter the protein functionality. This has not been applied commercially.

Addition of lysozyme chlorohydrate (derived from eggs) is a permitted treatment to prevent "blowing" because of outgrowth of clostridium spores during ripening of hard and semi-hard cheeses. However, this is a limited application.

Addition of high levels of sodium chloride (3–12%) reduces water activity (A_w) of milk sufficiently to arrest bacterial growth. The technique is employed in some middle-eastern countries in the traditional manufacture of local brined cheese. Although it is a traditional process, there are many negative effects including very salty taste, micelle disruption, coagulation problems and corrosion of processing equipment. Hence the application is extremely limited.

4.6 IMPACT OF THE ADOPTION OF THE LACTOPEROXIDASE SYSTEM ON THE USE OF NON-APPROVED METHODS OF MILK PRESERVATION

A number of non-approved milk preservation methods are applied in some countries, including:

- Addition of high (300–800 milligrams/litre) levels of hydrogen peroxide, which leads to a direct bactericidal effect, but causes problems in processing because of disruption of proteins, and from a nutritional perspective it reduces the levels of vitamin A and carotenoids.
- Direct addition of antibiotics.
- Addition of ice (from water which may be contaminated), which clearly dilutes the milk.
- Transfer of chemicals from burnt wood containers to the milk.
- Alkalisation with sodium hydroxide or calcium dihydrate.
- Addition of other chemicals, including formalin or chlorine.

It is clear that lack of, or limited effectiveness of quality control procedures in developing countries leads to lack of detection of these non-approved methods. While adoption of

the LP-s has the potential to reduce the use of these non-approved methods, and hence reduce potential risk to consumer health, there is currently little available evidence to illustrate this. However, evidence from extensive studies in Cuba and Latin American countries (Ponce, 2005) suggests that use of LP-s activation has reduced the utilisation of some of the non-approved practices mentioned above.

4.7 CONCLUSIONS AND RECOMMENDATIONS

The meeting concluded based on numerous observations from laboratory and field studies that the LP-s does not induce adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products. Therefore, LP-s is an efficient alternative for preservation of raw milk that will be subjected to further processing. It does not preclude the need for pasteurization and does not negatively impact on, or interfere with, subsequent processing.

The LP-s can be used alone when refrigeration is not available, or in synergy with cooling or chilling and can be considered to be an efficient tool to improve the quality and quantity of milk and dairy products by maintaining the microbiological quality of raw milk.

Considering that the LP-s is technically considered as an effective method of milk preservation for further milk processing the meeting recommended that:

- The LP-s be considered as suitable to extend milk collection distances particularly in developing countries and thereby increase the amount of milk available for further processing and subsequent marketing.

- The LP-s is be used to improve the quality of processed products because of its proven bacteriostatic effect from milk collection to final processing.

5. Economic Value and Trade

In addition to the nutritional benefits of milk and its contribution to household food security, particularly in developing countries, dairying can also provide a major contribution to income generation. This is particularly important in areas where up to 80% of the total milk marketed goes through informal channels.

Refrigeration is the preferred means of milk preservation but requires high capital investment and can incur high running and maintenance costs. Use of LP-s is a reliable and economical method of preserving raw milk as compared to cooling in small-scale dairy enterprises, coupled with good hygiene and sanitation.

There is increasing regional and international trade in milk and dairy products from countries which were, in the past, net milk importers. Regional standards and equivalence are therefore of increased importance, particularly due to regional trade blocks and global trade agreements.

5.1 CURRENT SITUATION

In 2004, the total world milk output was 613 million metric tons of which 263 million metric tons was produced by developing countries – contributing about 30% share of the total world milk production, with small dairy farmers contributing about 70% of the total (NDA, 2004). The small farmer contribution to milk production may be conservative considering their share of the informal market. There was a 10.4% growth in global sales of milk and milk products recorded in 2003 (NDA, 2004). The group noted that one of the contributing factors is the rapid growth of emerging markets such as China, the Philippines and Saudi Arabia.

In 2003, FAO conducted a rapid appraisal of milk post harvest losses in five countries, including the Near East and Eastern Africa (FAO, 2004b). In Kenya, for example, the study found that a total of 15.4% of milk was lost at the farm and market level. The total national loss was estimated at 95 million litres, valued at about US$22.4 million. The losses at farm level are equivalent to US$15.4 million. Viewed against the poverty level where almost 60% of the population survive on less than US$1 a day, the loss at farm level alone is equivalent to the annual salary for 32,000 rural wage earners on US$40 per month (FAO, 2003).

Although milk production costs are low in developing countries, there can be high milk losses where ambient temperatures are high and the milk market chain lacks infrastructure and resources for refrigeration, and where there are problems with electricity

supply. The World Bank estimates that 20% of milk in developing countries is wasted. The opportunity to increase milk production and create additional income to farmers is also constrained by limited capacity for market absorption, lack of facilities to store milk (morning and evening milk) and difficulties to deliver milk on time to processing plants/collection centres.

Milk prices range from US$13 to US$50 per 100 kilograms, with a total cost of production from US$18 to US$28 per 100 kilograms of milk (IFCN, 2002). Due to low input production systems and the exchange rates, cost of milk production and milk prices are lower in developing countries. According to the FAO Dairy Outlook (FAO, 2002) the farm gate prices of milk were highest in Japan and lowest in developing countries such as Kenya, Malawi, Pakistan and Colombia (Table 3).

Table 3. Farm gate prices (cows milk) in US$/kilogram (October, 2002)

Range US$	Country (Price US$ per kilogram)
0.61 – 0.70	Japan (0.62)
0.51 – 0.60	Switzerland (0.53)
0.41 – 0.50	Mauritania (0.42)
0.31 – 0.40	Malta (0.37), Canada/Italy/Mauritius (0.35) France/Ireland/Germany (0.33), Sweden (0.31)
0.21 – 0.30	Costa Rica/Thailand/USA (0.28), Philippines/UK (0.27) Ecuador/Netherlands (0.26), Egypt (0.24), Nepal (0.22)
0.11 – 0.20	Kenya/Malawi (0.20), Pakistan/Colombia (0.18)

Source: *Calculation from FAO Dairy Outlook* (Muriuki, 2002)

Preserving milk using the most practical and economical method while maintaining its initial quality is deemed necessary to increase total milk production and marketing. This is especially relevant to developing countries through the reduction of post harvest losses of milk, promoting afternoon milking collection and the capture of more milk volume from informal markets.

5.2 THE COST OF REFRIGERATION AND THE LACTOPEROXIDASE SYSTEM

When considering the cost effectiveness of the LP-s, it should be borne in mind that it is difficult to compare with other methods applied throughout the world because costs, such as energy, vary widely and have increased significantly in recent years. It is important that such an evaluation be done on a case-by-case basis.

In the Philippines, initial investment in small-scale chilling equipment is between US$3000 and US$5000, and with the on-going cost of electricity it would not be viable to operate such equipment in a cooperative society with a 100 litre per day collection. In 1994, the total cost to cool 100 litres of milk was approximately US$0.5 compared to

US$0.35 if the LP-s is applied. LP-s preservation is cheaper and does not require a large outlay for equipment and cooling facilities (Barraquio *et al.,* 1994).

In Kenya, the cost of cooling a litre of milk ranged from US$0.017 (large scale coolers) to US$0.032 (small scale) while LP-s application was lower at US$0.014 (Wanyoike *et al.,* 2005). However, large scale milk cooling is not a solution to the problem considering the high cost of equipment, from US$197,000 to US$4 million, in addition to maintenance costs and the costs of milk collection.

In Cuba, more than 50% of the milk is not refrigerated due to, among other reasons, the high cost of cooling equipment and lack of electricity. However, the use of the LP-s has allowed significant quantities of milk, valued at US$100 million over 13 years, which would otherwise have been lost, to enter the food chain. The LP-s has proved to be effective in the dairy chain in maintaining the initial quality of the milk from the farm level through to the dairy plant. In Latin America, 30 million litres of milk was activated using the LP-s between 2000 and 2005. Fifty percent of the milk that would otherwise be lost is saved through the LP-s, amounting to a value of around US$3 million. In the Latin American region the cost of cooling a litre of milk can range from US$0.05 to US$0.1 per litre compared to a cost of US$0.0025 to US$0.05 per litre for LP-s application, again without considering the large capital outlay for investing in the cooling equipment and its maintenance.

The cost of using the LP-s compares favourably with that of cooling, particularly for smallholder dairy farmers. It has been shown that the LP-s is more cost effective than cooling in areas where milk quantities are small or there is irregular or no power supply. This is also the best way to improve the flow of milk from the farm to markets thereby creating additional income for dairy households.

5.3 INTERNATIONAL TRADE

Although milk production costs in the developing countries are lower than in developed countries, the developing countries have been net importers of milk and dairy products. However, this is slowly showing signs of change with some development of regional trade, for example among a number of the regional trade blocks in Africa including the East African Community (EAC), Common Market for Eastern and Southern Africa (COMESA), Inter-Governmental Authority on Development (IGAD) and the Southern African Development Community (SADC). Due to increased international trade in countries like Kenya in the EAC and South Africa in the SADC area, there is need for harmonisation in milk and dairy product standards to facilitate trade. Most of these countries have their national standards based on the Codex standards. It is therefore easy to harmonise their standards, although it is important that in the development of Codex standards, regional differences are taken into consideration if the standards are to continue to be of relevance to those countries.

It is difficult to estimate the loss in trading opportunities as a result of the Codex provision that the LP-s should not be used for products intended for international trade. However, the issue is not only related to trade, but also that the LP-s is not adopted in the first place because of a fear of being excluded from international markets. If products treated with the LP-s are not considered suitable for international trade then this raises doubts as to whether it is appropriate and safe to use for milk and dairy products in the domestic market. Despite this, the LP-s is applied in some countries where it is the most practical option for raw milk preservation. Kenya, for example, exports dairy products worth over US$4 million (2003 estimate) to the immediate region, and this is rising. This is the trade that could potentially be lost if they were to officially adopt the use of the LP-s and abide to the condition of not trading the milk treated with the LP-s. The meeting noted that it is likely that similar situations exist in Africa, Latin America and other developing countries.

5.4 DAIRY STANDARDS, POLICY AND THE LACTOPEROXIDASE SYSTEM

The standards developed by the Codex Alimentarius Commission are, under the WTO SPS agreement, the recognised international benchmark standards for food safety. Codex has developed a number of standards for milk and dairy products. These standards inform many of the dairy standards adopted in both developed and developing countries. National governments adopt or modify these standards depending on their national needs and dairy development policy and implementation strategies. It is important that developing world conditions are borne in mind in standard development. This would contribute to the ease with which standards are understood and can be adopted by governments and adapted under prevailing conditions within the national legal framework governing the dairy industry and milk and dairy products.

Smallholder dairy farmers play an important role in the supply of fresh milk and dairy products to growing urban centres in developing countries. To ensure the supply of the quantity of milk needed, dairy development policies need to have a choice of suitable options for milk preservation, which can be adopted by the national milk industry (Muriuki *et al.,* 2003). There are currently only two Codex approved means of preserving raw milk, i.e. refrigeration and the lactoperoxidase system of raw milk preservation. The LP-s is recognised as a cost efficient means of raw milk preservation and can be effective in reducing milk losses and expanding milk collection systems. In addition, it also appears to have significant potential for use with refrigeration as a complementary means of milk preservation. The consideration of the use of the LP-s within a national dairy development policy and strategy is therefore essential to meet the needs of producer groups, milk collectors and processors, particularly in developing and transitional countries where refrigeration is not an immediately feasible and practical option.

5.5 ECONOMIC VALUE AND IMPACT

The World Bank reported that in West Africa approximately 5 million litres of milk is

thrown away annually due to spoilage. Cuba has reported that the use of LP-s system has produced a wide range of benefits over a 13-year period. It has enabled them to get total volumes exceeding 1000 million litres of milk into the market. A conservative estimate indicated that the use of the LP-s has prevented the loss of approximately 50,000 tons of milk, which is equivalent to the annual dairy imports for the country in foreign currency. In addition it has led to the creation of employment and improvement in dairy farmers incomes (P. Ponce, personal communication, 2005).

A functional system of raw milk preservation can stimulate increased milk production to be benefit of both producers and consumers. In a country like Kenya, milk production fluctuates between seasons and, mainly only the morning milk gets into the market chain. During a high production season, there are very high milk losses due to collection logistics, exacerbated by lack of preservation systems. Evening milk is not collected due to a lack of feasible preservation systems. It has been estimated that the total amount of marketed milk would increase by about 30% through collection of evening milk. This would translate to an annual increase of over 100 million litres. An FAO study (FAO, 2005) however estimated a lower level of losses. A conservative estimate by Muriuki (H. Muriuki, personal communication, 2005) is that there would be an increase of 68 million litres of milk from market growth.

Milk markets usually pay a premium for quality milk. In Kenya, the processors pay about US$0.06 per litre for high quality milk over the going standard milk price. An increase in marketed milk, especially from the smallholder sector, would also improve livelihoods through employment, increased incomes and improved nutrition. Other issues that will need to be addressed with an increase in marketed milk include whether this will take milk away from home consumption and whether it will shift incomes from women to men. In some communities, income from milk sold within the immediate neighbourhood is controlled by women and the income from the formal sector is controlled by men.

5.6 AVAILABILITY OF THE LACTOPEROXIDASE SYSTEM COMPONENTS

Most countries with pharmaceutical facilities have the capacity to produce activators as long as they meet the specifications stipulated in the Codex guidelines and account for the purity and hygroscopic nature of percarbonate. Currently, only a few countries produce the LP-s activators, such as Sweden, Cuba and France. It would be expected that the LP-s would be more economical if the activators were made in the countries applying the system. The cost of packaging also needs to be considered given that the package alone constitutes around 40–60% of the total cost of the product.

5.7 CONCLUSIONS AND RECOMMENDATIONS

Economic benefits of dairying include household income generation that can be a major contribution to regular income and household food security and nutrition, particularly for vulnerable groups, e.g. children and women, in developing countries. Small-scale

dairy production, collection, processing and marketing are a major source of off-farm rural employment. Nevertheless, post harvest losses are a major issue in dairying in developing counties. Smallholder dairy farmers could increase their participation in worldwide milk production, processing and marketing if they could reduce their losses using any approved milk preservation method. The potential increase in the quality and shelf life of milk and dairy products may have a considerable social and economic benefit at local level. While refrigeration is the preferred means of milk preservation it does require high capital investment and can incur high running and maintenance costs for expensive equipment. Thus the use of the LP-s provides a reliable and economical alternative for preserving raw milk, particularly in small-scale dairy enterprises when coupled with good hygiene and sanitation. Its economically viablity, either as a standalone system or in combination with refrigeration, and its potential to significantly reduce milk losses and thereby increase the amount of milk collected leads to direct benefits for both milk producers and consumers.

There is increasing regional and international trade in milk and dairy products from countries which were, in the past, major milk importers. With an increasing demand and milk production growth in developing and transitional countries, regional standards are of growing importance coupled with proper hygiene and sanitation practices along the dairy chain. Such standards are often based on Codex standards as these are considered the benchmark standard under WTO for foods in international trade. However, the provision relating to the use of the LP-s makes this somewhat of an exception and is an important limitation to the adoption of the system because of the potential of being shut out of regional and international trade in these products.

Based on these conclusions the meeting recommended that:

- Small-scale dairying be promoted given its contribution to household nutrition, food security, and poverty alleviation.

- Codex Alimentarius develop milk and dairy product standards that can be easily adopted at regional or national level. Active participation of a representative range of country members should be supported in the development of standards.

- The current Codex limitation related to the use of LP-s in milk or dairy products intended for international trade be removed.

6. Overall Conclusions and Recommendations

The meeting sought to take a holistic approach to its review of the LP-s as a system of raw milk preservation taking into consideration the relevant microbiological, human health and nutrition, processing and technology and economic value and trade aspects.

The antimicrobial activity of the LP-s against a wide variety of milk spoilage and pathogenic microorganisms including bacteria, viruses, moulds, yeasts, mycoplasma and protozoa has been well documented in both laboratory and practical settings. The overall activity is primarily bacteriostatic, the extent of which is dependent on the initial total bacterial load, species and strains of contaminating bacteria and the temperature of milk. While its effectiveness against well-known milk spoilage and pathogenic microorganisms is well established, it was concluded that further studies would be useful on the efficacy of the LP-s against milk-borne viruses and emerging pathogenic microorganisms.

The efficacy of the system in raw milk from different species and under different ambient conditions was also considered. The Codex guidelines focus on the application of the LP-s to cow and buffalo milk. However, the meeting concluded that the same time-temperature combination as outlined in the Codex guidelines (CAC, 1991b) can also be applied to goat and sheep milk. The LP-s has also been shown to be effective in camel milk although the presence of other antimicrobials in this milk mean that a different pattern in terms of the level of activity at various temperatures may be observed.

An important consideration of the meeting was the impact of the LP-s on pathogenic microorganisms in milk. Based on the available evidence the meeting concluded that the LP-s does not promote the growth of pathogenic microorganisms after completion of the bacteriostatic effect[6] and there is no evidence to show that the long-term use of the LP-s would lead to any such microbiological risks, e.g. development or accumulation of toxin-producing bacteria. Furthermore, the meeting concluded that the application of the LP-s is not likely to stimulate the development of resistance to the LP-s itself or other antimicrobial agents but due to the dynamic nature of microorganisms ongoing monitoring of the situation would be reasonable.

[6] Under laboratory conditions.

The meeting gave particular consideration to data on the effectiveness of the LP-s at time-temperature combinations outside those outlined in the Codex document. It concluded that the LP-s also has a positive impact on the keeping quality of raw milk at ambient temperatures of 31– 35 °C albeit only for 4 to 7 hours. Nevertheless, this was considered important as it may mean the difference in terms of getting milk to a refrigerated collection point in a good condition particularly in areas of warm or very warm ambient temperatures. The impact of the LP-s at refrigeration temperatures was also considered, especially the ability of the system to minimise the growth of psychrotrophic bacteria. The effectiveness of the LP-s at lower temperatures led the meeting to conclude that the application of the system could be broadened to extend the period of refrigerated storage of raw milk.

The kinetics of thermal inactivation of the lactoperoxidase enzyme are well established and the time and temperature of heat treatment will determine the level of destruction of the lactoperoxidase enzyme. The meeting noted suggestions that residual lactoperoxidase activity plays a role in the keeping quality of pasteurized milk and dairy products generally. With regard to further processing of milk it was noted that while there is the potential for the LP-s to have an impact on the organoleptic quality of milk and the manufacture of products, this has not been observed in practice. Numerous observations from laboratory and field studies indicate that the LP-s does not induce adverse effects on the chemical, physical or sensory characteristics of raw milk and processed dairy products. In considering the potential impact of the LP-s on fermented products it was noted that the data on this issue was somewhat inconsistent, which appears to relate to the difference in susceptibility of the various starter culture strain to the LP-s. Where negative effects have been reported they were limited.

The meeting concluded that the LP-s has a role to play as part of an integrated system to improve milk quality and safety. It was strongly emphasised that the LP-s cannot be used to disguise poor quality milk and that the system is most effective when implemented in conjunction with good hygienic practices. While cooling and heat treatment are well recognised as effective means of milk preservation, and numerous other systems are used on a smaller scale or being developed, the expansion of milk production particularly in developing countries where appropriate infrastructure and equipment for cooling, heat treatment or other physical processes are not always possible, means that it is important that cost effective alternatives are available. The application of naturally occurring preservation systems, of which the LP-s is one, is an area that is currently being widely investigated for application in a range of different foods and at different points in the food chain. Their application is not being considered as a replacement of existing well serving technologies, such as cooling and heat treatment, but to provide complimentary alternatives, particularly at the primary production stage when the other approaches are not available, feasible or suitable.

In this context, the meeting considered that the LP-s provides a real alternative in terms of short-term raw milk preservation. The fact that it can be used without any expensive

infrastructure or equipment makes it a potentially viable option especially for many small rural milk producers. The ability to extend the shelf-life of raw milk, in a regulated way, is critical to ensuring that safe milk is made available for consumers and there is an economic benefit for the small dairy holder. Extension of the shelf-life of raw milk can ensure that it is still in a good condition when it reaches the processing facility despite long distances or poor transport infrastructure under warm or very warm ambient conditions. Milk losses are reduced again benefiting both producer and consumer.

Noting the increasing regional and international trade in milk and dairy products from countries which were, in the past, major milk importers and the increasing demand and milk production growth in developing and transitional countries, the meeting emphasised that the implementation of standards that fulfil obligations under the WTO agreements are of growing importance. Such standards are often based on Codex standards as these are considered the benchmark standard under WTO for foods in international trade. However, the provision relating to the use of the LP-s makes this somewhat of an exception and is an important limitation to the adoption of the system because of the potential of being shut out of regional and international trade in these products.

In this context the health and nutritional aspects of milk, particularly milk that had been subjected to the LP-s was considered. In terms of human health and nutrition it was firstly concluded that the advantages of the LP-s mainly result from significantly reduced spoilage losses of milk and thus improved availability of milk as a good nutrient source in the diet and benefiting both milk producers and consumers. Milk improves health and reduces morbidity and mortality from childhood disease. Therefore, the application of the LP-s could be considered as part of a system to improve public health by increasing the availability and safety of milk. The meeting reviewed the available toxicological data on the LP-s and confirmed the evaluation of the 35th JECFA that the LP-s does not present a toxicological hazard when implemented according to established Codex guidelines. The meeting also noted that very few new data have become available since the JECFA evaluation. Nevertheless, the meeting recognised the significance of iodine deficiency and emphasised that where iodine deficiency is common, public health measures to rectify this situation are needed whether or not the LP-s is used.

Overall the meeting concluded that the LP-s has numerous advantages to offer when used as part of an integrated system to improve milk quality and safety, reduce milk losses and enhance its availability. Based on the available data and an evaluation thereof, the technical meeting considered the LP-s to be a safe method of raw milk preservation. When implemented according to established Codex guidelines the meeting concluded that there is currently no scientific basis for continuing the provision related to the limitation on the international trade of LP-s treated milk and dairy products.

RECOMMENDATIONS

In making its recommendations the meeting reiterated the safety of the lactoperoxidase system of raw milk preservation when used according to the existing guidelines (CAC 13/91), recommending its use in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities. Based on its deliberations the following specific recommendations were made.

To Codex

Consider expanding the guideline for the use of this system with regard to temperature of application of the LP-s to also include the temperature range from 31 °C to 35 °C for 4–7 hours and down to 4 °C for 5–6 days.

Develop milk and dairy product standards that can be easily adopted at regional or national level through the encouragement and support of active participation of a representative range of country members in the development of standards.

Remove the current provision regarding the restriction on the use of LP-s in milk or dairy products intended for international trade as the meeting found no scientific or technical basis or economic justification for the provision.

To member countries, FAO, WHO, Codex, NGOs and the dairy industry

Acknowledge the LP-s as an effective and feasible method of raw milk preservation that does not display a negative impact on the further processing of milk.

Owing to its bacteriostatic effect, give consideration to the application of the LP-s as part of a programme to improve milk hygiene and safety along the milk chain.

Consider the application of the LP-s to complement cooling in order to extend the keeping quality of raw milk and halt proliferation of milk spoilage and pathogenic microorganisms.

Use the LP-s to improve the quality of processed products based on its proven bacteriostatic effect from milk collection to final processing and in particular to extend milk collection distances in developing countries, thereby increasing the amount of milk available for marketing. This can have significant direct benefits for both milk producers and consumers.

Recognise that the use of the LP-s is an economically viable option (either standalone or in combination with refrigeration) to significantly reduce milk losses and increase milk availability.

In addition to those recommendations specific to the use of the LP-s a number of other

related issues were discussed, based on which the technical meeting made the following recommendations.

Promote the consumption of milk as a valuable source of human nutrition contributing to healthy development and growth.

Promote the contribution of small-scale dairying to household nutrition, food security, and poverty alleviation.

Implement measures to rectify iodine deficiency in recognised IDD areas accompanied by appropriate monitoring of its prevalence. Milk can also be a valuable source of iodine, providing there is adequate iodine in the diet of the milk-producing animals.

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Appendix C - Summary table comparing LP-s, refrigeration and the combination of LP-s with refrigeration

	Safety	Microbiological performance	Applicability	Cost/benefit
LP-s	No safety concern for public health when used in accordance with the Codex guidelines.	1. Primarily bacteriostatic for many milk-borne and other human pathogenic microorganisms. 2. Maintains initial milk quality for 4–7 hours (at 30 to 35°C) and up to 24–26 hours at 15°C. 3. Does not improve milk quality. 4. No long-term microbiological resistance expected.	Milk of all species. May interfere with fermentation when milk is not adequately heat treated. No significant adverse effects on the chemical, physical or sensory characteristics of raw milk and dairy products.	1. Low start-up and maintenance costs. 2. No energy requirements. 3. Can be applied in areas where refrigeration is not a viable option. 4. May increase availability of milk and dairy products. 5. Requires appropriate training of personnel for use.
Refrigeration	No safety concern for public health.	1. Primarily bacteriostatic for many milk-borne and other human patho genic microorganisms. 2. Maintains initial milk quality for several days (depending on temp. of refrigeration and initial microbial quality of milk). 3. Does not improve milk quality	Milk of all species. Limited negative physical and chemical effects.	1. Extends keeping time of milk by several days. 2. Nothing added to milk. 3. Requires electricity. 4. Relative high cost for initial investment and maintenance.
Refrigeration with the LP-s	No safety concern for public health when used in accordance with the Codex guidelines.	1. Primarily bacteriostatic for many milk-borne and other human pathogenic microorganisms. 2. Maintains initial milk quality for 5–6 days at 4°C. 3. Does not improve milk quality. 4. No long-term micro biological resistance expected.	Milk of all species.	1. Increases shelf-life of milk and dairy products as compared to refrigeration alone 2. Minimal increase in cost.

Appendix D - Thiocyanate exposure based on the GEMS/Food regional diets both with and without lactoperoxidase treated milk

THIOCYANATE EXPOSURE WITHOUT LP-S USING FOOD SUPPLY OF GEMS/FOOD REGIONAL DIETS IN MILLIGRAMS/YEAR

GEMS/Food Consumption Cluster Diets[7]	A	B	C	D	E	F	G	H	I	J	K	L	M
Brassica vegetables	87.8	1001.5	379.1	1761.9	1267.1	1125.5	1071.2	203.5	492.0	80.4	173.3	2240.2	814.4
Tomato	19.6	370.0	236.0	121.4	63.2	74.4	47.1	63.1	29.9	25.0	71.2	19.9	180.5
Cassava	971.2	0.0	0.1	0.0	0.0	0.0	62.4	96.5	685.3	1128.7	230.9	79.2	2.6
Lima beans, dry	0.0	4.8	4.7	17.4	0.0	0.0	9.5	32.9	0.0	0.0	0.0	15.4	2.9
Milk only	344.2	953.3	396.6	1512.6	898.0	1189.4	330.1	604.0	408.1	511.6	1038.4	285.5	1439.7
Total thiocyanate exposure	1422.9	2329.7	1016.5	3413.4	2228.3	2389.3	1520.3	999.9	1615.3	1745.7	1513.9	2640.2	2440.1
Total thiocyanate exposure (milligrams/day)	**4.0**	**6.5**	**2.8**	**9.5**	**6.2**	**6.6**	**4.2**	**2.8**	**4.5**	**4.8**	**4.2**	**7.3**	**6.8**

THIOCYANATE EXPOSURE ADDING LP-S USING FOOD SUPPLY OF GEMS/FOOD REGIONAL DIETS IN MILLIGRAMS/YEAR[8]

Brassica vegetables	87.8	1001.5	379.1	1761.9	1267.1	1125.5	1071.2	203.5	492.0	80.4	173.3	2240.2	814.4
Tomato	19.6	370.0	236.0	121.4	63.2	74.4	47.1	63.1	29.9	25.0	71.2	19.9	180.5
Cassava	971.2	0.0	0.1	0.0	0.0	0.0	62.4	96.5	685.3	1128.7	230.9	79.2	2.6
Lima beans, dry	0.0	4.8	4.7	17.4	0.0	0.0	9.5	32.9	0.0	0.0	0.0	15.4	2.9
Milk only	1307.9	3622.5	1507.2	5747.9	3412.3	4519.6	1254.3	2295.0	1550.7	1944.0	3945.8	1084.9	5470.7
Total thiocyanate exposure*	2386.6	4998.9	2127.1	7648.7	4742.6	5719.5	2444.5	2691.0	2757.9	3178.2	4421.4	3439.5	6471.2
*incl. 100% LP-s treated milk (milligrams/year)													
Total thiocyanate exposure (milligrams/day)	**6.6**	**13.9**	**5.9**	**21.2**	**13.2**	**15.9**	**6.8**	**7.5**	**7.7**	**8.8**	**12.3**	**9.6**	**18.0**

[7]For complete list of country assignment codes (listed A-M above) see http://www.who.int/foodsafety/chem/gems/en/index1.html

[8]Mean exposure of sodium thiocyanate has been estimated by multiplying the mean consumption of the 13 GEMS/Food regional diets with the mean concentration in selected foods.

Appendix E - Food supply according to GEMS/Food regional[9] diets in kilograms/year

CODE	GEMS	NOTES	A	B	C	D	E	F	G	H	I	J	K	L	M	Sodium thiocyanate or HCN in milligram/ kilogram
VB 40	Brassica vegetables[10]	(14)	2.2	25.0	9.5	44.0	31.7	28.1	26.8	5.1	12.3	2.0	4.3	56.0	20.4	40[11]
VO 448	Tomato[12]	(9)	9.8	185.0	118.0	60.7	31.6	37.2	23.5	31.6	15.0	12.5	35.6	9.9	90.3	2
VR 463	Cassava[13]	(1)	242.8	0.0	0.0	0.0	0.0	0.0	15.6	24.1	171.3	282.2	57.7	19.8	0.7	4[14]
VD 534	Lima bean (dry)[15]		0.0	0.2	0.2	0.7	0.0	0.0	0.4	1.3	0.0	0.0	0.0	0.6	0.1	25[16]
ML 106	Milk[17]	(1)(2)	68.8	190.7	79.3	302.5	179.6	237.9	66.0	120.8	81.6	102.3	207.7	57.1	287.9	5 (19 with LP-s)
A0 31	**Total Milk & Milk Products**		**70.5**	**223.4**	**87.9**	**317.4**	**249.7**	**301.4**	**66.6**	**136.2**	**85.6**	**103.5**	**211.7**	**63.9**	**6.8**	

[9] For complete list of country assignment codes (listed A-M above) see http://www.who.int/foodsafety/chem/gems/en/index1.html

[10] Food Standards Australia and New Zealand, 2002

[11] Cooked (60% leaking into cooking water).

[12] Tonacchera, *et al.*, 2004

[13] WHO, 1993

[14] Cooked (1% of raw)

[15] WHO, 1993

[16] Cooked (1% of raw)

[17] Introduction 1.1 of document CAC/GL 13-1991 (CAC, 1991b)

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Lactoperoxidase is an enzyme that is naturally present in milk. The activation of the lactoperoxidase in the presence of hydrogen peroxide and thiocyanate, both of which are naturally present in milk in varying concentrations has a bacteriostatic effect on raw milk and effectively extends the shelf life of raw milk for 7–8 hours under ambient temperatures of around 30°C or longer at lower temperatures. Such an extension in shelf life particularly under warm ambient conditions can allow adequate time for the milk to be transported from the collection point to a processing centre without refrigeration.

Codex adopted the "Guidelines for the preservation of raw milk by use of the lactoperoxidase system in 1991 to facilitate the application of its use in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities.

However, at that time the Codex Alimentarius Commission also agreed to emphasise that the system should not be used for products intended for international trade. In the intervening years concerns have been raised by numerous countries as to why the system can be applied for products in domestic but not international trade.

In order for Codex to reconsider this issue they requested FAO and WHO to convene a technical meeting to review the most recent scienticic information on the risks and benefits of the lactoperoxidase system.

This report provides the output of that meeting including a summary of the most recent information relating to the use of the lactoperoxidase system for raw milk preservation and the discussions and recommendations of the technical meeting.

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Annex 5

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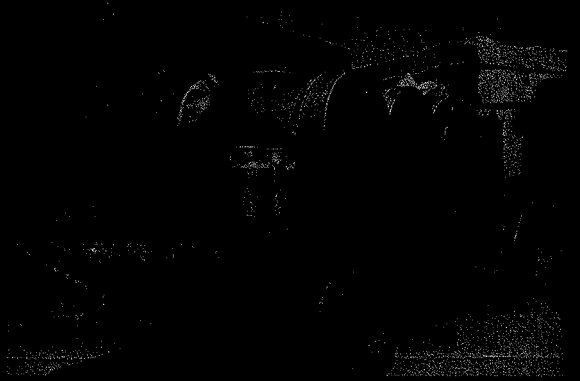

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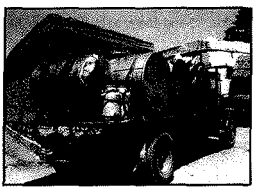
CODE OF PRACTICE FOR THE PRESERVATION OF RAW MILK BY THE LACTOPEROXIDASE SYSTEM

CONTENTS



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PREFACE

At the 21st Session of the FAO/WHO Milk Committee (Committee of Government Experts on the Code of Principles concerning Milk & Milk Products) in Rome (2-6 June 1986), the technical advice of IDF regarding the use of the lactoperoxidase system for the preservation of raw milk was submitted and discussed.

The decision reached at that meeting was to ask IDF to prepare a Code of Practice for raw milk preservation using the lactoperoxidase system. A draft for such a Code of Practice was prepared by Group F19 (Indigenous antibacterial systems in milk) on the initiative of Dr L. Björck of the Swedish University of Agricultural Science.

The first draft (D-Doc 150) was submitted to IDF Commission D at the 70th Annual Sessions of the IDF in The Hague in September 1986, and subsequently amended by the author in the light of the discussion.

Commission D then decided to circulate this amended draft to all NC with the following questions (questionnaire 1787/D):

1. Please give general comments on the Code of Practice.

2. Is it, in your opinion, advisable that the method should be used at individual farms or should it be used only at collection centres as stated in the present text ?

3. Are you aware of any other antibacterial system that can be used for the preservation of raw milk ? Please send details, literature, references, etc.

Replies were received from 19 countries and the replies were considered and analyzed by the Group. In this light, it was concluded that the following amendments should be introduced in the first draft:

1. In Section 1 - Scope

The following sentence should be added: "It should be stressed that this method should only be utilized when refrigeration of the raw milk is not feasible".

2. In Section 3 - Intended utilization of method

In para. 3.6 the following sentence should be added: "Neither does it exclude the normal precautions and handling routines applied to ensure a high hygienic standard of the raw milk".

3. Section V - Control of usage

To be added in this paragraph: "The dairy processing plant should also be responsible for the control of the chemicals to be used at the collection centres for the activation of the lactoperoxidase system".

4. Technical specification of sodium percarbonate - Appendix II

The following footnote should be inserted:

"Information where sodium percarbonate can be obtained commercially can be obtained from the IDF General Secretariat, 41, Square Vergote, B-1040 Brussels, Belgium".

5. Analysis of thiocyanate - Appendix III

The following should be added: "The minimum level of detection by this method is 1 to 2 ppm of SCN^-.

..... should be stored dark and cool (in refrigerator). It is then stable for a minimum of 30 days".

It was also concluded that, regarding the second question, all answers were in favour of the method being used at collection centres as stated in the suggested text.

It was also obvious from the answers that any other antibacterial system to be used for preservation of raw milk is not known.

As a next step, the draft Code was revised and the revised version was submitted by Dr L. Björck as paper D-Doc 157 to Commission D at its next Session in Helsinki (Finland) in September 1987 and the following is taken from the minutes of that meeting:

In reply to Dr J. Nichols (US), who had drawn attention to the difficulties encountered in USA resulting from the formation of histamine in cheese consequent upon protein breakdown mediated by the addition of significant amounts of hydrogen peroxide to the cheese milk, Dr Björck remarked that when the lactoperoxidase system (LPS) was used the very small quantity of hydrogen peroxide added reacted in its entirety with the enzyme (lactoperoxidase) and the proteins were not affected. In reply to Mr I.M.V. Adams (GB), who asked if there was further evidence in relation to the activity of LPS against pathogens, Dr Björck explained that LPS was not intended as a substitute for pasteurization and furthermore there was no evidence that the system favoured the development of pathogens - it had a negative effect on all common pathogenic bacteria occurring in milk, which should be pasteurized.

The Commission approved the Code of Practice appended to D-Doc 157 for publication.

The thus amended Code of Practice is appended. It was also submitted to the FAO/WHO Codex Committee on Food Hygiene (CCFH) meeting in March 1988 and the following is quoted from the minutes of that CCFH meeting:

The following views were presented at the 23rd Session of the Codex Committee on Food Hygiene (ALINORM 89/31, paras. 74-80) in consideration of a Code of Hygienic Practice for Raw Milk Preservation by the Use of the Lactoperoxidase System.

***74.** The Observer from IDF introduced a report, document CX/FH 88/12 (Conference Room Document No. 9), on a Code of Practice for the Preservation of Raw Milk by the Lactoperoxidase System. He informed the Committee that the 21st Session of the Joint FAO/WHO Committee of Government Experts on the Code of Principles Concerning Milk and Milk Products had considered the technical advice IDF had given on the use of the Lactoperoxidase System for the preservation of raw milk. That Committee had requested IDF to prepare the draft of a Code of Practice. The draft document had been amended at the 70th Annual Session of IDF (The Hague, 1986) and subsequently circulated to IDF members for comments. Comments had been received from 19 countries and had been incorporated in a revised text of the draft code of practice for submission to the IDF Session in Helsinki (1987) and to the Milk Committee (IDF Working Paper D-Doc 157/1987).*

***75.** The representative of IDF noted that the additional comments received from Thailand and Denmark had been taken into consideration during the preparation of the present wording of the Code.*

***76.** Several delegations inquired about the implications of elaborating a Code of Practice which might be used to discourage efforts to improve the use of refrigeration of raw milk. The delegation of Cuba expressed the view that, supported by such a Code adopted at the international level, the lactoperoxidase system might be used on a continuous basis for an indefinite period of time and questioned whether that was the intent of the Committee. The delegation of Australia questioned whether the holding of raw milk at ambient temperatures had effects on the chemical composition and*

nutritional qualities of the milk. Concern was also expressed about adequate control of the use of the chemical additives under practical conditions.

77. *The Observer of IDF indicated that IDF had considered nutritional and safety aspects of the lactoperoxidase system especially in regard to the use of other chemicals which may be used to preserve milk. He stated that the final goal for good hygienic practices remained the use of refrigeration. He informed the Committee that the process was being introduced in certain areas of India and China, and that the draft document contained adequate provisions for the control of the chemicals used. The delegation of the United Kingdom stated the lactoperoxidase system operated naturally in raw milk and that the proposed application was an enhancement of the naturally-occurring bacteriostatic action.*

78. *It was proposed that the draft Code should be given only the status of a Guideline, in view of the opinion of several delegations that the procedure should be used only under specified conditions, and that this should also be indicated in the title and in the introductory sections. The Committee agreed that the following alternative title:*

"Draft Guidelines for the Preservation of Raw Milk by Use of the Lactoperoxidase System where Refrigeration is Virtually Impossible"

should be included in the document, and that both titles should be placed in square brackets for further government comments.

79. *Concerning the status of the Code/Guidelines within the Codex Procedures, it was agreed to advance the draft document to Step 3 for government comments and to request the Commission to decide whether further elaboration of the Code/Guidelines should be referred to this Committee in view of the extended period of time between sessions of the Milk Committee. In the meantime, the Secretariat was requested to seek authorization from the Executive for obtaining comments at Step 3. The document distributed for comments would carry references to both the Committee on Food Hygiene and the Milk Committee.*

80. *The Committee expressed its appreciation to IDF for the extensive work carried out in relation to these agenda items.*

The Code/Guidelines was issued by the Codex Alimentarius Commission (CAC) to governments and International Organizations for comments, in August 1988 (CL 1988/22 - FH/MDS) - Comments were invited for 31 March 1989.

The General Secretariat was informed that the lactoperoxidase system was approved by the National Expert Committee on Food Additives in the People's Republic of China as "an acceptable preservative used for milk preservation" in certain localities in China. Experiments on the use of the system appear to being done in several countries: India, Mexico, Fidji. Field trials have been published from Kenya, India, Pakistan, Mexico, Sri Lanka, Egypt, People's Republic of China, Poland.

In IDF, consideration is being given to the development of a standardized method for the determination of lactoperoxidase activity. IDF Group F19, Chairman, Dr L. Björck, is following up on international developments in relation to indigenous antibacterial systems in milk and a copy of the report (F-Doc 96) of the Group, issued in 1983, on "temporary preservation of raw milk by activation of the lactoperoxidase system" is attached, for information.

Finally, attention should be called to the publication in 1985, of the Proceedings of a Symposium on "Antimicrobial Systems in Milk" held in Bath (UK) in September 1985 under the joint auspices of IDF, BSPP and FEMS. A copy of the Proceedings is available from IDF Brussels for £15.-

IDF General Secretariat
November 1988.

CODE OF PRACTICE FOR THE PRESERVATION OF RAW MILK BY USE OF THE LACTOPEROXYDASE SYSTEM

INTRODUCTION

Milk is an easily perishable raw material. Contaminating bacteria may multiply rapidly and render it unsuitable for processing and/or unfit for human consumption. Bacterial growth can be retarded by refrigeration, thereby slowing down the rate of deterioration. Under certain conditions refrigeration may not be feasible due to economical and/or technical reasons. Difficulties in applying refrigeration are specially a problem for certain areas in countries setting up or expanding their milk production. In these situations, it would be beneficial to have access to a method, other than refrigeration, for retarding bacterial growth in raw milk during collection and transportation to the dairy processing plant.

In 1967 the FAO/WHO Expert Panel on Milk Quality concluded that the use of hydrogen peroxide might be an acceptable alternative in the early stages of development of an organized dairy industry, provided that certain conditions were complied with. However, this method has not achieved any general acceptance as it has several drawbacks, most important of which is the difficulty of controlling its use: it may be misused to disguise milk of inferior basic hygienic quality produced under poor hygienic conditions. The toxicological aspects of the use of relatively high concentrations of hydrogen peroxide in milk have also been questioned.

A chemical method for preserving milk would still be of great advantage in certain situations. The search for such a method has therefore continued. Interest has recently been focused on the indigenous antibacterial systems in milk to determine if these could be applied practically to preserve raw milk. During the last decade, basic and applied research has demonstrated that one of these systems, the lactoperoxidase/thiocyanate/hydrogen peroxide system (LP-system) can be used successfully for this purpose.

1. SECTION I – SCOPE

This Code of Practice describes the use of the lactoperoxidase system for preventing bacterial spoilage of raw milk (bovine and buffalo) during collection and transportation to a dairy processing plant. It describes the principles of the method, in what situations it can be used, its practical application and control of the method. It should be stressed that this method should only be utilized when refrigeration of the raw milk is not feasible.

2. SECTION II – PRINCIPLES OF THE METHOD

The lactoperoxidase/thiocyanate/hydrogen peroxide system is an indigenous antibacterial system present in milk and human saliva. The enzyme lactoperoxidase is present in bovine and buffalo milk in relatively high concentrations. It can oxidize thiocyanate ions in the presence of hydrogen peroxide. By this reaction, thiocyanate is converted into hypothiocyanous acid (HOSCN). At the pH of milk HOSCN is dissociated and exists mainly in the form of hypothiocyanate ions ($OSCN^-$). This agent reacts specifically with free sulphydryl groups, thereby inactivating several vital metabolic bacterial enzymes, consequently blocking their metabolism and ability to multiply. As milk proteins contain very few sulphydryl groups and those that are present are relatively inaccessible to $OSCN^-$ (masked), the reaction of this compound is in milk quite specific and is directed against the bacteria present in the milk.

The effect against bacteria is both species and strain dependent. Against a mixed raw milk flora, dominated by mesophilic bacteria, the effect is bacteriostatic (predominantly inhibitory). Against some gram-negative bacteria, i.e. pseudomonads, *Escherichia coli*, the effect is bactericidal. Due to the mainly bacteriostatic effect of the system it is not possible to disguise poor quality milk, which originally contained a high bacterial population, by applying this method.

The antibacterial oxidation products of thiocyanate are not stable at neutral pH. Any surplus of these decomposes spontaneously to thiocyanate. The velocity of this reaction is temperature dependent, i.e. more rapid at higher temperatures. Pasteurization of the milk will ensure a complete removal of any residual concentrations of the active oxidation products.

Oxidation of thiocyanate does not occur to any great extent in milk when it has left the udder. It can, however, be initiated through addition of small concentrations of hydrogen peroxide (see Section IV). The high concentrations of hydrogen peroxide used to preserve milk (300-800 ppm), destroy the enzyme lactoperoxidase and thereby preclude the oxidation of thiocyanate. With this method the antibacterial effect is thus an effect of hydrogen peroxide itself.

The antibacterial effect of the LP-system is, within certain limits, proportional to the thiocyanate concentration in the milk (provided than an equimolar amount of hydrogen peroxide is provided). The level of thiocyanate in milk is related to the feeding of the animals and can thus vary. The practical use of the method consequently requires addition of some thiocyanate to ensure that a level necessary to achieve the desired effect, is present in the milk.

The levels of thiocyanate resulting from this treatment are within the physiological levels reported to occur in milk under certain circumstances and feeding regimes. They are also far below the thiocyanate levels known to exist in human saliva and certain common vegetables, e.g. cabbage and cauliflower. In addition, results from clinical experiments have clearly demonstrated that milk treated according to this method will not cause any interference of the iodine uptake of the thyroid gland, neither in persons with a normal iodine status nor in cases of iodine deficiency.

3. SECTION III – INTENDED UTILISATION OF METHOD

3.1 The method should be used in situations when technical, economical and/or practical reasons do not allow the use of cooling facilities for maintaining the quality of raw milk. Use of the LP-system in areas which currently lack an adequate infrastructure for collection of liquid milk, would ensure the production of milk as a safe and wholesome food, which otherwise would be virtually impossible.

3.2 The method should not be used by the individual farmers but at a suitable collecting point/centre. These centres must be equipped with proper facilities for cleaning and sanitizing the vessels used to hold and transport milk.

3.3 The personnel responsible for the collection of the milk should be in charge for the treatment of the milk. They should be given appropriate training, including training in general milk hygiene, to enable them to fulfill this in a correct way.

3.4 The dairy processing the milk collected by use of the lactoperoxidase system should be made responsible for ensuring that the method is used as intended. This dairy should set up appropriate control methods (see Section V) to monitor usage of the method, raw milk quality and quality of the milk prior to processing.

3.5 The method should primarily be used to prevent undue bacterial multiplication in raw milk during collection and transportation to the dairy processing plant under conditions stated in 3.1. The inhibitory effect of the treatment is dependent on the temperature of the stored milk and has been found to act for the following periods of time in laboratory and field-experiments carried out in different countries with raw milk of an initial good hygienic standard:

Temperature, °C	Time, hrs
30	7 - 8
25	11 - 12
20	16 - 17
15	24 - 26

3.6 The use of the lactoperoxidase method does not exclude the necessity of pasteurization of the milk before human consumption. Neither does it exclude the normal precautions and handling routines applied to ensure a high hygienic standard of the raw milk.

4. SECTION IV – PRACTICAL APPLICATION OF THE METHOD

4.1 The lactoperoxidase system can be activated in raw milk to give the above stated antibacterial effect by an addition of thiocyanate as sodium thiocyanate and hydrogen peroxide in the form of sodium percarbonate by the following procedure:

14 mg of NaSCN is added per litre of milk. The milk should then be mixed to ensure an even distribution of the SCN^-. Plunging for about 1 minute with a clean plunger is normally satisfactory.

Secondly, 30 mg of sodium percarbonate is added per litre of milk. The milk is then stirred for another 2 - 3 minutes to ensure that the sodium percarbonate is completely dissolved and the hydrogen peroxide is evenly distributed in the milk.

4.2 It is essential that the sodium thiocyanate and sodium percarbonate are added in the order stated above. The enzymatic reaction is started in the milk when the hydrogen peroxide (sodium percarbonate) is added. It is completed within about 5 minutes from the addition of H_2O_2; thereafter, no hydrogen peroxide is present in the milk.

4.3 The activation of the lactoperoxidase system should be carried out within 2 - 3 hours from the time of milking.

4.4 Quantities of sodium thiocyanate and sodium percarbonate needed for the treatment of a certain volume of milk, for example 40 or 50 litre milk churns, should be distributed to the collecting centre/point in prepacked amounts lasting for a few weeks at a time. The technical specifications of the thiocyanate and sodium percarbonate which should be used are stated in Appendix I and II.

5. SECTION V – CONTROL OF USAGE

The use of the lactoperoxidase system for preserving raw milk must be controlled by the dairy processing plant receiving the milk. This should be a combination of currently used acceptance tests, e.g. titratable acidity, methylene blue, resazurin, total viable count, and analyses of the thiocyanate concentration in the milk. Since the thiocyanate is not consumed in the reaction, treated milk arriving at the dairy plant would contain approximately 10 mg above the natural amount of thiocyanate (the latter can be determined by analysing untreated milk from the same area) per litre of milk. The analytical method for SCN^- is described in Appendix 3. Testing should be undertaken at random. If the concentration of thiocyanate is too high (or too low), investigation must be carried out to determine why the concentration is outside specification. The dairy processing plant should also be responsible for the control of the chemicals to be used at the collection centre for the activation of the lactoperoxidase system.

Analysis of the bacteriological quality of the milk (methylene blue, resazurin, total plate count) should also be carried out to ensure that good hygienic standards are not neglected. Since the effects of the system are predominantly bacteriostatic, an initial high bacterial population in the milk can still be revealed by such tests.

TECHNICAL SPECIFICATION OF SODIUM THIOCYANATE — APPENDIX I

DEFINITION

Chemical name	Sodium thiocyanate
Chemical formula	NaSCN
Molecular weight	81.1
Assay content	98-99%
Humidity	1-2%

PURITY (according to JECFA* specification)

Heavy metals (as Pb)	< 2 ppm
Sulphates (SO_4)	< 50 ppm
Sulphide (S)	< 10 ppm

* *Joint FAO/WHO Expert Committee on Food Additives*

TECHNICAL SPECIFICATION OF SODIUM PERCARBONATE — APPENDIX II

DEFINITION

Chemical name	Sodium percarbonate (*)
Chemical formula	$2Na_2CO_3.3H_2O_2$
Molecular weight	314.0
Assay content	85%

Commercially available sodium percarbonate recommended to be used has the following specification

Sodium carbonate peroxyhydrate	> 85%
Heavy metals (as Pb)	< 10 ppm
Arsenic (as As)	< 3 ppm

(*) For information where sodium percarbonate could be obtained commercially, please apply to IDF General Secretariat, 41 Square Vergote, B–1040 Brussels, Belgium.

ANALYSIS OF THIOCYANATE IN MILK — APPENDIX III

PRINCIPLE: Thiocyanate can be determined in milk, after deproteinisation with trichloroacetic acid (TCA), as the ferric complex by measuring the absorbance at 460 nm. The minimum level of detection by this method is 1 to 2 ppm of SCN^-.

REAGENT SOLUTIONS:

1. 20% (w/v) trichloroacetic acid: 20 g TCA is dissolved in 100 ml of distilled water and filtered.

2. Ferric nitrate reagent: 16.0 g Fe $(NO_3)_3$. 9 H_2O is dissolved in 50 ml 2 M HNO_3* and then diluted with distilled water to 100 ml. The solution should be stored dark and cold.

3. Determination: 4.0 ml of milk is mixed with 2.0 ml of 20% TCA solution. The mixture is blended well and then allowed to stand for at least 30 minutes. It is thereafter filtered through a suitable filter paper (Whatman no 40). 1.5 ml of the clear filtrate is then mixed with 1.5 ml of the ferric nitrate reagent and the absorbance measured at 460 nm. As a blank, a mixture of 1.5 ml of ferric nitrate solution and 1.5 ml of water is used. The measurement must be carried out within 10 minutes from the addition of the ferric nitrate solution as the colored complex is not stable for any length of time. The concentration of thiocyanate is then determined by comparison with standard solutions of known thiocyanate concentration, e.g. 10, 15, 20 and 30 mg/ml of thiocyanate.

* *2M HNO_3 is obtained by diluting 138.5 ml 65% HNO_3 to 1000 ml with distilled water.*

APPENDIX IV

TEMPORARY PRESERVATION OF RAW MILK BY ACTIVATION OF THE LACTOPEROXIDASE SYSTEM

(Report F-Doc 96 submitted by Group F19 to the IDF Sessions in Oslo, July 1983)

1. INTRODUCTION

In many countries which are in the process of starting up and/or expanding their milk production, the collection and transportation of raw milk to processing centres present many problems. Frequently, it is impossible to refrigerate the milk during collection and transportation, which often take place at high ambient temperatures, with the result that the milk often has an inferior hygienic quality when it arrives at the dairy plant. It is well-known that large quantities of milk are spoiled in this way. Although the long-term objective of these countries is to set up and maintain adequate refrigeration facilities, this will, for both practical and economic reasons, take a number of years to accomplish. In the meantime, an alternative method of preserving raw milk would certainly be of advantage and enable larger intakes of raw milk with better hygienic quality.

In countries with modern dairying, the collection and storage of raw milk is today more or less totally dependent on the supply of electricity for the cooling equipment. Consequently, a widespread power failure will have a dramatic effect on the hygienic quality of raw milk due to insufficient cooling. In such situations, access to an alternative way of preserving the raw milk would be beneficial also to developed countries.

Today, the only alternative method available is the use of hydrogen peroxide as a preservative. The hydrogen peroxide treatment of raw milk was approved by the FAO in 1957 (1): "when technical and/or economic reasons do not allow the adoption of cooling facilities for maintaining the quality of raw milk, hydrogen peroxide may be an acceptable alternative in the early stages of development of an organised dairy industry". The hydrogen peroxide treatment of raw milk is today officially used only in a few countries. The method has not achieved general acceptance since it involves a number of disadvantages, which mainly are due to the high concentrations, i.e. 300-800 ppm, of hydrogen peroxide required to obtain the necessary preservative effect.

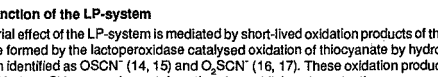
2. THE LACTOPEROXIDASE SYSTEM

In recent years the idea of using an antibacterial system in milk to prevent bacterial spoilage has been put forward (2, 3). The antibacterial system used is the so-called lactoperoxidase / thiocyanate / hydrogen peroxide system (LP-system). This system is not only present in milk (4, 5), but has also been found to be active in saliva (6, 7). In saliva it is believed to be a factor that regulates the bacterial metabolism but serves also as a mechanism that protects the cells of the mucous membrane in the oral cavity against hydrogen peroxide toxicity (8).

In the following only a brief presentation of the basic mechanism of the LP-system and its use as a temporary milk preservative will be given, since several review articles have recently covered this in detail (9, 10, 11).

2.1 Basic function of the LP-system

The antibacterial effect of the LP-system is mediated by short-lived oxidation products of thiocyanate (12, 13). These are formed by the lactoperoxidase catalysed oxidation of thiocyanate by hydrogen peroxide, and have been identified as $OSCN^-$ (14, 15) and O_2SCN^- (16, 17). These oxidation products react rather specifically with free SH-groups in proteins, thereby oxidising them to the corresponding sulfenyl derivatives, which in turn undergo hydrolysis to yield sulfenic acids (18). The over-all reaction can be illustrated by the following reaction:



Functional SH-groups are destroyed in this way by the LP-system, thereby causing an interference with the metabolism of the bacteria. In some bacteria, such as streptococci and lactobacilli, this results in a temporary inhibition, after which the bacteria recover (19, 20). In other bacteria, such as most strains of *Escherichia coli*, Salmonella and Pseudomonads spp, it leads to an irreversible inhibition, i.e. the killing of the bacteria (21, 22).

The antibacterial effect is proportional to the formation of the oxidation products of thiocyanate. This is, in turn, dependent on the available concentrations of thiocyanate and hydrogen peroxide. Lactoperoxidase is always present in milk in non-limiting concentrations.

2.2 Preservation of milk by the LP-system

Activation of the LP-system in raw milk in order to achieve a temporary preservative effect is accomplished by supplementing the thiocyanate level to about 15 ppm, i.e. an addition of about 10 ppm thiocyanate, and an addition of 8 to 9 ppm of hydrogen peroxide. This initiates the enzymatic reaction and the antibacterial agents are formed *in situ*. It is important to note the following:

- the added hydrogen peroxide is consumed within minutes in the enzymatic reaction - it does not persist in the milk
- the active oxidation products of thiocyanate formed are unstable compounds that decompose spontaneously or during pasteurization of the milk.

The length of the antibacterial effect achieved by activation of the LP-system is inversely related to the storage temperature of the milk. The following approximate length of time has been reported (23):

storage temperature °C	duration of effect hours
30	7 - 8
25	11 - 12
20	16 - 17
15	24 - 26

At still lower storage temperatures the effect is prolonged, viz. at 10°C-48 hours; at 5°C-96 hours.

Field experiments in Kenya (23) and Sri Lanka (24) have demonstrated that a substantial improvement of the hygienic quality of the raw milk can be achieved during collection and transportation after an activation of the LP-system at the collecting point or collecting centre. An example of the improvements that can be achieved is shown in Table 1 (24).

Table 1. Analyses of milk samples from Giriulla & Siringapatha collection centres. Cows were milked at 4.6 a.m. and samples were stabilized at 7:30-8 a.m. Ambient (= samples) temperature: 30-32°C.

Quality test	Treatment [1]	Percentage accepted samples at				
		10 a.m.	12 noon	2 p.m.	4 p.m.	6 p.m.
10 min. resazurin	LP	100	100	70	50	30
	C	80	60	10	0	0
Acidity [2]	LP	100	100	80	60	50
	C	70	60	20	0	0
Alcohol stability	LP	100	100	90	60	50
	C	70	60	30	10	10
Clot-on-boiling	LP	100	100	100	100	80
	C	100	100	90	30	30

[1] LP = Samples stabilized by activation of the LP-system; C = Controls
[2] Samples with an acidity > 0.16% recorded as rejected.

3. LEGAL AND TOXICOLOGICAL ASPECTS

To use the LP-system in raw milk as a temporary bacterial preservative, it is necessary to supplement the thiocyanate level in the milk and to add a source of hydrogen peroxide. Although these additions are minute, they interfere with the Code of Principles concerning milk and milk products developed by the FAO and WHO, which states that the term milk means "exclusively the normal mammary secretion obtained from one or more milkings without either addition thereto or extraction therefrom". However, as such a method could be of substantial practical use in certain situations it should be evaluated in an objective way regarding its efficiency and any potential health hazards.

In Sweden, the National Food Administration has evaluated the efficiency of the method and existing toxicological data and has decided to allow the use of LP-activation in milk in situations when raw milk cannot be properly cooled (25).

To evaluate any toxic risks with the utilization of the LP-system as a temporary milk preservative, the toxicity of added hydrogen peroxide and thiocyanate as well as the oxidation products formed, have to be evaluated.

A. Hydrogen peroxide

With the traditional way of using hydrogen peroxide as a milk preservative, i.e. additions of 300-800 ppm of H_2O_2, the hydrogen peroxide persists for long periods in the milk. The milk has therefore to be treated with catalase before processing to ensure that any residual hydrogen peroxide is destroyed. At these levels of hydrogen peroxide the lactoperoxidase is destroyed. The antibacterial effect obtained in this case is thus not related to the LP-system, it is only due to the antibacterial effect of hydrogen peroxide itself. This method has been approved by the FAO/WHO (1) and thus is not considered to constitute any health hazards.

With the LP-method, the small amounts of hydrogen peroxide added to milk are rapidly utilised in the enzymatic oxidation of thiocyanate, whereby it is reduced to water. In this context, it should also be recalled that milk itself contains several enzymes capable of producing hydrogen peroxide, viz. xanthine oxidase and sulphydryl oxidase. It is therefore likely that low levels of hydrogen peroxide are produced in milk although detectable amounts are not normally found since they are continuously reduced by enzymes such as catalase or lactoperoxidase. These facts indicate that the hydrogen peroxide used to activate the LP-system in milk does not imply a health risk.

B. Thiocyanate

Thiocyanate ingested in very high concentrations has an acute toxic effect. The LD_{50} dose of orally administrated sodium thiocyanate in rats is reported to be 764 mg/kg (26). On the other hand, thiocyanate is also considered to be a normal electrolyte in mammalian blood. Human plasma levels are 2-3 ppm in non-smokers and 9-12 ppm in smokers (27). The thiocyanate is largely of exogenous origin, being derived through the ingestion of various glucosinolates such as sinigrin, glucobrassicin and neoglucobrassicin, which release thiocyanate upon hydrolysis. Common sources of these glucosinolates are plants belonging to the cruciferous family, e.g. cauliflower, cabbage and kale. Another important source of thiocyanate is the enzymatic detoxification of cyanide by the enzyme thiosulfate sulfur transferase (E.C. 2.8.1.1) (rhodanase). This enzyme is present in most mammalian tissues although the highest concentrations are found in liver, kidney, adrenals, thyroid and pancreas (28). Cyanide intake in man is essentially due to ingestion of cyanogenetic glucosides such as amygdalin and limarin, which are present in bitter almonds, linseed and cassava. In smokers, tobacco smoke is an important source of cyanide (29).

Thiocyanate is secreted by the mammary and salivary glands and by the gastric mucosa. The levels of thiocyanate found in milk are fairly variable. Levels of 10 to 15 ppm have been reported (30, 31) but normally the concentrations are in the range between 2-7 ppm (32). Saliva is rich in thiocyanate, levels between 50-300 ppm having been reported (33).

Although thiocyanate is a normal electrolyte in many secretions, it is well-established that high serum levels result in disturbances in the thyroid function (hypothyroidism) (34). It has also been suggested that thiocyanate in milk may be a factor causing goiter. Finnish investigations (35), on the other hand, have demonstrated that doses between 200 to 400 mg were necessary to give a thyrostatic effect. More recent studies of the pharmaco-kinetics of nitro-prusside, which is used in hypertension treatment, have clearly demonstrated that serum levels above 18-20 ppm of thiocyanate are necessary to cause impaired thyroid function (36).

These investigations indicate that the levels of thiocyanate utilised in LP-activated milk would not cause any disturbances of thyroid function. However, to confirm this, a clinical experiment was carried out in Sweden during 1982 in collaboration between the National Food Administration, the University of Uppsala (Department of Medicine) and the Swedish University of Agricultural Sciences, Uppsala. The results of this experiment have been submitted to the American Journal of Clinical Nutrition for publication (Dahlberg, P.A. et al., Intake of thiocyanate by way of milk and its possible effect on the thyroid function). In this trial, 43 persons were given 0.4 l milk containing 20 ppm thiocyanate per day during 3 months. The increased intake of thiocyanate was reflected in increased serum concentrations. The highest values were obtained after 4 weeks (7.8 mg/l in non-smokers; 10.7 mg/l in smokers). Thereafter the concentrations decreased and at the end of the experimental period were (7.0 mg/l and 8.9 mg/l in the non-smoking and smoking group, respectively. No apparent effect was observed on the thyroid function, i.e. no significant changes were found in the serum levels of thyroxine-4, triiodothyronine and thyrothropic hormone (TSH). These results are thus in agreement with other investigations and indicate that the levels of thiocyanate used in LP-activated milk will not cause any disturbances in thyroid function.

C. Oxidation products of thiocyanate

The antibacterial effect of the LP-system is mediated by short-lived oxidation products of thiocyanate. These intermediates are very unstable and those not reacting with bacteria decompose spontaneously or in connection with pasteurization of the milk. When the milk reaches the consumer, none of the active agents will thus be present in the milk. On the other hand, it has also to be considered whether there is any potential risk if the milk is consumed shortly after an inactivation of the LP-system and residual levels of the oxidation products of thiocyanate still are present and ingested via the milk. Any toxic risks of the intermediate oxidation products present in freshly activated LP-activated milk have to be considered as low. These oxidation products normally are present in human saliva (37). Recent studies in which certain mammalian cell types, viz. Hela cells, fibroblasts and chinese hamster ovary cells, were exposed to the complete LP-system (utilizing concentrations up to 50 ppm of thiocyanate and an equivalent concentration of hydrogen peroxide) also failed to reveal any adverse effects (J. Carlsson, personal communication). Consumption of freshly LP-activated milk would therefore not present any additional risks apart from those generally connected with consumption of unpasteurized milk.

4. EFFECTS ON THE PROCESSING PROPERTIES AND NUTRITIVE VALUE OF MILK

In contrast to the general oxidative effect of hydrogen peroxide, which is known to affect adversely the processing properties of milk, the antibacterial effect of the LP-system is much more specific. The active agents of the system react primarily with free SH-groups in proteins. Milk proteins, however, contain few free SH-groups, implying that there is little risk of negative effects.

So far the rennet coagulation and acid production of starter cultures in LP-activated pasteurized milk have been investigated (2). Negative effects could not be found in this investigation. Regarding growth of starter cultures in LP-activated milk it should, however, be pointed out that if the starter strains used produce hydrogen peroxide, as do some strains of streptococci and lactobacilli, they may show an impaired growth rate in LP-activated milk, as the hydrogen peroxide they produce completes the LP-system. Nevertheless, this can easily be overcome by using starter strains that produce no or little hydrogen peroxide.
Although effects of the LP-activation on the vitamins in milk are not to be expected, this aspect has yet to be investigated.

5. PRACTICAL USE OF THE LP-ACTIVATION UNDER FIELD CONDITIONS

Although the optimal way of applying the LP-activation has to be decided from case to case, the following principles are recommended. The raw milk should not be treated by the individual farmers but at a collecting centre or a similar place. The main reason for this is that if the activation is carried out on the farm, the dosage of the activators will be complicated as widely variable volumes of milk are to be treated. Neither should it be overlooked that usage on the farms will be difficult to control and the risks of misuse thereby enhanced. If the treatment, on the other hand, is carried out at a collecting centre, correct dosage of the activating substances wil be easier as the milk is handled in churns of a certain volume, i.e. the activators can be distributed to give the correct concentration for this volume. At a collecting centre it will also be easier to supervise the correct use of the method. Another important factor that indicates that the activation should take place at a collecting centre, is that most of the deterioration of the raw milk often occurs during the transport from the collecting centre to the dairy plant.

The practical use of the LP-activation must also always be combined with proper control measurements to ensure that the method is correctly used and that the basic hygiene is not neglected. The correct dosage of the activators can be controlled by analysing the thiocyanate concentration of the delivered milk. This can be achieved by a simple colorimetric test. If the thiocyanate level is found to be substantially higher than 15 ppm, overdosage is likely to have occurred. If the ordinary basic hygienic precautions are neglected, it will automatically be revealed in the usual quality control carried out on the delivered milk. The LP-activation causes only an inhibition of most of the raw milk flora. Consequently, if the milk had high bacterial numbers at the time of LP-activation, high bacterial numbers will also be present at delivery to the dairy plant and will be reflected in a dye reduction test or plate count.

6. CONCLUDING REMARKS

The use of the LP-system as a temporary preservative for raw milk represents a new approach to the old problem of finding a suitable temporary preservative for milk when adequate cooling is not available.

The basic biochemical mechanism of this antibacterial system, which not only has been investigated in connection with milk but also by oral bacteriologists (the system is also present in saliva) is by now fairly well documented.

Field experiments carried out in Kenya and Sri Lanka have clearly demonstrated that a substantial improvement of the hygienic quality of raw milk can be achieved by using the method. To activate the system it is necessary to supplement milk with about 10 ppm of thiocyanate followed by an addition of 8 to 9 ppm hydrogen peroxide. Any toxicological risks of these additions have to be most carefully evaluated. The main concern is the effect that the enhanced thiocyanate levels may have in milk. Clinical experiments with LP-activated milk for 3 months have indicated that the levels of thiocyanate used in LP-activated milk do not interfere with the thyroid function or give any other clinical effects. The subjects in this trial were healthy individuals with a normal iodine status. The effect of an increased thiocyanate intake should, however, also be investigated in subjects with a low iodine status. Such an experiment has recently been carried out in Sudan. The results from this trial are expected to be available in July 1983.

The use of the LP-system as a temporary milk preservative seems to offer several advantages over the use of high concentrations of hydrogen peroxide:

- the LP-system has a much more specific antibacterial effect than the general oxidative effect of hydrogen peroxide. This will minimize the risks of any negative effects on the processing and nutritive properties of the milk;
- the effect of the LP-system on the bacterial flora in raw milk is largely of a bacteriostatic nature. Therefore, it is not possible to "improve" the hygienic quality of an initially low quality milk by use of the LP-system. This is a most important aspect as good basic hygiene is thus a prerequisite for successful use of the LP-system;
- the residual thiocyanate makes it possible to monitor the usage of the method; overdosage, etc. can easily be revealed by excessively high thiocyanate levels in the milk;
- the practical application of the method is simple as no liquid substances are required.

An alternative method to refrigeration as a way of preserving raw milk would be beneficial in many countries where adequate cooling facilities cannot always be provided, either for economic or practical reasons. Although the long-term goal in these countries is to establish complete cooling facilities throughout the milk handling system, it must be realised that this will take a considerable time to accomplish. Until then, there is an urgent need for an alternative method of preserving raw milk, primarily during collection and transportation to processing centres. If such a method becomes available, it will be possible to collect milk from more remote areas without risking an inferior hygienic quality upon arrival at the dairy plant. In turn, this would not only increase the intake of milk for the dairy industry, but also lead to a more rapid development for many small-scale dairy farmers.

The use of the LP-system as an alternative method seems to offer several advantages over the use of concentrated hydrogen peroxide. It is therefore the view of Group F19 that IDF should encourage further research and development in this area and that the FAO/WHO should be recommended to evaluate the use of the LP-system as a temporary preservative for milk when adequate cooling facilities are not available.

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Annex 6

Exponent®

Center for Chemical Regulation and Food Safety

D R A F T

Estimated Daily Intake of Thiocyanate

D R A F T
Estimated Daily Intake of Thiocyanate

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March 25, 2016

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List of Acronyms

DHHS	U.S. Department of Health and Human Services
EDI	Estimated Daily Intake
FARE®	Foods and Residues Evaluation Program
FDA	U.S. Food and Drug Administration
FNDDS	Food and Nutrient Database for Dietary Studies
FPED	Food Patterns Equivalents Database
NCHS	National Center for Health Statistics
NHANES	National Health and Nutrition Examination Survey
WWEIA	What We Eat In America
U.S.	United States
USDA	U.S. Department of Agriculture

Introduction

At the request of Morgan, Lewis & Bockius LLP (Morgan, Lewis & Bockius), Exponent, Inc. (Exponent) conducted an intake assessment to estimate the total daily intake of thiocyanate proposed for use in the following five milk-based foods and beverages: fresh cheese including mozzarella and cottage cheeses, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. The estimated daily intake (EDI) of thiocyanate was based on food consumption data from the 2009-2012 National Health and Examination Survey (NHANES) and provided for the total U.S. population. The data and methods used to conduct the intake assessment and results are summarized in this report.

Data and Methods

Proposed Use and Levels

Thiocyanate is proposed for use in the following five milk-based food and beverage categories: fresh cheeses (including mozzarella and cottage cheese), frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. Table 1 lists the proposed food use categories and their corresponding thiocyanate concentration that is naturally occurring, proposed for use in food, and the total maximum thiocyanate levels in proposed foods which accounts for both the naturally occurring thiocyanate levels in food plus the proposed use levels. The data on thiocyanate levels in the proposed food categories were provided by Morgan, Lewis & Bockius.

Table 1. Proposed Food Uses and Levels[1]

	Thiocyanate (mg/kg)		
Food Category	**Naturally Occurring**	**Proposed Use**	**Total Maximum (Naturally Occurring + Proposed Use)**
Fresh cheese			
Mozzarella	15	0	15
Cottage cheese	15	15	30
Frozen dairy desserts	3	1.5	4.5
Fermented milk	15	15	30
Flavored milk drinks	15	15	30
Yogurt	30	15	45

[1] Data provided by Morgan, Lewis & Bockius

Consumption Data

Thiocyanate intakes from proposed uses in food were derived using the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES) 2009-2012. This continuous survey is a complex multistage probability sample designed to be representative of the civilian U.S. population (NCHS 2014, 2013). The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the U.S. To produce reliable statistics, NHANES over-samples adults 60 years of age and older, African Americans and Hispanics. Statistical

weights are provided by the National Center for Health Statistics (NCHS) for the surveys to adjust for the differential probabilities of selection. As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24-hour time period (midnight to midnight). A second dietary recall is administered by telephone three to ten days after the first dietary interview, but not on the same day of the week as the first interview. The dietary component of the survey is conducted as a partnership between the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). DHHS is responsible for the sample design and data collection, and USDA is responsible for the survey's dietary data collection methodology, maintenance of the databases used to code and process the data, and data review and processing. A total of 16,011 individuals in the survey period 2009-2012 provided two complete days of dietary recalls.

Analysis

Using the WWEIA NHANES consumption data, Exponent estimated the 2-day average daily intake on a *per capita* and *per user* basis. In this analysis, a *user* is anyone who reported consuming any of the proposed foods on either of the survey days. We identify each participant who reported consuming a proposed food on either of the survey days, and we use that individual's responses for both survey days. Zero consumption days are included in calculating that individual's average daily intake. For example, if someone reported consuming 100 grams (g) of yogurt on day 1 and 225 g of yogurt on day 2, his/her 2-day average yogurt consumption would be 162.5 g ([100 + 225]/2). The analysis was limited to individuals who provided two complete and reliable dietary recalls as determined by NCHS. The 2-day average intakes by each individual were estimated using Exponent's Foods and Residues Evaluation Program (FARE® version 11.14) software. Exponent uses the statistically weighted values from the survey in its analyses. The statistical weights compensate for variable probabilities of selection, adjusted for non-response, and provide intake estimates that are representative of the U.S. population.

In the analysis, the 2-day average intake of thiocyanate was estimated by multiplying the reported intake of foods from the 24-hr recall with the proposed corresponding thiocyanate use level (see Table 1) and the cumulative sum over the two 24-hr recalls was divided by two. This was then repeated using the maximum levels of thiocyanate (i.e., naturally occurring level plus proposed use level). Intake estimates of thiocyanate were derived from all proposed uses combined for the total U.S. population and expressed in units of milligram per day (mg/day).

Consumption data in the NHANES survey are reported on an "as consumed basis". That is, if a survey participant consumed a roast beef sandwich, the consumption amount reported in the survey for that subject would be for the total amount of the whole sandwich consumed, and not for the ingredients (bread, meat, lettuce, tomato, and mayonnaise) used to make that sandwich. Exponent identified foods reported consumed in NHANES with proposed uses of thiocyanate (see Table 1). The list of NHANES codes (and their description) that was captured in determining the foods with thiocyanate proposed uses are provided in Appendix I. Baby foods were excluded from the analysis.

When only a component of a food consumed was proposed for thiocyanate use, Exponent utilized USDA's Food and Nutrient Database for Dietary Studies (FNDDS), version 2011-2012 (USDA, 2014), which translates the food as consumed into its corresponding ingredients (and gram amounts) or recipes. For example, USDA recipes were used to identify the cottage cheese component in gelatin desserts mixtures and the yogurt component in a gyro sandwich and curry meat dishes. Thus, for foods containing an ingredient that is proposed for thiocyanate use, only the proportion corresponding to that ingredient was captured in the analysis.

In several cases, the USDA recipes did not have a complete breakdown of ingredients and an alternate approach was taken to identify food components with thiocyanate proposed use. A summary of the alternate approaches taken are presented below:

- <u>Mozzarella:</u>
    - Pizza: The amount of mozzarella cheese per 100 grams of food in pizzas was determined based on the Food Patterns Equivalents Database (FPED) 2011-2012

and 2009-2010 (Bowman et al. 2015, 2014). The FPED converts WWEIA foods to their respective number of cup equivalents of various food groups including dairy (including cheese). The cup equivalents of cheese per pizza food were converted to grams of cheese per 100 gram food for pizza codes that did not have a full recipe breakdown in the USDA recipes. Additionally, it was conservatively assumed that the cheese in the pizza was entirely mozzarella.

  - Turnovers: The amount of mozzarella cheese per 100 grams of food in cheese-filled turnovers was based on the average mozzarella amount per 100 grams of turnovers with complete USDA recipe breakdowns. It was conservatively assumed that the cheese in the cheese-filled turnovers was entirely mozzarella.
  - Lasagna: The amount of mozzarella cheese per 100 grams of food in lasagna foods was based on similar lasagna foods and USDA recipes that indicated the amount of mozzarella was approximately 7 grams per 100 grams food.

- Fermented milk: The amount of fermented milk per 100 grams of food in several buttermilk biscuits which had incomplete USDA recipe breakdowns was assumed to be 35% of the biscuit. This percentage was based on a similar food that had a recipe breakdown.
- Yogurt: As was done in determining the mozzarella amount in pizza, the amount of yogurt per 100 grams of food in coated snacks (i.e., bars, pretzel), candy not containing chocolate, and margarine products was also based on FPED 2011-2012 and 2009-2010.

Results

Two-day average thiocyanate intake estimates from the proposed use in five food categories were calculated based on food consumption data collected in NHANES 2009-2012. Both the *per capita* and *per user* mean and 90th percentile results for the total U.S. population in mg/day are provided in Table 2 from all proposed foods combined based on proposed use levels and total maximum levels (i.e., naturally occurring + proposed use levels).

Table 2. Two-day average estimated daily intake (EDI) of thiocyanate (mg/day) based on proposed and total maximum levels in milk-based foods by the total U.S. population; NHANES 2009-2012

	Total U.S. Population					
			Per Capita (mg/day)		Per User (mg/day)	
Thiocyanate EDI based on[1]	Un-wtd N[2]	% User	Mean	90th Percentile	Mean	90th Percentile
Proposed use levels	7,576	49	0.59	2.00	1.20	3.33
Total maximum levels (i.e., naturally occurring +proposed use)	10,208	67	1.63	5.52	2.44	7.24

[1] Thiocyanate use levels provided in Table 1.
[2] Unweighted number of users; % user, *per capita* and *per user* estimates for NHANES derived using the statistical weights provided by the NCHS.
Note: Baby foods were excluded from the analysis.

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Appendix I: Foods Included In Analysis

Food Category	Food code	Food description
Mozzarella	14010000	Cheese, NFS*
	14100100	Cheese, natural, NFS*
	14107010	Cheese, Mozzarella, NFS
	14107020	Cheese, Mozzarella, whole milk
	14107030	Cheese, Mozzarella, part skim
	14107040	Cheese, Mozzarella, reduced sodium
	14107060	Cheese, Mozzarella, nonfat or fat free
	14620300	Topping from cheese pizza*
	14620310	Topping from vegetable pizza*
	14620320	Topping from meat pizza*
	14620330	Topping from meat and vegetable pizza*
	14660200	Cheese, nuggets or pieces, breaded, baked, or fried*
	27135110	Veal parmigiana*
	27146300	Chicken or turkey parmigiana*
	27460510	Antipasto with ham, fish, cheese, vegetables*
	27510700	Meatball and spaghetti sauce submarine sandwich*
	28113110	Salisbury steak, baked, with tomato sauce, vegetable (diet frozen meal)*
	28140730	Chicken patty, breaded, with tomato sauce and cheese, fettuccine alfredo, vegetable (frozen meal)*
	58106200	Pizza, cheese, prepared from frozen, thin crust*
	58106205	Pizza, cheese, prepared from frozen, thick crust*
	58106210	Pizza, cheese, from restaurant or fast food, NS as to type of crust*
	58106220	Pizza, cheese, from restaurant or fast food, thin crust*
	58106225	Pizza, cheese, from restaurant or fast food, regular crust*
	58106230	Pizza, cheese, from restaurant or fast food, thick crust*
	58106233	Pizza, cheese, stuffed crust*
	58106235	Pizza, cheese, from school lunch, thin crust*
	58106236	Pizza, cheese, from school lunch, thick crust*
	58106250	Pizza, extra cheese, thin crust*
	58106255	Pizza, extra cheese, regular crust*
	58106260	Pizza, extra cheese, thick crust*
	58106300	Pizza, cheese, with vegetables, prepared from frozen, thin crust*
	58106305	Pizza, cheese with vegetables, prepared from frozen, thick crust*
	58106310	Pizza, cheese, with vegetables, NS as to type of crust*
	58106320	Pizza, cheese, with vegetables, thin crust*
	58106325	Pizza, cheese, with vegetables, regular crust*
	58106330	Pizza, cheese, with vegetables, thick crust*
	58106345	Pizza with cheese and extra vegetables, thin crust*
	58106347	Pizza with cheese and extra vegetables, regular crust*
	58106350	Pizza with cheese and extra vegetables, thick crust*
	58106358	Pizza, cheese, with fruit, thin crust*

58106359	Pizza, cheese, with fruit, regular crust* -
58106360	Pizza, cheese, with fruit, thick crust* -
58106411	Pizza with chicken, thin crust* -
58106412	Pizza with chicken, regular crust* -
58106413	Pizza with chicken, thick crust* -
58106441	Pizza with chicken and vegetables, thin crust* -
58106442	Pizza with chicken and vegetables, regular crust* -
58106443	Pizza with chicken and vegetables, thick crust* -
58106462	Pizza with chicken and fruit, regular crust* -
58106500	Pizza with meat, prepared from frozen, thin crust* -
58106505	Pizza with meat, prepared from frozen, thick crust* -
58106540	Pizza with pepperoni, from restaurant or fast food, NS as to type of crust* -
58106550	Pizza with pepperoni, from restaurant or fast food, thin crust* -
58106555	Pizza with pepperoni, from restaurant or fast food, regular crust* -
58106560	Pizza with pepperoni, from restaurant or fast food, thick crust* -
58106565	Pizza with pepperoni, stuffed crust* -
58106570	Pizza with pepperoni, from school lunch, thin crust* -
58106580	Pizza with pepperoni, from school lunch, thick crust* -
58106610	Pizza with meat other than pepperoni, from restaurant or fast food, NS as to type of crust* -
58106620	Pizza with meat other than pepperoni, from restaurant or fast food, thin crust* -
58106625	Pizza with meat other than pepperoni, from restaurant or fast food, regular crust* -
58106630	Pizza with meat other than pepperoni, from restaurant or fast food, thick crust* -
58106633	Pizza, with meat other than pepperoni, stuffed crust* -
58106635	Pizza, with meat other than pepperoni, from school lunch, thin crust* -
58106636	Pizza, with meat other than pepperoni, from school lunch, thick crust* -
58106640	Pizza with extra meat, NS as to type of crust* -
58106650	Pizza with extra meat, thin crust* -
58106655	Pizza with extra meat, regular crust* -
58106660	Pizza with extra meat, thick crust* -
58106700	Pizza with meat and vegetables, prepared from frozen, thin crust* -
58106705	Pizza with meat and vegetables, prepared from frozen, thick crust* -
58106710	Pizza with meat and vegetables, NS as to type of crust* -
58106720	Pizza with meat and vegetables, thin crust* -
58106725	Pizza with meat and vegetables, regular crust* -
58106730	Pizza with meat and vegetables, thick crust* -
58106733	Pizza with extra meat and extra vegetables, prepared from frozen, thin crust* -
58106734	Pizza with extra meat and extra vegetables, prepared from frozen, thick crust* -
58106735	Pizza with extra meat and extra vegetables, NS as to type of crust* -
58106736	Pizza with extra meat and extra vegetables, thin crust* -
58106737	Pizza with extra meat and extra vegetables, thick crust* -
58106738	Pizza with extra meat and extra vegetables, regular crust* -

	58106750	Pizza with meat and fruit, thin crust*
	58106755	Pizza with meat and fruit, regular crust*
	58106760	Pizza with meat and fruit, thick crust*
	58106820	Pizza with beans and vegetables, thin crust*
	58106910	Pizza with seafood, thin crust*
	58106915	Pizza with seafood, regular crust*
	58107220	White pizza, thin crust*
	58107225	White pizza, regular crust*
	58107230	White pizza, thick crust*
	58108000	Calzone, with cheese, meatless*
	58108010	Calzone, with meat and cheese*
	58108050	Pizza rolls*
	58126130	Turnover, meat- and cheese-filled, no gravy*
	58126150	Turnover, meat- and cheese-filled, tomato-based sauce*
	58126160	Turnover, cheese-filled, tomato-based sauce*
	58126290	Turnover, meat- and cheese-filled, lower in fat*
	58126300	Turnover, meat- and cheese-filled, tomato-based sauce, lower in fat*
	58126400	Turnover, filled with egg, meat and cheese*
	58130011	Lasagna with meat*
	58130013	Lasagna with meat, canned*
	58130020	Lasagna with meat and spinach*
	58130140	Lasagna with chicken or turkey*
	58130150	Lasagna, with chicken or turkey, and spinach*
	58130310	Lasagna, meatless*
	58130320	Lasagna, meatless, with vegetables*
	58133110	Manicotti, cheese-filled, no sauce*
	58133120	Manicotti, cheese-filled, with tomato sauce, meatless*
	58133130	Manicotti, cheese-filled, with meat sauce*
	58134110	Stuffed shells, cheese-filled, no sauce*
	58134120	Stuffed shells, cheese-filled, with tomato sauce, meatless*
	58134130	Stuffed shells, cheese-filled, with meat sauce*
	58134160	Stuffed shells, cheese- and spinach- filled, no sauce*
	58301020	Lasagna with cheese and sauce (diet frozen meal)*
	58301030	Veal lasagna (diet frozen meal)*
	58301110	Vegetable lasagna (frozen meal)*
	58301150	Zucchini lasagna (diet frozen meal)*
	58302050	Beef and noodles with meat sauce and cheese (diet frozen meal)*
	58304200	Ravioli, cheese-filled, with tomato sauce (diet frozen meal)*
	75412060	Eggplant parmesan casserole, regular*
	75412070	Eggplant with cheese and tomato sauce*
Cottage cheese	14200100	Cheese, cottage, NFS
	14201010	Cheese, cottage, creamed, large or small curd
	14201200	Cottage cheese, farmer's
	14202010	Cheese, cottage, with fruit
	14202020	Cheese, cottage, with vegetables
	14203010	Cheese, cottage, dry curd
	14203020	Cheese, cottage, salted, dry curd

	14204010	Cheese, cottage, lowfat (1-2% fat)
	14204020	Cheese, cottage, lowfat, with fruit*
	14610200	Cheese, cottage cheese, with gelatin dessert*
	14610210	Cheese, cottage cheese, with gelatin dessert and fruit*
	27212050	Beef and macaroni with cheese sauce (mixture)*
	28110660	Meatballs, Swedish, in gravy, with noodles (diet frozen meal)*
	53400200	Blintz, cheese-filled*
	53400300	Blintz, fruit-filled*
	53511500	Danish pastry, with cheese, fat free, cholesterol free*
	58122320	Knish, cheese (pastry filled with cheese)*
	58301080	Lasagna with cheese and meat sauce, reduced fat and sodium (diet frozen meal)*
	72125310	Palak Paneer or Saag Paneer (Indian)*
Frozen dairy desserts	11459990	Yogurt, frozen, NS as to flavor, NS as to type of milk
	11460000	Yogurt, frozen, flavors other than chocolate, NS as to type of milk
	11460100	Yogurt, frozen, chocolate, NS as to type of milk
	11460160	Yogurt, frozen, chocolate, lowfat milk
	11460170	Yogurt, frozen, flavors other than chocolate, lowfat milk
	11460190	Yogurt, frozen, NS as to flavor, nonfat milk
	11460250	Yogurt, frozen, flavors other than chocolate, with sorbet or sorbet-coated
	11460300	Yogurt, frozen, flavors other than chocolate, nonfat milk
	11460400	Yogurt, frozen, chocolate, nonfat milk, with low-calorie sweetener
	11460410	Yogurt, frozen, flavors other than chocolate, nonfat milk, with low-calorie sweetener
	11460430	Yogurt, frozen, chocolate, whole milk
	11460440	Yogurt, frozen, flavors other than chocolate, whole milk
	11461260	Yogurt, frozen, cone, flavors other than chocolate
	11461270	Yogurt, frozen, cone, flavors other than chocolate, lowfat milk
	11461280	Yogurt, frozen, cone, chocolate, lowfat milk
	11541110	Milk shake, homemade or fountain-type, chocolate*
	11541120	Milk shake, homemade or fountain-type, flavors other than chocolate*
	11541400	Milk shake with malt*
	11541500	Milk shake, made with skim milk, chocolate*
	11541510	Milk shake, made with skim milk, flavors other than chocolate*
	11542100	Carry-out milk shake, chocolate*
	11542200	Carry-out milk shake, flavors other than chocolate*
	13110000	Ice cream, NFS
	13110100	Ice cream, regular, flavors other than chocolate
	13110110	Ice cream, regular, chocolate
	13110120	Ice cream, rich, flavors other than chocolate
	13110130	Ice cream, rich, chocolate
	13110140	Ice cream, rich, NS as to flavor
	13110200	Ice cream, soft serve, flavors other than chocolate
	13110210	Ice cream, soft serve, chocolate
	13110220	Ice cream, soft serve, NS as to flavor

13110310	Ice cream, no sugar added, NS as to flavor
13110320	Ice cream, no sugar added, flavors other than chocolate
13110330	Ice cream, no sugar added, chocolate
13120050	Ice cream bar or stick, not chocolate covered or cake covered
13120100	Ice cream bar or stick, chocolate covered
13120110	Ice cream bar or stick, chocolate or caramel covered, with nuts
13120120	Ice cream bar or stick, rich chocolate ice cream, thick chocolate covering
13120121	Ice cream bar or stick, rich ice cream, thick chocolate covering
13120130	Ice cream bar or stick, rich ice cream, chocolate covered, with nuts
13120140	Ice cream bar or stick, chocolate ice cream, chocolate covered
13120300	Ice cream bar, cake covered
13120310	Ice cream bar, stick or nugget, with crunch coating
13120400	Ice cream bar or stick with fruit
13120500	Ice cream sandwich
13120550	Ice cream cookie sandwich
13120700	Ice cream cone with nuts, flavors other than chocolate
13120710	Ice cream cone, chocolate covered, with nuts, flavors other than chocolate
13120720	Ice cream cone, chocolate covered or dipped, flavors other than chocolate
13120730	Ice cream cone, no topping, flavors other than chocolate
13120750	Ice cream cone with nuts, chocolate ice cream
13120760	Ice cream cone, chocolate covered or dipped, chocolate ice cream
13120770	Ice cream cone, no topping, chocolate ice cream
13120780	Ice cream cone, chocolate covered, with nuts, chocolate ice cream
13120790	Ice cream sundae cone
13120800	Ice cream soda, flavors other than chocolate*
13120810	Ice cream soda, chocolate*
13121000	Ice cream sundae, NS as to topping, with whipped cream
13121100	Ice cream sundae, fruit topping, with whipped cream
13121300	Ice cream sundae, chocolate or fudge topping, with whipped cream
13122100	Ice cream pie, no crust
13130300	Light ice cream, flavors other than chocolate (formerly ice milk)
13130310	Light ice cream, chocolate (formerly ice milk)
13130330	Light ice cream, no sugar added, flavors other than chocolate
13130340	Light ice cream, no sugar added, chocolate
13130600	Light ice cream, soft serve, flavors other than chocolate (formerly ice milk)
13130610	Light ice cream, soft serve, chocolate (formerly ice milk)
13130620	Light ice cream, soft serve cone, flavors other than chocolate (formerly ice milk)
13130630	Light ice cream, soft serve cone, chocolate (formerly ice milk)
13130700	Light ice cream, soft serve, blended with candy or cookies
13135000	Ice cream sandwich, made with light ice cream, flavors other than chocolate
13135010	Ice cream sandwich, made with light chocolate ice cream
13136000	Ice cream sandwich, made with light, no sugar added ice cream
13140100	Light ice cream, bar or stick, chocolate-coated (formerly ice milk)
13140110	Light ice cream, bar or stick, chocolate covered, with nuts (formerly ice milk)

	13140450	Light ice cream, cone, NFS (formerly ice milk)
	13140500	Light ice cream, cone, flavors other than chocolate (formerly ice milk)
	13140550	Light ice cream, cone, chocolate (formerly ice milk)
	13140580	Light ice cream, no sugar added, cone, chocolate
	13140660	Light ice cream, sundae, soft serve, chocolate or fudge topping (without whipped cream) (formerly ice milk)
	13140680	Light ice cream, sundae, soft serve, not fruit or chocolate topping (without whipped cream) (formerly ice milk)
	13140700	Light ice cream, creamsicle or dreamsicle (formerly ice milk)
	13140900	Light ice cream, fudgesicle (formerly ice milk)
	13142000	Milk dessert bar or stick, frozen, with coconut
	13150000	Sherbet, all flavors
	13160150	Fat free ice cream, no sugar added, chocolate
	13160160	Fat free ice cream, no sugar added, flavors other than chocolate
	13160400	Fat free ice cream, flavors other than chocolate
	13160410	Fat free ice cream, chocolate
	13161000	Milk dessert bar, frozen, made from lowfat milk
	13161500	Milk dessert sandwich bar, frozen, made from lowfat milk
	13161520	Milk dessert sandwich bar, frozen, with low-calorie sweetener, made from lowfat milk
	13161600	Milk dessert bar, frozen, made from lowfat milk and low calorie sweetener
	13161630	Light ice cream, bar or stick, with low-calorie sweetener, chocolate-coated (formerly ice milk)
	13170000	Baked Alaska*
	53112000	Cake, ice cream and cake roll, chocolate*
	53112100	Cake, ice cream and cake roll, not chocolate*
	53366000	Pie, yogurt, frozen
	91611050	Ice pop filled with ice cream, all flavor varieties
	92510730	Fruit punch, made with soda, fruit juice, and sherbet or ice cream*
Fermented milk	11112130	Milk, cow's, fluid, acidophilus, 2% fat
	11115000	Buttermilk, fluid, nonfat
	11115100	Buttermilk, fluid, 1% fat
	11115200	Buttermilk, fluid, 2% fat
	11115300	Buttermilk, fluid, whole
	52101000	Biscuit, baking powder or buttermilk type, NS as to made from mix, refrigerated dough, or home recipe*
	52101100	Biscuit, baking powder or buttermilk type, made from mix*
	52102040	Biscuit, baking powder or buttermilk type, made from refrigerated dough*
	52103000	Biscuit, baking powder or buttermilk type, commercially baked*
	52104010	Biscuit, baking powder or buttermilk type, made from home recipe*
	53341500	Pie, buttermilk*
Flavored Milk Drinks	11511000	Milk, chocolate, NFS
	11511100	Milk, chocolate, whole milk-based
	11511200	Milk, chocolate, reduced fat milk-based, 2% (formerly "lowfat")

	11511300	Milk, chocolate, skim milk-based
	11511400	Milk, chocolate, lowfat milk-based
	11519040	Milk, flavors other than chocolate, NFS
	11519050	Milk, flavors other than chocolate, whole milk-based
	11519105	Milk, flavors other than chocolate, reduced fat milk-based
	11519200	Milk, flavors other than chocolate, lowfat milk-based
	11519205	Milk, flavors other than chocolate, skim-milk based
	11531000	Eggnog, made with whole milk
	11531500	Eggnog, made with 2% reduced fat milk (formerly eggnog, made with "2% lowfat" milk)
	11551050	Milk fruit drink
	11552200	Orange Julius
	11553000	Fruit smoothie drink, made with fruit or fruit juice and dairy products
	11553100	Fruit smoothie drink, NFS
	11560000	Chocolate-flavored drink, whey- and milk-based
Yogurt	11410000	Yogurt, NS as to type of milk or flavor
	11411010	Yogurt, plain, NS as to type of milk
	11411100	Yogurt, plain, whole milk
	11411200	Yogurt, plain, lowfat milk
	11411300	Yogurt, plain, nonfat milk
	11420000	Yogurt, vanilla, lemon, or coffee flavor, NS as to type of milk
	11421000	Yogurt, vanilla, lemon, or coffee flavor, whole milk
	11422000	Yogurt, vanilla, lemon, maple, or coffee flavor, lowfat milk
	11422100	Yogurt, vanilla, lemon, maple, or coffee flavor, lowfat milk, sweetened with low calorie sweetener
	11423000	Yogurt, vanilla, lemon, maple, or coffee flavor, nonfat milk
	11424000	Yogurt, vanilla, lemon, maple, or coffee flavor, nonfat milk, sweetened with low calorie sweetener
	11425000	Yogurt, chocolate, NS as to type of milk
	11426000	Yogurt, chocolate, whole milk
	11427000	Yogurt, chocolate, nonfat milk
	11430000	Yogurt, fruit variety, NS as to type of milk
	11431000	Yogurt, fruit variety, whole milk
	11432000	Yogurt, fruit variety, lowfat milk
	11432500	Yogurt, fruit variety, lowfat milk, sweetened with low-calorie sweetener
	11433000	Yogurt, fruit variety, nonfat milk
	11433500	Yogurt, fruit variety, nonfat milk, sweetened with low-calorie sweetener
	11446000	Fruit and lowfat yogurt parfait
	27116100	Beef curry*
	27120160	Pork curry*
	27130100	Lamb or mutton curry*
	27146150	Chicken curry*
	27150100	Shrimp curry*
	27150320	Fish curry*
	27213010	Biryani with meat*
	27243100	Biryani with chicken*

27516010	Gyro sandwich (pita bread, beef, lamb, onion, condiments), with tomato and spread*
32101530	Egg curry*
53104580	Cheesecake -type dessert, made with yogurt, with fruit*
53441210	Basbousa (semolina dessert dish)*
53540500	Breakfast bar, date, with yogurt coating*
53540902	Nature Valley Chewy Granola Bar with Yogurt Coating*
53710902	Nature Valley Chewy Granola Bar with Yogurt Coating*
53714230	Granola bar, oats, nuts, coated with non-chocolate coating*
54408250	Pretzel, yogurt-covered*
54430010	Yogurt chips*
58124500	Pastry, filled with potatoes and peas, fried*
63401015	Apple and grape salad with yogurt and walnuts*
75440600	Vegetable curry*
81103041	Margarine-like spread, made with yogurt, stick, salted*
81104011	Margarine-like spread, reduced calorie, about 40% fat, made with yogurt, tub, salted*
83115000	Yogurt dressing*
91708150	Yogurt covered fruit snacks candy, with added vitamin C*
91731150	Peanuts, yogurt covered*
91739600	Raisins, yogurt covered*

* Only the food category component for proposed thiocyanate food use was included in the analysis.
Note: Excludes baby foods.

Annex 7

CHAPTER 3

Pharmacology and Toxicology of inorganic Thiocyanates

A. Grisk, A. Kramer and W. Weuffen

Thiocyanates have been used prophylactic and therapeutically in human and veterinary medicines since the beginning of last century. However, the indications were varied based on empirical observations but also theoretical considerations. Thiocyanates were used for:

- Fighting of microorganisms, as well as episomatic in hand and skin disinfectants or in antiseptics as well as endosomatic with the goal of a chemotherapy,
- Reduction of blood pressure,
- Therapy of polycythemia vera rubra,
- Treatment of immune pathologies or diseases that involve the immune system,
- Reduction of vasomotor pain.

During many clinical studies using thiocyanates, its effectiveness and its compatibility or toxic side effects have been collected. Also studies in pharmacokinetics were performed. Results from animal experiments complete the picture of the pharmacological and toxicological properties of thiocyanate.

1. Past use in human medicine

1.1. Application in antiseptics and disinfectants

SCN^--containing preparations have been and partially still are used for skin and mucous membrane antisepsis or disinfection.

Skin and wound antisepsis. Boro-Weidnerit [1)], Rhodocrema and Rhodobazid have been used successfully for the treatment of festering wounds, ulcera cruris, pyodermia, seborrhoic and intertriginous eczema, paronychia and so on (*Rose, 1897; Joseph, 1899; Steiner, 1900; Jacobi, 1901; Notthaft, 1902; Schwab, 1902)*. Rhodocrema, a SCN^--containing weak acidic plant mucilage, was used as a preoperative hand antiseptic (*Christiansen, 1948*).

Oral cavity antisepsis. Cevidan-Gel used as teeth cleaning substance not only proved to be pleasant to use, but also was obviously effective preventively against some gums diseases, particularly in gingivitis (*Christiansen, 1948*).

Several reports are present about the preventative effect e.g. of Rhodalzid in caries (*Männich, 1912; Meyer, 1912; Nerking, 1912; Freyvogel, 1917; Blessing, 1922; Keßler, 1942*). However *Jacoby* (1923) assumed for this application that a deficit of SCN^- in saliva could often be found

in caries patients. Under the aspect of modern understanding of the genesis of caries and the microbial population of the oral cavity (*Prickler, 1980*), especially the anti-microbial effect of the lactoperoxidase-peroxide-thiocyanate system in saliva (see *Thomas*, Chapter 6) and the optimization of the immune response through the SCN^- -ion (see *Weuffen*, Chapter 14), these empirical observations appear in a new light.

Rhodalzid, given as lozenge 3 times a day (1 tablet contained 48mg bound HSCN), reached always good, sometimes drastic therapeutic successes for *Wetzel* (1923) in 120 patients with stomatitis or gingivitis. The medication had to be continued after the conclusion of the localized therapy for an extended period (up to 1 year) in order to obtain long-running success, or otherwise a trend to reoccurrence existed.

Mucidan-Tinktur® is still considered as therapeutically active to this date and is recommended for stomatitis, gingivitis (*Dietrich, 1935, 1946*) and alveolar pyorrhea. The tincture reacts neutral, is harmless to the enamel and prevents the rot of meat residues. It is suitable as oral cavity antiseptic for daily mouth hygiene because of its smell-erasing and anti-bacterial effect (*N.N., 1976a*).

Antisepsis in the nose-throat cavity. This took also place with Mucidan® -preparations. Angina (*Burkardt, 1931*), pharyngitis, local therapy of diphtheria (*Paschlau, 1935; Goecker, 1937*), flu and scarlet fever prevention are named as indications for Mucidan-Tinktur®. Brush application and spray treatment with 1:5 diluted solutions are practicable for children that cannot yet gargle (*N.N., 1976a*).

The manufacturer selected the KSCN-additive because of the secretolytic, secretomotoric, and especially the sialogen effect (*Brockmann and Niepolt, 1977*). The KSCN- effect is seen as a sliding rail for formaldehyde in that it allows an easier access to the disease process through the thinning of secretion and swelling of membranous plaque (*N.N., 1967a*). To what extent the anti-microbial effect of KSCN and the immune optimization take part in the effect development remains open since respective studies are not present.

Mucidan ® was recommended for the treatment of ozaena, chronic catarrhs of the upper airways and rhinitis atrophicans by *Weiss* (1923) and *Jacoby* (1923). Even then the SCN^- -administration was based on the observation that in ozaena patients SCN^- is not present in the nasal secretion which was confirmed more recently by *Jurato and Tenaglia* (1958). The latter authors reported the effect of Mucidan® in chronic atrophic processes of the upper airways. *Pollack* (1934) and *Lembcke* (1960) recommend based on good therapeutic results the commercially available Mucidan®-tablets (since 1924) for chronic pharyngitis with dryness (sucking 5 times/day 1 tablet) and also for dryness in the mouth following X-ray treatments and operations.

Also here again it is remarkable that SCN^- -administration is active in syndromes with an assumed participation of the immune system.

Coughs of any kind, acute and particularly chronic bronchitis, tracheitis and pharyngitis are named as indications for Mucidan® - Cough Tea (*N.N., 1976a*).

Ear antisepsis. *Pichler* (1927), *Bamberger* (1937) and *Joseph* (1924) successfully used Mucidan® for festering ear infections.

Antiseptsis for bladder and vagina. *Dikomeit* (1933, 1935) used SCN^--containing preparations as antiseptic flush for bacterial infections of the bladder and vagina. Otrhomin-Viganal Swaps often proved reliable in fluor albus (*Christiansen, 1948*).

Hand and skin disinfection. Here, Rhodobazid® was used for its kindness towards the skin and its acid-coat (*Schmidt-Hoensdorf, 1936; Kiwit, 1939; Dötzer, 1940*). Sepso-Tinktur® contains thiocyanate to this date (*Vierthaler, 1940*).

1.2 Application with the goal of an anti-microbial chemotherapy activity

Infectious intestine diseases. *Mitchel and Goltmann* (1935) gave NaSCN as a therapy against the bacteria Ruhr (Shiga dysentery) at a dose of 20 mg/kg, which means a single dose is administrated of about 1400 mg. The immediate administration after the outbreak of the illness was important. *Keßler* (1942) and *Christiansen* (1946) observed a surprisingly fast healing of bacteria-induced Ruhr and of unspecified enteritis after SCN^--application.

Orthomin proved itself as a preventative of infectious intestine disease in human (*Christiansen, 1946*) and domestic animal (*Behrens, 1948*).

Based on the good results, *Christiansen* (1948) extends his considerations as far as suggesting the addition of SCN^- to bread dough – with the respective approval and declaration – during threatening Typhus- and Ruhr-epidemics to sharply increase the resistance of the population. Baking tests with Rhodobazid proceeded favorable.

We also were able to obtain good successes in the protective application of thiocyanates in the commercial animal production. New results from animal experiments for the optimization of the immune response through thiocyanate were the basis for this application (see *Weuffen, Blohm and Rotermund*, Chapter 16).

Tuberculosis. *Keßler* (1942) achieved a significant activity with the administration of 1g NaSCN i.v. in bone-tuberculosis. *Keßler* administered for 24 days a dose of 1 g NaSCN per day (maximum dose possible from the current toxicity studies) in a bone-tuberculosis of the sternum with fistula, after 20 days the closing of the fistula was observed with healing. Likewise, *Keßler* reported therapeutic successes in tuberculous glands at the angle of the jaw with fistula, tuberculous pyonephorsis, tuberculous infiltrations of the lungs, fingers – and hands phlegmona.

In the search for basic combinations that were suitable for salt formation with HSCN one fell back to the azo compound Chrysoidine. Its thiocyanate-hydrogen acidic salt was recommended under the name Dairin ® as tuberculosis therapy for some time.

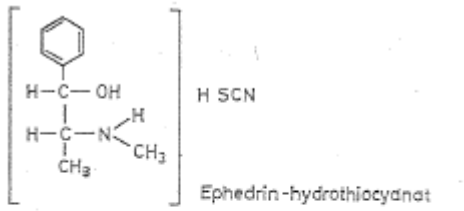
Mastitis. The therapy of mastitis with SCN^- -infusion in cattle didn't always lead to clear results (*Wittholz and Loch, 1934; Seelemann, 1936; Tapken, 1936; Lamm, 1937; Böttger, 1938; Elsner, 1938; Kipp, 1938; Schmidt-Hoensdorf, 1938; Eylman, 1939*). On the other hand SCN^- -containing milking-fat proved suitable for the prevention of the transmission of mastitis (*Seelemann, 1936; Schmidt, 1937; Schmidt-Hoensdorf, 1938*).

In humans, the administration of 3 times 2 tablets Rhodovet ® (equals 450 mg SCN^-/d) over 6 days suggests a tendency towards a protective effect in Mastitis puerperalis, where at this dose no side effects were observed (*Prott and colleagues, 1978*).

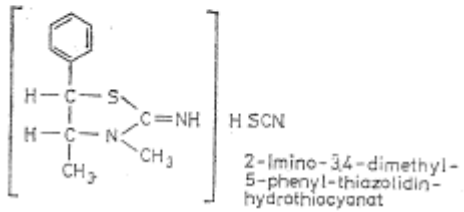
Other infections. *Keßler* (1942) reports about therapeutic successes through the administration of NaSCN in staphylococcal phlegmona of the lower leg, lower arm and face, boils on the upper lip, osteomyelitis and lymphangitic sepsis (dose 1 g/d i.v.). *Westphal* (1926) found that at a medium dosage of 3 times 0.2 g NaSCN/d, syphilitic gums (rubber nodes) and periostitis were not present any longer within a few days. *Dalmady* (1912) used NaSCN for luetic tabes. With a daily dose of 0.45 to 1.25 g a pain reducing effect has been reported after the 3rd to the 5th day of the treatment. The NaSCN- medication was discontinued at the latest after 4 weeks to avoid addiction. The favorable effect continued for 2 to 3 weeks after the end of the application. After discountinuing the treatement for a cetain period of time, he carried out another 'treatment cure'.

1.3 Treatment of immunopathy

It is interesting that thiocyanates were used already directly for the treatment of immunopathy. *Denichou* (1931) obtained a reduction of pain and joint swelling through the administration of 0.3 g KSCN 3 times/d in rheumatoid arthritis. *Loutchich* (1932) and *Maire-Amero* (1933) were able to confirm this observation. *Schau-Levantis* (1953) observed a favorable effect of Otrhomin 3 times 2 tablets per day for 3 to 8 days in allergies caused by plants, allergies with focal origin, in urticaria and neurodermatitis with and without asthma; additionally pruritus vulvae et senilis, anal eczema and infected eczema responded to the therapy. *Oesterreicher* (1915) saw good therapeutic results in keratitis eccematosa, in which the SCN^- concentration in saliva of patients was reduced before the application of SCN^-.



Formula 3-1



Formula 3-2

The combination of basic medicines with HSCN is still common in medicinal use. The same is true for cough and asthma medications, which are made based on for instance paracodin-hydrothiocyanate, ephedrine-hydrothiocyanate (formula 3-1) and 2-imino-3.4-dimethyl-5-phenylthiazolidin-hydrothiocyanate (formula 3-2) present. The two last named substances are contained in the much-used asthma medication Asthmolytan ®.

Based on results from animal experiments especially a favorable influence of KSCN in experimental asthma in guinea pigs (*Lachman and colleguas, 1977*), it is suggested that the effect of such preparations, with multiple administrations per day achieved in single cases resulting in a high SCN^- supply, is connected with an additional influence on the immune reaction. With these preparations, not only the symptoms will be affected.

1.4 Application in hypertension

Before the introduction of the modern highly effective and better tolerated medicines for hypertension, thiocyanates were commonly used medicines in hypertension. NaSCN or KSCN (in Rhodan-calcium-diuretin) and betain-hydrothiocyanate (in Rhodapurin) were the most commonly preparation.

Organic thiocyanate compounds, e.g. 2-thiocyan-4-nitrobenzoic acid and 2-thiocyanbenzoic acid, also proved themselves as strong reducers of blood pressure in animal experiments (*Cannava and Chiarlo, 1951*).

Different doses were required for the reduction of blood pressure in essential hypertension:

- 300 mg 3-4 times/week, meaning a blood level of 80 to 120 mg SCN/l (*Barker, 1936*)

- 1st week 3 times/day 100 mg, 2nd week 2 times/day 100 mg, 3rd week 1 times/day 100 mg, 1 week treatment free interval, possible start of new course, for month-long continued cures 50-100 mg/day (*Westphal, 1926*)
- 326 mg SCN^-/day for 14 to 78 days long (*Goldring and Chasis, 1932*)

In contrast, *Egloff, Hoyt and O'Hare* (1931) obtained in only 2 out of 25 patients a minimal reduction of blood pressure with the following high dose: 1st week 1 g NaSCN/day, 2nd week 0.6 g NaSCN/day, 3rd week 0.3 g NaSCN/day.

We were unable to find any indication of a blood pressure reducing effect of NaSCN in normotension in the literature. Today's treatments of hypertension are made without thiocyanate for the benefit of better preparations.

1.5 Anti-tumor therapy

Beickert and Jorke (1952, 1952/1953, 1953) reported an interesting therapeutic activity with KSCN in polycythaemia vera rubra in end-stage. It came to an extensive regression of the spleen tumor, a coalescence of neoplastic infiltrates on the finger bones and a significant reduction of unripe erythrocyte precursors in the peripheral blood. This effect was achieved with the administration of 600 to 1200 mg/day for 14 days per bone. The therapy was interrupted after 14 days because of transient side effects in form of diarrhea and a 2nd treatment was administered after 5 weeks. The leucopoiesis was not attacked, but only an elective erythrostatic effect observed. In the same way a life-threatening effect was not demonstrated.

The cause for the erythrostatic effect was not resolved. *Beickert and Jorke* (1952/1953) discussed a blockage of erythropoiesis through the formation of SCN^- heavy metal complexes. This could be favored because erythrocyte membranes possess a particular permeability for SCN^- (*Eichler, 1950*). The heavily reduced nuclear reaction during the Feulgen-staining, particularly in erythrocyte precursors, could be caused by the intervention of SCN^- in the nuclear protein metabolism. An influence on the immune response is not excluded.

Beickert and Siering (1952) could also observe a regression in the ascites tumor of the mouse during SCN^- treatment. In contrast to Gallic acid and caffeine, SCN^- added to food had no protective effect in lung adenoma of the mouse that was induced through administration of Morpholine and sodium nitrite (*Mirvish and colleagues, 1975*). Otrhomin® was ineffective for the Jensen-sarcoma of the white rat (*Lührs and Kindermann, 1953*).

1.6 Other uses

Administration in sickle cell anemia. Cyanate inhibits the formation of sickle cells of erythrocytes *in vitro* (*Cerami and Manning, 1971*) and extends the life span of treated sickle cells *in vivo* (*Gilette, Manning and Cerami, 1971, Cerami, 1972*). In a clinical study, *Gilette and colleagues* (1972 a, b) were able to achieve an improvement of the blood status with a reduction of the number of hemolytic crises through the oral administration of cyanate (KOCN 1000 to 1200 mg/d) in patients with sickle cell anemia. Analog, KSCN unfolded a favorable effect at even lower dosages (500 mg/d, *Torrance and Schnabel, 1932*).

During a comparison of the alimentary absorption of SCN^- or SCN^- precursors in Africans and African-Americans a deficit of SCN^- came to light for the latter. African-Americans ingested alimentary 25 mg SCN^- daily which compared to about ¼ of the SCN^- taken up by Africans. A reciprocal correlation existed between the SCN^- quantity taken and the frequency of sickle cell anemia. This supports that the genetically-determined sickle cell anemia can be influenced in its prominence through alimentary SCN^- ingestion (*Houston, 1973*).

Pain syndromes. At least temporary relieve was achieved with SCN^- administration in arteriosclerotic pain, angiospastic migraine, Tabes and sympathetic neurosis (*v. Dalmady, 1912*), in gastric crisis, cholecystitis, ulcus ventriculi and duodeni (*Christiansen, 1948*). SCN^- is not used for these indications anymore today. Obviously these favorable experiences do not lend themselves to generalization.

Further clinical observations showed that thiocyanate can reduce the pulse frequency in the Morbus Basedow (*Paschkis, 1885; Scheurer, 1912; Takacs, 1926*) and increase the reflex reaction and intestine peristaltic (*Paschkis, 1885*).

1.7 Newer findings on the effect of thiocyanates in animal experiments

The effect of SCN^- on the immune response in animal experiments was summarized in table 3-1 (further details see *Weuffen*, Chapter 14).

Since a connection between immune defense and anti-mutagenic effect could exist, the influence of NaSCN on experimentally created bone marrow cell mutations through cyclophosphamide in the mouse was investigated. There was an ascertain and significant reduction in mutation rate (*Kramer and colleagues*, in preparation).

Finally, a liver protective effect of NaSCN was observed in the rabies immunization (guinea pig) (*Kramer and colleagues, 1980*). The past examination of this effect in experimental carbon tetrachloride poisoning of the guinea pig confirmed the results (*Weuffen and colleagues*, in preparation).

1.8 Conclusions

Even if some of the older reports about the therapeutic or preventive effect of SCN^- administration need to be considered with caution, it remains without doubt that the administration of SCN^- causes a manifold of effects in the organism. 1948 *Christiansen* generalized the current results of SCN^- administration in his representative overview work on 'the Rhodan in medicine' as follows: 'Several facts like the expressed cycle of Rhodan in the organism, its constant serum level, its delayed excretion, the ability to use high dosages as well as the strong colloidal-chemical effect of the anion speak together with clinical experiences for a general cell physiological effect are the results of the parasympathetic nervous system toning and the regulation of the permeability conditions of the cell membranes.'

In vitro studies (table 3-2) showed that SCN^- can only unfold a directly damaging effect on microorganisms at concentrations significantly higher than can be reach in an organism (*Weuffen, 1963*). The effectiveness of Al^{+++}, Cu^{++}, Ag^{+}, Hg^{++} and Ni^{++}-chlorides and -thiocyanites may be caused through the cations.

An antimicrobial effect is still thinkable in this respect since the most varied functions of the body are influenced by SCN^- (e.g. enzyme systems, proteins, membrane activity) and with that also the entire immune defense (unspecific and specific) and indirectly the microorganisms themselves (see *Weuffen* Chapter 1). Moreover the good therapy results in microbial syndromes shall not be forbidden.

For the optimization of the immune response significantly smaller doses are sufficient than for a direct antimicrobial effect (see *Weuffen* Chapter 14). As described in the section 1.2 where favorable results were observed without any explanation about a real chemotherapeutic effect, a favorable influence on the immune response was observed particularly since thiocyanate was directly used in immunopathy (see section 1.3). The preventive effectiveness and the favorable effect particularly by application in an early stage of infection also suggested this.

2. Pharmacokinetics

A detailed representation of normal values in man and animal is given in *Weuffen* and colleagues see Chapter 9.

Table 3-1 Effect of Thiocyanate on the immune response in animal experiments

Dosis/Application	Animal Species	Biological effect	Literature
--	Guinea pig (GP)	During sensitization without SCN^- -supply highest antibody (ab) titer (passive hemaggl.) with serum thiocyanate level of 20-30 mg/l, meaning 'good thiocyanate formers' = 'good ab formers'	*Weuffen, Jülich and Bohnenstengel (1973)*
32 mg NaSCN/kg from 1^{st} -20^{th} experimental day 20^{th} – 40^{th} day	GP	Sensitization on 3^{rd}, 5^{th} and 15^{th} day with horse serum: raised hem agglutination titer by 1-2 titer levels on the 21^{st}, 28^{th}, 36^{th} and 44^{th} day Only small influence on titer	*Weuffen and colleagues (1974)*
32 mg from 1^{st} to 11^{th} and 12^{th} to 21^{st} day NaSCN/kg KM	Suprarenal body-less GP	Sensitization on the 5^{th} + 7^{th} day with horse serum: in the spleen piece-migration-inhibition test significant inhibition of the cell mediated immune answer (IA)	*Weuffen and colleagues (1979)*
32 mg NaSCN/kg KM 1^{st} – 20^{th} day	GP	Sensitization on the 3^{rd}, 5^{th} and 7^{th} day with horse serum: significant inhibition of the cell mediated IA	*Weuffen, Jost and Grulich (1969); Weuffen and colleagues (1974)*
32 mg KSCN/kg KM 1^{st} – 20^{th} day	GP	Sensitization on the 2^{nd}, 5^{th} + 8^{th} day with ovalbumin i.p., on the 19^{th}, 50^{th} + 70^{th} day aerogen: on the 19^{th} day clinically stronger reaction as in control, on the 50^{th} + 70^{th} day significant reduction of the asthma attack (quotient of maximum esophagus pressure amplitude and breath volume) compared to control, obviously reached a reaction readiness compared to SCN^- - booster administration a maximum earlier and fades away earlier	*Lachmann and colleagues (1977)*
32 mg NaSCN/kg KM 100 mg NaSCN/kg KM	GP Mouse	During rabies immunization improved IA IA stronger as in control, maximum of plaque forming cells 1 day earlier, titer increase hemaaggl. Ab ≥1 level	*Sinnecker and colleagues (in preparation)* *Nieber and colleagues (1982)*
64 mg NaSCN	Mouse	In the erysipelas septic infected mouse significant reduction of the number of sickness days and deaths	*Kramer and colleagues* (in preparation)

Dosis/Application	Animal Species	Biological effect	*Literature*
96 mg NaSCN/kg KM	Chick	Sensitization with horse serum: titer increase by 2-2.5 titer levels (hemaggl. Ab)	*Berling and colleagues* (in prepration) *Weuffen, Jülich and Rotermund (1975*)
50 mg NaSCN/kg KM	Sheep	Earlier appearance of 7-S-ab and significant titer increase by immunization with E. coli and Salm. Newport	*Weuffen, Jülich and Szugs* (in press)
32 mg NaSCN/kg KM during 21 days after transfer to stable	Calf	Reduction of morbidity by 60% during easier disease progression and nutritive effect	*Weuffen, Jülich and Rotermund* (1975)
	Calf	Reduction of morbidity by 50% during easier disease progression and nutritive effect, also OTC could be left out in the pre-mixture	*Blohm and colleagues (1978)*
20 mg NaSCN/kg KM 14 days before and after having a litter	Pig	Improvement in the raising performance, meaning less puerperal sicknesses, less raising losses, disease progression during piglet infection less severe with reduced mortality and possible nutritive effect	*Bredereck and colleagues (1977)*
5 days long 4 times 0.15 g NaSCN/kg KM oral	Human	Influence on the properdine- and complement serum level	*Weuffen and colleagues (1980)*

Table 3-2 Characteristic numbers for fungi-static (KfW_{10}) and the bacteria-statistic (KbW_5) effectiveness of chlorides and thiocyanate (*Weuffen, 1963*)

Cation	Anion			
	Chloride		Thiocyanate	
	KfW_{10} [1)]	KbW_5 [1)]	KfW_{10}	KbW_5
Sodium	3.1	4.5	3.2	8.1
Potassium	2.5	3.2	3.1	5.6
Ammonia	3.8	3.5	4.4	5.5
Calcium	3.1	5.0	3.8	5.6
Barium	2.7	6.0	4.2	6.3
Aluminum	4.3	5.0	9.6	6.5
Copper	9.6	9.5	8.7	6.4
Silver	5.9	12.4	8.6	14.2
Mercury	13.3	14.6	11.9	12.7
Nickel	10.3	10.7	11.6	11.5

[1)] it is a geometrical average of the $^{-3}$logarithm of the effective substance dilution determined in 10 fungi or 5 bacteria colonies

2.1 Absorption

Absorption experiments in rabbits and dogs (*Anderson and Chen, 1940*) showed that in oral application a maximum blood level was only reached after 6-8 hours, suggesting a slow absorption. On the reversed small intestine sack of the rat, SCN^- is taken up from the jejunum as well as the ileum in contrast to several other anions (*Clarkson, Rathstein and Cross, 1961*). Within the group of Cl^-, Br^-, I^-, $Citrate^-$ and SCN^- in which size and lipid solubility achieves the penetration through water permeable pores of membranes, SCN^- belongs to the ions with the slowest permeation speed. A generally similar absorption behavior can be concluded for the human intestine based on therapeutic experiments (*Schreiber, 1925; Westphal, 1926*). The relative anion permeability of SCN^- shows a dependency on species and the investigated organ and stands, therefore depend to the fine architecture of the membrane: The isolated choroid plexus of the rabbit is able to accumulate it in a concentration range of $5x10^{-7}$ to $5x10^{-6}$ mol SCN^-/l against a concentration gradient. This mechanism can be hindered by ClO_4^-, BF_4^-, I^-, F^-, 2,4-dinitrophenol and Digoxin (*Davson, Kleeman and Levin, 1962; Welch, 1962*). SCN^- has a medium relative permeability on spinal motor neurons of frogs (*Matsuura and Endo, 1971*) and neuromuscular end organ of crustaceans (*Takeuchi and Takeuchi, 1967*). Meanwhile it has the highest permeability among several tested anions on the muscle fiber of balanidae (*Hagiwara, Toyama and Hayashi, 1971*).

2.2. Distribution

SCN^- distributes itself mainly in the extracellular space (ECS). It reaches distribution equilibrium within 50 to 60 min, which remains constant for the duration of 1 h. During this time SCN^- can be used for the determination of the size of the ECS. The so determined space is found about 15% larger then as with Inulin due to a gradual transition of SCN^- into the tissue (*Biewald and Billmeier, 1978*).

In transudates inflammatory tissues and tumor tissues, SCN^- has about the same concentration as in plasma; a preferred uptake could not be found (*Wallace and Brodie, 1937*).

Ullberg and colleages (1964) compared the distribution of F^-, Cl^-, Br^-, I^- and SCN^- after i.v. or i.p. administration in auto radiographic studies in adult mice and rats. Br^-, I^- and SCN^- accumulated in the walls of blood vessels particularly of the aorta and A. carotis. The penetration into the CNS declines in the following order $Br^- > I^- > SCN^- > F^-$, whereby Br^-, SCN^- and I^- occur in the pituitary gland at higher concentrations than in the CNS. I^- and Br^- are present into the eyes to a fairly large degree, SCN^- less, where retina, sclera, cornea and peripheral lens portion contain the relatively highest concentrations. Furthermore SCN^- was observed in bone and cartilage but not in bone marrow, in teeth, in the lymphatic tissue and only in a very small concentration in muscle with a relative increase in the myocardium. The concentration of SCN^- was only slightly higher in the thyroid than in blood. On the other hand, a strong SCN^-

accumulation was present in the salivary gland particularly the submaxillary gland. SCN^- was also enriched in the mucosa of the large intestine. The last named finding stands in conformity with results of *Stuber and Lang* (1934), in a simultaneous analysis of peripheral and portal vein blood where they showed that SCN^- is re-absorbed in the intestine which results in a reduced SCN^- excretion in stool (5-10% of the entire excretion). Particularly SCN^- but also I^- and Br^- are subject to intensive gastric secretion where the highest concentration is present in the epithelium of mucous membranes.

The distribution of i.v. administered investigated in dogs shows the influence of plasma SCN^- concentration on the SCN^- concentration in cerebrospinal fluid (CSF). Below a plasma concentration of 1 mmol SCN^-/l only small quantities can be seen in the CSF. At higher plasma concentrations, starting at about 1.5 mmol SCN^-/l a relatively constant plasma-CSF-difference of about 1.2 mmol SCN^-/l can be observed. Based on investigations of *Weir* (1942) with Br^-, *Streicher, Rall and Gaskins* (1964) also demonstrate for SCN^- serum concentrations between 25 and 96 mmol SCN^-/l a SCN^--serum-CSF-difference of about 6 mmol SCN-/l is maintained. The existence of a blood-CSF-barrier is usually assumed as explanation for these findings. However since it was shown by *Welch* (1962) that the choroid plexus transports SCN^- actively, it seems possible that this mechanism is responsible for the concentration gradient (*Streicher, Rall and Gaskins, 1964*).

In *Bödigheimer, Nowak and Schoenborn* (1979) described a partial binding activity to serum protein receptors, already supported by *Lavietes, Bourdillion and Klinghoffer* (1936) who observed incomplete equilibration of SCN^- with the extra cellular environment. In the course of this distribution I^- is displaced by SCN^- in the I^- storing organs (thyroid glands, but also salivary glands, peptic glands, mammary glands and bronchial glands) where I^- enrichment through a common mechanism is prevented (*Höbel and colleagues, 1967*). This SCN^- -dependent I^- deposition reflects substantially the ability for hydrophobic interactions. The binding possibilities and the association are greater for SCN^- than for I^- and therefore this mechanism could be put down to such a physicochemical behavior (*Azzone and Massari, 1973*).

SCN^- penetrates the placenta barrier. The fetal SCN^- concentration is about 1/3 of the mothers in the distribution equilibrium. Investigations in this respect brought the confirmation that i.v. injections of 20 mg NaSCN did not have a damaging influence on the fetus (*Boulos and colleagues, 1973*) and also were not teratogenic (*Ivankovic, 1979*).

In general, a serum level of 50 to 70 mg SCN^-/l can be determined after the administration of 300 mg NaSCN/d in human according to *Barker* (1936); 0.6 to 1g were necessary for this (serum level 80 mg SCN^-/l) in 2 out of 45 patients and in one patient only 60 mg/d.

In experiments by *Weuffen and colleagues* (1980) the SCN^- serum level after oral application of 600 mg NaSCN daily for 5 days showed the following course:

- Starting value: 3.4 ± 1.6 mg/l
- 5^{th} day: 55.3 ± 20 mg/l
- 10^{th} day: 28.7 ± 22.8 mg/l
- 15^{th} day: 13.3 ± 14.6 mg/l

2.3 Elimination

Renal excretion and to a lesser extend biotransformation take part in the elimination of SCN^-. The elimination occurs slowly which could be the cause for cumulative poisoning.

2.3.1 *Excretion*

Animal experimental results. Rabbits, guinea pigs and rats excrete about 80-95% of the administered dose within 5 to 14 days via the kifneys.

Baumann, Springson and Metzger (1933) determined in the rabbit that administered KSCN is eliminated by the kidney to 90% within 5 days. Guinea pigs excrete 88 to 96% NaSCN vi athe kidneys after s.c. administration of 200 mg NaSCN/kg, while in oral administration only 72% are eliminated (*Hunt, 1907*).

Wood, Williams and Kingsland (1947) injected rats with 9 to 15 mg labeled SCN^-/kg. During a course of 23 days 81% were excreted unchanged in urine and less than 1% in feces. The large dose led to an overloading of the renal SCN^- -excretion. Retention of SCN^- in serum is caused by kidney re-absorption and distribution of SCN^- into other compartments (*Funderburk and van Middlesworth, 1971*). *Streicher, Rall and Gaskins* (1964) were able to show in this respect that the organism is able to maintain a certain SCN^- concentration gradient between serum level and other compartments. The renal re-absorption is dependent on the total amount of ion in serum. ClO_4^-, NO_3^-, I^- and Cl^- for example increase the SCN^- -clearance (*Nickerson and Thomas, 1951; Walser and Rahill, 1965; Funderburk and Middlesworth, 1967*).

After application of 725 µg labeled $NaS^{14}CN$ in rats, 2/3 of the dose were eliminated by the kidneys and the rest exhaled as metabolized CO_2 (*Boxer and Rickards, 1952*). For dogs *Nickerson and Thomas* (1951) determined a clearance of 0.1 to 7.3 ml/min for a SCN^- plasma concentration of 10 mg/l.

Funderburk and van Middlesworth (1971) determined the half-life for serum-SCN^- for administrations in small doses (50µg SCN- i.p.) to be 4.42 days and for larger doses (5 mg) on the other hand 0.31 days.

Jirousek (1956) was able to show that high doses (100 mg/rat) of arginine, alanine, glutamic acid, methionine and cysteine caused a transient excretion of SCN^- and to a smaller extend also acetamine, methylamine or diethylamine. Hyperthyroidism caused by thyroxin or tri-iodine-thyronin resulted in an increase of serum SCN^- as well as increase of the urine SCN^- in long-term experiments. The SCN^- concentration in serum is reduced to normal levels when these animals are given methyl-thiouracil.

Findings in humans. *Takacs* (1926) names 48 to 80 mg as the quantity of SCN^- daily excreted through urine. *Pollack* (1902) determined that 0.25 to 2.2 g of oral or subcutaneously administered SCN^- is excreted within 4 to 5 days. 96 to 99% of a single dose of 1.2 to 1.5 g NaSCN was eliminated by the kidneys within 5 to 14 days in investigations of *Moister and Freis* (1949). 3 to 7 mg per day were excreted in feces and only traces through sweat. Over a course of 4 to 11 weeks it was impossible to find 20 to 36% of the administered dose possibly due to an increased oxidation of the ion and increased microbial metabolism particularly from the intestinal flora.

With increased administration of sodium nitroprusside in modern anti-hypertension therapy, reliable data gathered for the SCN^- elimination and its limited toxicity, the following conversion can be observed: 1mmol = 298 mg sodium nitroprusside and 5 mmol = 290 mg SCN^-. An elimination half-time of 1 to 5 days and on average 3 days was found for SCN^- in healthy subjects. The developed SCN^- was found almost completely in urine. In contrast to older investigations, it was not possible to increase SCN^- excretion by doubling Cl^- excretion (*Schulz, Bonn and Kindler, 1979*). In kidney insufficiency metabolism, the half-time elimination is extended to 7 to 9 days in approximate correlation with the creatine-clearance and is responsible for cumulative poisoning (*Bödingheimer, Nowak and Schoenborn, 1979; Schulz, Bonn and Kindler, 1979*). The so-called ‘therapeutic blood level’ of 80 to 120 mg SCN^-/l blood serum increases by a continuous administration to 150% within 2 weeks of treatment and to 200% within 4 weeks and equals the toxic concentrations (*Schulz, Bonn and Kindler, 1979*).

2.3.2 *Biotransformation*

SCN^- was regarded as metabolically inert for a long time since in fact the largest part is eliminated unchanged by the kidneys (see paragraph 2.3.1). In investigations with radioactively labeled SCN^- or CN^- prove was obtained that part of the administered SCN^- is metabolized to CN^- and SO_4^{--}. A metabolic equilibrium exists between SCN^- and CN^- meaning part of the CN^- supply in the organism is converted to SCN^- (detoxification). The sulfur for this form is of endogenous origin (S-supply). The carbon of CN^- is partially converted to CO_2 and partially entered the C-supply. A separate metabolism exists for the S and C in SCN^-. The metabolic cycle of SCN^-/CN^- is called thiocyanate-cyanide cycle (figure 3-1).

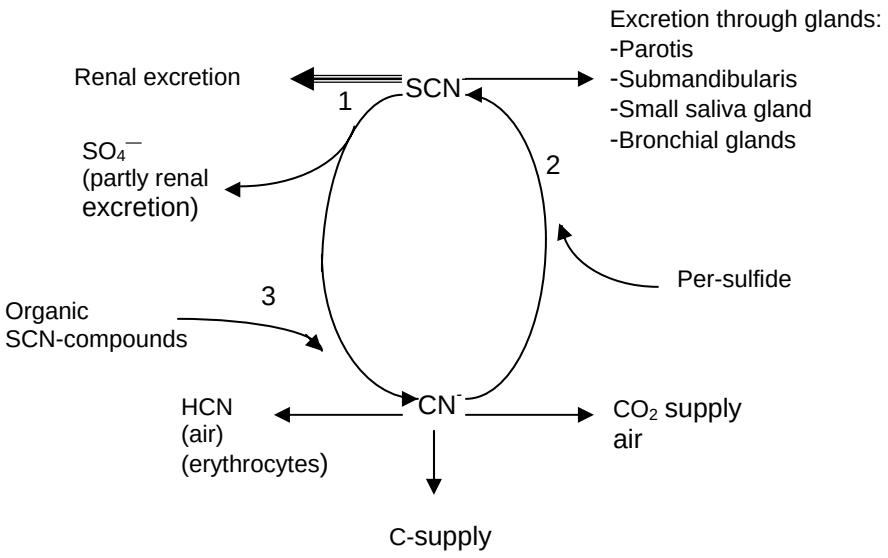


Figure 3-1 Thiocyanate-Cyanide Cycle (modified from Wood, 1975)
1 Thiocyanate-oxidase, identical to oxy-hemoglobin-, myo-, thyro- or lacto-peroxidase
2 Thiosulfate: Cyanate-sulfur-transferase (EC 2.8.1.1), 'Rhodanese' (Rhodan-generation)
3 S-acetyl-glutathione-hydroxylase (EC 3.1.2.7)
Glandular elimination: see *Stephen and colleagues* (1973), metabolism of organic SCN- -compounds: see *Ohkawa and Casida* (1971), Cyan-hydrogen in erythrocytes: see *Vesey and Wilson* (1978)

Wood, Williams and Kingsland (1947) proved in the rat that 4.5% of administered KSCN is eliminated by the kidneys as SO_4^{--}. HCN was found in breath where the maximum of the specific radioactivity of CN^- from HCN was detectable within 3 to 4 hours. Regarding the carbon of SCN^- 1/3 was exhaled as CO_2 and 2/3 were eliminated as SCN^-. Its biologic half- life in SCN^- amounted to 3 to 6 days and it was replaced by about 19% per days in the rat (*Boxer and Rickards, 1952*). The thyroid gland metabolizes SCN^- with a maximum rate of 65 nmol/g of thyroid gland tissue/h (*Maloof and Soodak, 1959*).

3. Thoughts on the mechanism of action

3.1 General expositions

Many of the pharmacodynamic effects of SCN^- can be traced back to *Hofmeister* (1888) who described the effects of anions on colloid systems. The anions listed in the so-called Hofmeisterschen lyotropic series have a similar order of other physicochemical values like surface tension, compressibility, viscosity, electrophoretic mobility of proteins, ability to activate and urea-stability of enzymes or their affinity to serum albumin and can be observed. In this

respect the effects of organic thiocyanate are significantly different from the effects of the SCN^- anion and the hydro-thiocyanates of organic bases. The actual mechanism of action, particularly the effect on the immune response, is to date inexplicable. Therefore only a few properties shall be put out that could be important for the effect of SCN^- (further details see *Böhland*, Chapter 2):

- The SCN^- ion is an elongated linear molecule with lengths of 2.32 Å (*Araki, Ito and Oscarsson, 1961*)
- It has a prominent dipolar character
- The negative charge is distributed over the entire molecule through mesomerism. The distance between the two possible binding positions is relatively large. A polarization of the molecule should be more difficult in comparison to I^- based on the CN-triple binding (*Kröhnke, 1955*)
- It influences the sol-gel-condition of protein
- It influences the hydrogen bindings (loosening)
- It has a high tendency to bind on protein
- It influences enzyme activity
- Finally it has a clearly demonstrable membrane activity (see *Redmann*, Chapter 4)

The influence of electrolytes on biological processes needs to be regarded highly since they change the water structure significantly. A serie of biological effects of SCN^- accompanies a change in the water structure as a result of the anion-binding with the cationic binding position in the receptor area of enzymes (*Fridovich, 1963*). SCN^- similar to ClO_4^{--} and in contrast to practically all other anions takes a privileged position.

Table 3-3 Influence on the water structure

Influenced value	Electrolyte effect	
	SCN^-	SO_4^{--}
Structure temperature	Increased	Decreased
Number of hydrogen-bridge-bindings	Decreased	Increased
Number of free OH-groups	Increased	Decreased
Influence on the water lattice	Structure breaking	Structure forming
Cluster formation	Disturbed	Supported
Secondary hydration	Weak	Strong
Influence on organic molecules	In-salting	Salting out

In table 3-3 the SCN^- ions are compared with the SO_4^{--} ions – thus 2 anions to be arranged oppositely in the Hofmeisterschen anion series – with respect to their effects on the water structure. Both anions not only influence important base structures of water but also differentiate markedly themselves in their effects so that opposing effects occur.

Ambrosius and colleagues (see chapter 15) report about the experimental investigations on the influence of SCN^- on the affinity of antibodies and the respective conclusions.

Electrolytes influence apart from the structure of water also proteins. Also here, SCN^- may have a special position amongst the anions. In particular the spatial structure of proteins should change with a different degree of intensity in the following properties:

- Hydrophobic valence
- Hydrophilic valence
- Formation of the tertiary structure
- Hydrogen bridge formation
- Van der Waals force
- Electrostatic force

The immune regulation can be counted to the biological processes that are influenced by electrolytes. In the formation of a judgment about if and where SCN^- becomes effective one has to take into account a series of points of attack:

- The synthesis of immune proteins can be influenced directly by changes of enzyme activity.
- An influence on nucleic acids is conceivable.
- The uptake of antigens or antigen particles and release of immune proteins by cells may be dependent substantially on the state of cell permeability, the charge and surface tension of the cell. These again are values that are influenced by electrolytes whereas SCN^- can be counted to be the strongly effective electrolytes.
- The structure of immune protein certainly is influenced by SCN^- - as discussed above for proteins in general.
- Also, electrolytes have a regulatory effect in the antigen-antibody reaction which itself again depends on the structure of immune protein. The same is true for the course of events during the secondary immune answer. This effect was investigated extensively in vitro.
- Furthermore an influence of SCN^- on the resultant reaction is conceivable.

3.2 Special expositions

The SCN^- effects are to be regarded as specific and therefore biologically important to the extent of which the ion has the ability to change the conformation of bio-macromolecules. If the intra-molecular bonds within a low molecular substance (active substance, pharmaceutical) remain intact during the interaction with a bio-macromolecule (receptor protein) but result in a conformational change in the macromolecule level then a biological effect can be confirmed. It is quite possible to describe the existence of ‘thiocyante’ receptors in particular bio-macromolecules based on the criteria for a pharmaceutical-receptor from *Ehrenpreis, Fleisch and Mittag* (1969).

Some investigations that were carried out with thiocyanate on well characterized model-proteins such as hemo-proteins and other proteins formed the first basis for its mechanism of action on molecular level.

Investigations by *Scheler* (1968) as well as *Blanck and Scheler* (1968) on met-hemoglobin, met-myoglobin and hem-albumin complexes as conformation labile proteins showed that they change their conformation dependent on the kind of ligand on the iron-porphyrins. Here, SCN^- is capable of partaking in biomolecular exchange reactions of hemoproteins. The activation energy for these reactions increased in the investigated met-hemoglobin- and met-myoglobin-complexes in the following order: $HCOO^- < SCN^- < OCN^- < NO_2^- < SeCN^-$. Sodium salts of diverse anions also exercise conformation changing effects on the acidic ferri-cytochrome c. Chaotrope anions change the high-spin form of the enzyme into a mixed spectral type, where the order is: tri-bromide acetic acid > tri-chloride acetic acid > ClO_4^- > SCN^- > NO_3^- > Br^- > Cl^-. A correlation was found between the effect of the anion and their partial molar entropy (*Aviram, 1973*). The result of such a ligand binding in hemoproteins activity is the strengthening of the bridge function of the hemo group between E- and F-helix of the polypeptide chain which manifests itself not only in a higher stability in heat, acids and other denaturing agents but also in oxy-hemoglobin in a higher affinity to oxygen (P_{50}-value, *Laver and colleagues, 1977*). Such a thiocyanate-bond reduces to an insignificant extent a trans-reaction of hemoglobin at the millimolar concentration level (*Müller and Scheler, 1967*). Changes in the conformation of proteins are amongst other changes of fluorescence properties. The fluorescence quenching effect on proteins increases in the series SO_4^{--}, $CH_3COO^- \sim Cl^- < ClO_4^{--} < SCN^- < Br^- < I^- < NO_3^- < NO_2^-$ (*Altekar, 1977*). SCN^- distinguishes itself by a medium quenching effect and a shift of the fluorescence maximum toward longer wave lengths. This feature suggests an interaction with tryptophan and an unfolding of the proteins. The activity increasing effect at low concentrations and activity reducing effect at higher concentrations (3 to 4 mol/l) of SCN^- on a series of enzymes that metabolize pharmaceuticals such as hydroxylases, NADPH-cytochrome c-reductase, NADH-cytochrome c-reductase, NADPH-cytochrome-P 450-reductase or the UDP-glucuronyltransferase (*Vainio, 1973*). In addition, the ATP-ase activity changes that were observed to occur with a KSCN-mediated attachment or an exchange of the subunits g_1, g_2 or g_3 with myosin, are coupled to a 0.6 to 0.8 molar SCN^- concentration (*Okamoto and Yagi, 1977*). At a high concentration range (0.3 to 3.0 mol/l) SCN^- alkali salts reduce the activity of myosin-ATPase, of trypsin, of lactate-dehydrogenase and of fumarase stronger than other anions within a serie of neutral salts (*Warren, Strowring and Morales, 1966*). Alongside reducing the activity of different enzymes via interaction with neutral salts they also destroy the structure of macromolecules which can be shown with physical methods.

Some effects of thiocyanate like the anti-microbial and the thyrostatic effects can be traced back to metabolites of SCN^- like the thiocyanogen ($(SCN)_2$), hypothiocyanate ($OSCN^-$ ('poisoning') of which the formation can be traced to peroxidase-activity. The formed products are able to react strongly with the amino acids in proteins. The conversion of SH-groups and the peroxidase-

influence are described by *Thomas* (Chapter 6). This reactivity of SCN^- on functional groups in proteins makes it possible for a manifold of functional changes of particular specificity including the antimicrobial effect to appear plausible.

Within the lyotropic series ($SCN^- > I^- > NO_3^- > Br^-$), SCN^- is the most effective anion with a potential effect on muscle contraction causing stimuli (*Sandow, 1965*), with a loosening of the membrane structure comes a facilitated release and facilitated influx (*Kamm and Casteels, 1979*) or a blockage of microsomal uptake (*Ebashi, 1965*) of the activator-calcium. With this SCN^- proves itself as an antagonist for myo-relaxants (*Bowman, Khan and Savage, 1977*). SCN^- has a similar effect in the marrow of the suprarenal body where concentrations of $1.5x10^{-2}$ mol/l effect a 50% reduction in microsomal calcium uptake *(Poisner and Hava, 1970*). The result of the so increased intra-cellular calcium level is an increased secretion and release of transmitters by the sympathetic nervous system.

These examples show that SCN^- is certainly capable of triggering multiple and specific effects in complex biological processes that are of significant meaning.

3.3 Influence on transport processes in biological membranes

(*Heidrun Paetzelt*)

Mechanisms of ion and organic substance uptake into the cells are of fundamental significance for macroorganisms as well as microorganisms. Physiologically meaningful cations and molecules cannot generally diffuse freely through cytoplasmic membranes but are coupled to transport mechanisms that themselves are connected to the energy metabolism of the cell.

Anions are generally regarded as freely permeable, however HCO_3^{--} is an exception. The diffusion potential of SCN^- is higher than for other anions ($SCN^- > Cl^-$ > glutathione > SO_4^{--}; *Carter-Su and Kimich, 1979*). It is known that SCN^- can increase the transmembrane potential of cells (see *Redmann*, Chapter 4).

Particularly well investigated are the conditions that form the basis for the active transport of Na^+ and K^+ across the plasma membrane. The catalytic enzyme, the Na^+, K^+ ATPase, has also a significant meaning for the transport of monosaccharides and amino acids. It is found in membranes of the small intestine, the kidney and in mitochondria. There are indications in the literature that SCN^- influences such material transfer. In this way *Luecke and colleagues* (1978) were able to show in experiments on brush border membranes of vesicles of the human small intestine that SCN^- increases the transport of D-glucose in the presence of a Na^+-gradient. It remains uncertain here whether SCN^- has an effect through the enzyme or through a change in the transmembrane potential.

SCN^- is more permeable than Cl^- for instance and could cause a hyperpolarization of the cell membrane (*Murer and Hopfer, 1974; Evers, Murer and Kinne, 1976*). Other authors found too

that on the brush border membrane of small intestine epithelial cells D-glucose is taken up exceedingly in the presence of a NaSCN-gradient (*Ohsawa, Kano and Hoshi, 1979*). The rheogenic Na^+-pump causes, similar to SCN^-, an increase of the membrane potential and this could be the driving force for the Na^+-coupled transport of sugars and amino acids (*Carter-Su and Kimmich, 1979*).

As mentioned before, amino acids can also enter cells through a Na^+ dependent transport system. Basic amino acids, proline, hydroxyproline, neutral amino acids and amino acid derivates shall be subject to an active transport. Among the neutral amino acids only the system A and the system ALC are Na^+ -dependent whereas the transport of the system L is based on simple diffusion processes (*Robinson, 1976*). *Syme and Levin* (1976) could prove that under the influence of KSCN in fact the maximum transport potential difference for the electrogenic valine-transfer increases in the rat jejunum. However the amino acid was not taken up faster. Valine-uptake is not coupled to a Na^+-gradient. The uptake of gamma aminobutyric acid into membrane vesicles of rat brain is increased by SCN^- if the Na^+-gradient provides the main driving force for the transport (*Kanner, 1978*).

In plasma membranes occurs in addition to the Na^+, K^+ -ATPase an anion-sensitive ATPase that is particularly stimulated by HCO_3^-. This enzyme is inhibited by SCN^-. OCN^- and SCN^- possesses the same inhibitory effect for the enzyme originating in the small intestine of the rat (*Humphreys and Chou, 1979*). The anion-sensitive mitochondrial ATPase of the rat heart also experiences an inhibition through SCN^- (*Ivashchenko, 1978*).

So far it was assumed that SCN^- like other anions was diffusing into cells. This diffusion can be facilitated by changes in the electrical potential of the cell. During the cleavage of ATP on the plasma membrane of neurospora by a membrane bound Mg^{++}-dependent ATPase the cell potential increases significantly (inside +) and the uptake of SCN^- is increased (*Scarborough, 1976*).

SCN^- was taken up together with H^+ on brush border vesicles of the rat small intestine. *Liedtke and Hoper* (1977) see this as a guiding post for SCN^-. In connection with the Mg^{++}-, ATP-dependent transport of catecholamines a combined uptake of SCN^- and H^+ occurs in chromaffin vesicles in cow brain (*Holz, 1978*).

There are indications that SCN^- as a pseudo-halogenide competitively inhibits the uptake and transport of halogens. The inhibition of the Na^+, K^+-dependent iodine-uptake can possibly be based on that (*Wolf, 1964*). In the mucous membrane of the frog stomach SCN^- inhibits the transport of Cl^- (*Takeguchi and Horikoshi, 1976*). In microsomes of the mucous membrane of the pig stomach SCN^- inhibits the Mg^{++}-dependent, K^+-stimulated ATPase which catalyses the intra vesicular H^+ - ion transport. In contrast, SCN^- has a stimulating effect on the enzyme after pre-incubation with valinomycin (*Lee and colleagues, 1979*).

SCN^- can have an inhibitory effect on the active transport of Ca^{++} that is a prerequisite for muscle contractions. Under the influence of SCN^- intracellular Ca^{++} accumulates. A stable bound is produced in the overlapping region between actin and myosin fibrils which results in a prolonged contraction (*Lyudkowskaya and Samasudova, 1979*).

The lipophilic SCN^- is increased in its effectiveness if cholesterol is stored in the cell membrane. It is then in a position to effectively influence more the electro-chemical gradient of the cells. It was shown that the storage of cholesterol in the cell membrane of bacterio-rhodopsin vesicles destroys the H^+-gradient (*Labelle and Racker, 1977*). This opens the possibility to look for an explanation for the anti-bacterial effect.

In investigations of the mechanism of action of the lacto-peroxidase, SNC^- - H_2O_2 systems was proven that the sugar transport in bacteria which is catalyzed by a membrane bound phosphotransferase is inhibited by SCN^- possibly through a blockage of functional SH-groups (*Mickelson, 1977*).

4. Toxicology

4.1. Animal experimental findings

4.1.1 *Acute toxicity*

The minimal lethal dose of NaSCN varies with the animal species and is about 500 mg/kg for oral application (*Garvin, 1939*).

In the guinea pig the lethal dose is 200 to 400 mg/kg (*Nichols, 1925*), in the rabbit 200 mg/kg after 48 h in oral administration and 150 mg/kg after 4 days in subcutaneous administration (*zit. Herbst, 1953*).

Female dachshunds (body mass 8.5 kg) received Rhodalzid (1 g Rhodalzid = 192 mg HSCN) with the following dosage regime: on the 1^{st} day 1 g, on the 2^{nd} day 2g, on the 5^{th} day 3 g, the 6^{th} day 4 g and on the 8^{th} day 5 g (total dose therefore 2.88 g HSCN). At this dosage the body mass remained unchanged as well as the general condition and bowel movement. A single application of 33 g NaSCN lead to tranquilization, pulse increase and convulsive twitching lasting 3 to 4 h after which a full remission is achieved (*Podcopaew, zit. Dalmady, 1912*). The lethal dose for i.v. administration is 300 mg/kg (*Goldstein and Holburn, 1949*).

KSCN is 5 times more toxic in i.v. administration in the mouse than NaSCN (LD_{50} 88 mg/kg and 483 mg/kg, respectively) for which the burden lies with the toxic K^+. This relationship was not observed in oral administration (LD_{50} mouse 594 and 598 mg/kg, respectively, LD_{50} rat 854 and 765 mg/kg, respectively; *Nichols, 1925*).

Goldstein and Rieders (1951) determined an arterio-venous O_2 -difference of 7.0 to 1.6 mg/100ml during acute intoxication of dogs which suggests a respiratory blockade. According to their opinion the cause could release cyanide from the SCN-metabolism. A more recent study by *Smith* (1973) argues against this assumption. He administered mice sodium thiosulfate i.v. at a dose that should reduce the LD_{50} of NaCN to a quarter. A protective effect was not provable through the application of 526 mg NaSCN/kg i.p.. Obvisouly, SCN^- exercises a central neurotoxic effect (*Wood, 1975*).

4.1.2 *Chronic Toxicity*

The repeated oral administration of 100 mg SCN^-/kg or more in dogs leads to progressive weight loss, apathy, hanging of the head, ataxia and finally death (*Anderson and Chen, 1940*). Toxic SCN^- doses administered to dogs over 3 months lead to microcytic anemia, reduction of blood cholesterol and total serum protein, damages of the bone marrow and spleen (*Lindenberg, Wald and Barker, 1941*) as well as a blockage of iodine-uptake into the thyroid gland (see *Michajlowskij*, Chapter 12). *Jorke and Beickert* (1955) could definitively confirm the anemia causing effect (aplastic anemia) with daily dosages of 0.1 to 0.2 g KSCN/kg KM for applications over the course of 63 to 96 days in dogs. A clear left-shift is present in the leucopoiesis in the bone marrow whereas eosinophils are completely absent. At the same time the lymphatic reticulum cells are increased. These findings are a further indication that SCN^- can intervene in the immune system. Diffuse fine-drop adiposity with necrosis and minimal bleeding are seen in the liver. In the kidneys, epithelia showed an oblique swelling, isolated also a discharge of blood from the glomerulus into the capsule and the away leading sling. Additionally goiter developed.

Nowinski and Pandra (1946) injected chick eggs with 0.3 to 0.7 ml of a 0.1, 0.5 or 1 mol NaSCN/l solution and observed malformations in the embryos. Equally, abnormal larvae were induced through the treatment of immature eggs of ascidians (*Ortolani, 1969*). The SCN^- forming nitroprusside sodium showed no teratogenic effect in Wistar rats and rabbits (*Ivancovic, 1979*). It seems somewhat questionable if these findings can be transferred to human. The fact that NaSCN significantly reduces the through cyclophosphamide induced mutation rate of bone marrow cells in mice (*Kramer and colleagues*, in preparation) would indicate that the supply of SCN^- at physiological concentrations could unfold a protective effect against teratogenic poisons. This would also be in agreement with the anti-tumor effect (see section 1.5).

4.2 Side effects in humans

Most of the toxicological data originate in the era of the treatment of hypertension with thiocyanate.

The evaluation of older literature is difficult in that in clinical publications often data is missing regarding the applied method and the time point of SCN^--determination following the SCN^-

administration as well as base factors for example compared to a healthy subject group. Furthermore, it is often impossible to determine which SCN^--salt was administered. Taken together it is noticeable that the SCN^- levels are high after SCN^--administration in earlier investigations.

From the application of nitropursside sodium

$$Na_2[Fe(CN_5]NO \cdot 2\ H_2O$$

modern and reliable even if not completely consistent data are available for the SCN^- toxicity (*Palmer and Lasseiter, 1975; Höbel, Kreye and Raithelhuber, 1976; Japp, Wissler and Baumann, 1978; Schulz, Döhring and Rathsack, 1978; Bödigheimer, Nowak and Schoeborn, 1979*).

With respect to SCN^- developed through biotransformation result the following relationships between dose and toxicity for SCN^- (*Bödigheimer, Nowak and Schoeborn, 1979*):

SCN- daily dose [mg]	Effect
125	Harmless
250	Possible intoxication from 10th
500	After 3 to 4 d toxic, after 10 d deadly SCN^- levels
1000	After 1 to 2 d toxic, after 3 to 4 d deadly SCN^- levels

Here the delay of SCN^- elimination due to kidney insufficiency needs to be accounted for as well as the toxic effect of nitroprusside sodium itself.

The smaller therapeutic width of SCN^- in the treatment of hypertension is unequivocally confirmed. The following blood levels show this:

SCN- level in serum [mg/l]	Effective range for the reduction of blood pressure
80 to 120 or 50 to 100	'therapeutic range'
100 to 200	Toxic range
>200	Lethal range

Remarkable are the strong individual variations so that in the so-called 'therapeutic range' for treatment of hypertension side effects such as dizziness, weakness, nervousness, headaches, sleepiness, exanthemas, loss of appetite and nausea as well as tremor of the hands are widespread (toxic threshold concentration). No subjectively experienced side effects were found in 12 voluntary subjects after the daily administration of 600mg NaSCN for 5 days (*Weuffen and colleagues, 1980*).

Forms of expression of SCN^- intoxication in humans. The first signs of an intoxication are nausea and vomiting, diarrhea and nervousness with a dose: 1st week 1g/day, 2nd week 0.6 g/day, 3rd week 0.3 g/day, starting 2 to 5 days after the first administration. However all symptoms were reversible (*Egloff, Hoyt and O'Hare, 1941*). In general elderly and those with a hypertonia obviously react more sensitively in respect to side effects than younger subjects (*Barker, 1936*).

Skin and Mucous membranes (Rhodan-Acne, Rhodan-Cold). The so-called 'Rhodan-Acne' and 'Rhodan-Cold' can develop in isolated cases at a maximum daily dose of 1 g NaSCN but they are reversible (*Pauli, 1903*). No further side effects were seen in these patients despite the high dosage for one to several weeks. Dermatitis can develop after SCN^- administration even if this is seldom (*Weis and Ruedermann, 1929*). Respective observations are present from *Takacs* (1926) with the use of KSCN however at the high dosage of 1 g/day. Here, dermatitis was observed in some patients after 9 days. *Baker and Brunstig* (1935) observed dermatitis in 1 case by application of NaSCN. *Hollander, Evans and Krugh* (1949) observed hair loss, petechial bleeding of the gums and dermatitis in the genital-anal region after treatment of hypertension with KSCN for several weeks.

Thyrostatic Effects. The contributions of *Michajlovskij* (Chapter 12) as well as *Saarivirta and Kreula* (see Chapter 13) report extensively about the thyrostatic effect of SCN^-.

Effects on the liver. According to *Rothlin and Cerletti* (1949), clinically recognizable defects in the liver are a rarity in case of long-term treatment of hypertension with SCN^-. *Jorke and Beickert* (1955) never saw any liver defects during treatment of polycythemia with KSCN (several impact-therapies over 2 weeks with doses of 600 to 1200 mg/day).

Hematopoietic system. An erythrostatic (anemia causing) effect starts at a SCN^- serum level between 100 and 150 mg/l (*Jorke and Beickert, 1955*). *Barker, Lindberg and Wald* (1941) as well as *Forster* (1943) observed also toxic anemia connected with damages to the bone marrow during long-term therapy of hypertension but never agranulocytosis has been seen so far. Thrombopenia was not determined by *Jorke and Beickert* (1955).

Effects on the CNS. *Bancrofft and Rutzler* (1932) observed breath acceleration, nausea, hallucinations, spastic paresis and cramps at a NaSCN dose of 100 mg 3 times/day in the 1st week, and 100 mg 2 times/day for the 2nd and 3rd week. Furthermore, disturbances in vision and hearing can appear at SCN^- levels of more than 150 mg/l in blood serum.

Idiosyncrasies. *Garvin* (1939) gives a literature overview on unfavorable side effects following NaSCN medication that however are rare (6 cases). He leads the lethal incidents back to idiosyncrasy. Since SCN^- is physiologically present in the organism and the case descriptions speak for an intoxication we would not regard the concept of idiosyncrasy as justified.

Further side effects. As a side effect of hypertension therapy with thiocyanate osteoporosis was observed by *Hinchey* (1948), this was however reversible.

Recently was ascertained that thiocyanate catalyzes the formation of nitrosamine during the conversion of nitrites with secondary and tertiary amines *in vitro* and *in vivo* (*Rudell, Blendis and Walters, 1977; Tannenbaum and colleagues, 1979*). Their carcinogenic effect could be shown in almost all cases in animal experiments. The catalytic SCN^- effect becomes particularly obvious at acidic pH conditions with a pH between 1 and 2 like it is prevalent in the stomach (*Boyland and Walker, 1974*) and can also become particularly obvious in smokers that have a 3 to 4 times higher SCN^- concentration in saliva than non-smokers (*Borgers and Junge, 1979*). Reaction-kinetic studies for the SCN^- influenced nitrosamine formation are present for among others ephedrine by *Kinawi and Schuster* (1978), for tetracycline derivates by *Röper and Heyns* (1977) or for aminopyrine by *Boyland and Walker* (1974). Meanwhile a tumor-inducing effect through the feed of secondary amines with sodium nitrite through SCN^- was not found (*Schweinsberg, 1975*). Furthermore, if one takes into consideration the epidemic relationship between carcinoma and the habit of smoking then in truth the influence on the overall processes in the organism can hardly have strong effects.

The therapy of a nevertheless occurring SCN^- intoxication is treated with hemo-dialysis.

5. Conclusions

NaNSC is recommended for administration of thiocyanate to optimize the immune response. It is better tolerated than KNSC. In animal experiments NaSCN had a stronger effect on the cell-mediated immune response than KSCN and NH_4SCN (see *Weuffen*, Chapter 14).

The question regarding dosage for the immune optimization in humans is determined obviously by the envisaged goal of SCN^- administration. In immunization for example the following dosage scheme is possible: 1 time 150 mg NaSCN/day at 1 day before the immunization, on the day of the immunization and 2 days after. In long-term experiments in the sense of a therapeutic goal a dosage of 100 mg NaSCN/day over the course of weeks and in exceptions over months seems possible.

The perspectives of possible thiocyanate-application in human and veterinary medicine are described by *Weuffen* (see Chapter 1). The great advantage of SCN^- administration is that normally in the organism the present substrate is given and therefore sensitization is not possible and allergic side effects can not develop. Toxic side effects as well as resistance (habituation) hardly need to be feared in the application at dosages that influence the immune response favorably.

The protective effect on the liver and the anti-mutagenic effect as observed in animal experiments open further possibilities for the SCN^- administration that cannot be foreseen at this point in time.

6. Listing of the cited preparations

Asthmolytan ® (see also Chapter 1; VEB Pharmamed Naumburg)

DL-2-imino-3.4-dimethyl-5phenyl-thiazolidinhydrothiocyanate	0.015 g	
DL-ephedrine hydrothiocyanate	0.015 g	/ tablet
Caffeine	0.075 g	
Phenazone	0.3 g	

Boro-Weidnerit [1)]
Weidnerit-gel with the addition of boric acid

Cevidan-Gel [1)]
Citric acid-viscose, thiocyanate-containing and foam forming

Dairin [1)]
2.4-diamino azo benzol hydrocyanate 0.1 g/tablet

Jodana [1)]

Ammoniumthiocyanate	4.2 g
p-chlorine-o-xylenol	2.0 g
p-chlorine-m-xylenol	0.5 g
Iron (III) bromide	2.0 g
Ethanol (95%)	50.0 g
Distilled water	add 100.0g

Mucidan ® Cough-Tea (Kali-Chemie, Hannover, BRD)
Extracts of different therapeutic plants that are common for cough-tea preparation with 0.8 g ammonium thiocyanate, from that use 1/25 for 1 cup of tea

Mucidan ® Nose-Ointment (Kali-Chemi, Hannover, BRD)

Calcium salt of methenamine thiocyanate	3.0 g
Boric acid	3.0 g
Camphor	0.5 g
Menthol	0.05 g
Ointment base	add 100.0 g

Mucidan ® Tablets (Kali-Chem, Hannover, BRD)

Calcium salt of methenamine thiocyanate	0.05 g
Juice of sweet wood	0.02 g
Ammonium chloride	0.0063 g
Etheric oils	

Mucidan ® (Kali-Chem, Hannover, BRD)

Potassium thiocyanate	21.3 g
Gelatin-buffered formaldehyde	12.1 g
Solvent	add 100.0 g

Otrhomin [1], see **Rhodovet** ® (see Chapter 1, formula 1-1)

Reoxyl 'Tosse' ® [1]

Urea	4.5 g
Sodium thiocyanate	0.5 g
Ointment base free of mineral oils	add 100.0 g

Rhodan-Calcium-Diuretin ® [1]

Calcium-Diuretin	0.5 g
Potassium thiocyanate	0.1 g

Rhodapurin ® [1]

Theobromide	0.1 g
Betain – hydrothiocyanate	0.077 g

Rhodasept ® [1]

In the original company brochure it was named rhodan hydrogen acid preparation (powder, tablets at 0.5 g)

Rhodobazid-Weidner ® [1]

Thiocyane-hydrogen acid	1.0 g
Amylopektine	
Grape sugar	add 100.0 g

Rhodocrema ® [1]

Plant mucilage mixed with 4.2% thiocyan-hydrogen acid

Rhodoforman ® [1] see **Rhodovet** ® (see Chapter 1, formula 1-1)

Rhodovet ®-**Pulver** (powder, see also Chapter 1, formula 1-1; VEB Serumwerk Bernbug/DDR)
Octa-methenamine-octa-hydrothiocyanate-ammoniumsulfate

Sepso-Tinktur ® (Hoffmann and Summer, Koenigsee/DDR)

Aluminum chloride	10.0 g
Iron (III) – chloride	1.2 g
Ammonium thiocyanate	1.75 g
Salicylic acid	2.8 g
Camphor	0.6 g
Ethanol	48.2 g
Distilled water	add 100.0 g

Weidneritgel ® [1)]

Aluminumoxide-thiocyanate combination and plant mucilage

As thickener

Alkalisulfate	13.0 g
Alkali bisulfate	37.0 g
Alkali thiocyanate	36.0 g
Aluminum sulfate	14.0 g

[1)] no longer commercially available

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Ambrosius, H., W.-D. Jülich, H. Fiebig and W. Weuffen: Antigen-Antikörper-Reaktion in vitro. Chapter 15. 276-283

Weuffen, W., H. Blohm and L. Rotermund: Protektive Anwenund von Thiocyanaten bei der industriemäßigen Tierproduktion. Chapter 16. 284-302

Annex 8

Ninety-seven pages of Annex 8 have been removed in accordance with copyright laws. The removed reference citations are:

Chandler, J., and Day, B. (2012). Thiocyanate: A potentially useful therapeutic agent with host defense and antioxidant properties. *Biochemical Pharmacology*. 84(11): 1381-
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Wang, P., Lin, C., Wu, K., and Lu, Y. (1987). Animal safety testing on preservatives used in the natural lactoperoxidase system for milk preservation. *Scientia Agricultura*
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saliva of newborn infants. *Infect. Immun.*, 11, 1210

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Bafort, F., Parisi, O., Perraudin, J.-P., and Jijakli, M. H. (2014). Mode of action of lactoperoxidase as related to its antimicrobial activity: a review. *Enzyme Research*, vol.
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60

Annex 9

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OPTIMAL CONDITIONS FOR THE $OSCN^-$ IONS PRODUCTION

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Table of contents

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Chapter I
Basic evaluation for the mechanism of the Lactoperoxidase System

A) Introduction

The Lactoperoxidase System (LPS) present naturally in the milk has been widely described in the scientific literature. This system is constituted of one enzyme and two substrates: the lactoperoxidase and its substrates thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2). An antimicrobial activity of the LPS essentially conducted towards the Gram negative has been observed. This one would be linked to the presence of the $OSCN^-$ ions resulting of the oxidation of the thiocyanate ions by the hydrogen peroxide in presence of the lactopeoxidase (LP).
The equation, very simplified, of the reaction of SCN^- oxidation in this system has been proposed as follows:

$$H_2O_2 + SCN^- \xrightarrow{LP} H_2O + OSCN^-$$

The production of the $OSCN^-$ ion could be realized in two different systems

1st system:

H2O2 – Lactoperoxidase system: H_2O_2
Lactoperoxidase
Thiocyanate

2nd system:

Glucose/ Glucose oxidase – Lactoperoxidase system: Glucose
Glucose Oxidase
Lactoperoxidase
Thiocyanate

In order to understand the mechanism of $OSCN^-$ production, we have studied the influence of each ingredient present in both systems mentioned here above.

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B) 1st system:

Hypothiocyanite

The oxidation of the thiocyanate ions, in the H_2O_2 – Lactoperoxidase system is almost instantaneous. The oxidation product is unstable in water solution and decomposes quite rapidly.
The increasing of the H_2O_2 concentration has for foreseeable consequence to increase the oxidation product concentration. However, the increasing of the concentration of this last one is not in the same ratio that the concentration of H_2O_2, which has been added (figure 1).
The rate of the decomposition of the oxidation product of the SCN^- ion is almost the same for the three H_2O_2 concentrations used. Its value is 4,7 µM/min (figure 1)

Thiocyanate

The decomposition of the oxidation product, after initial adding of the H_2O_2 regenerates almost completely the SCN^- concentration of the beginning (figure 2). Nevertheless, for further additions of H_2O_2 to the same mixture, the SCN^- regeneration is not performed at 100% and decreases as one goes along that we add H_2O_2 in the same solution (figure 2). In fact, it was demonstrated the appearance of several oxidized species such as $OSCN^-$, O_2SCN^-, O_3SCN^-, $(SCN)_2$... These species are not determined by the DTNB technique as it is the case for the $OSCN^-$ ions (Annex IX).

The production of the $OSCN^-$ is in relation with the SCN^- concentration at the beginning. The $OSCN^-$ ions decompose with the same speed for the 3 assays (Figure 3) and the regeneration of the SCN^- ions is all the more important that the initial concentrations of the SCN^- ions is important such as 80%, 70%, and 50% for SCN^- for solutions containing 80, 60 and 30 ppm of SCN^- respectively.

Lactoperoxidase

The influence of the lactoperoxidase concentration is relatively independent on the increasing of the concentration of the $OSCN^-$ ions. These results are in agreement to the fact that lactoperoxidase is a catalyser (figure 4).
In this experiment, the decomposition of the $OSCN^-$ ions presents a rate of 65% in favor of SCN^-. A new addition of H_2O_2 show that the reoxidation of remaining SCN^- (from the first decomposition of $OSCN^-$) give a much lower concentration of $OSCN^-$ that the one obtained in the first assay (figure 4).

Figure 1: Influence of the H_2O_2 concentration on the production of the $OSCN^-$ ions

System:

lactoperoxidase 40 ppm

SCN^- 120 ppm

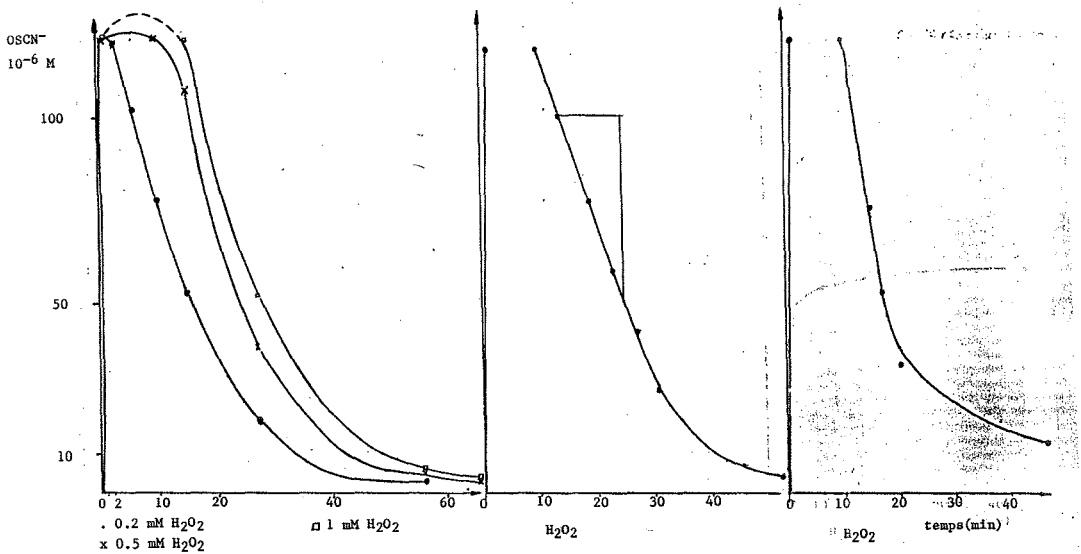


Figure 2: Influence of the H_2O_2 concentration on the oxidation of the SCN^- ions

System:

Lactoperoxidase 40 ppm

SCN^- 120 ppm

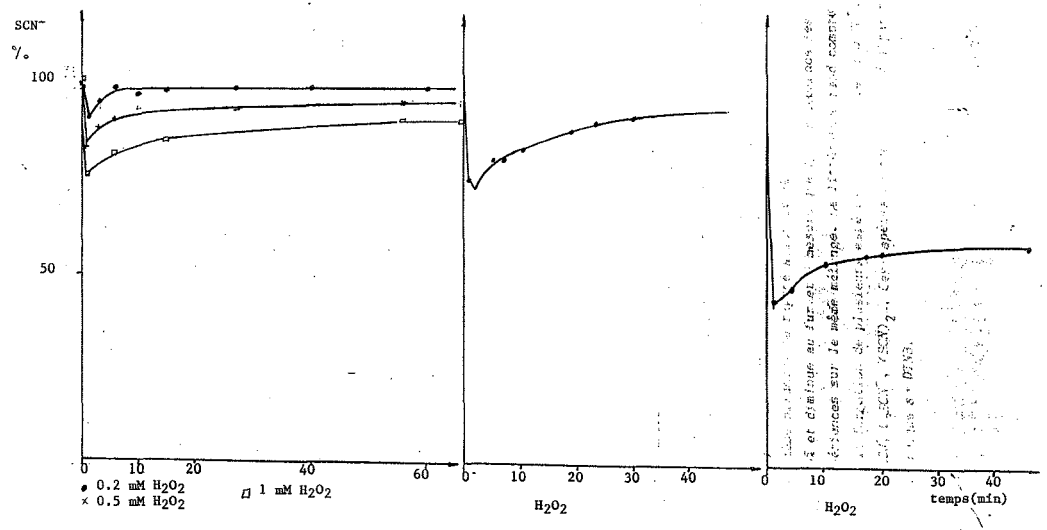


Figure 3: Influence of the SCN^- ion concentration on production of the $OSCN^-$ ions

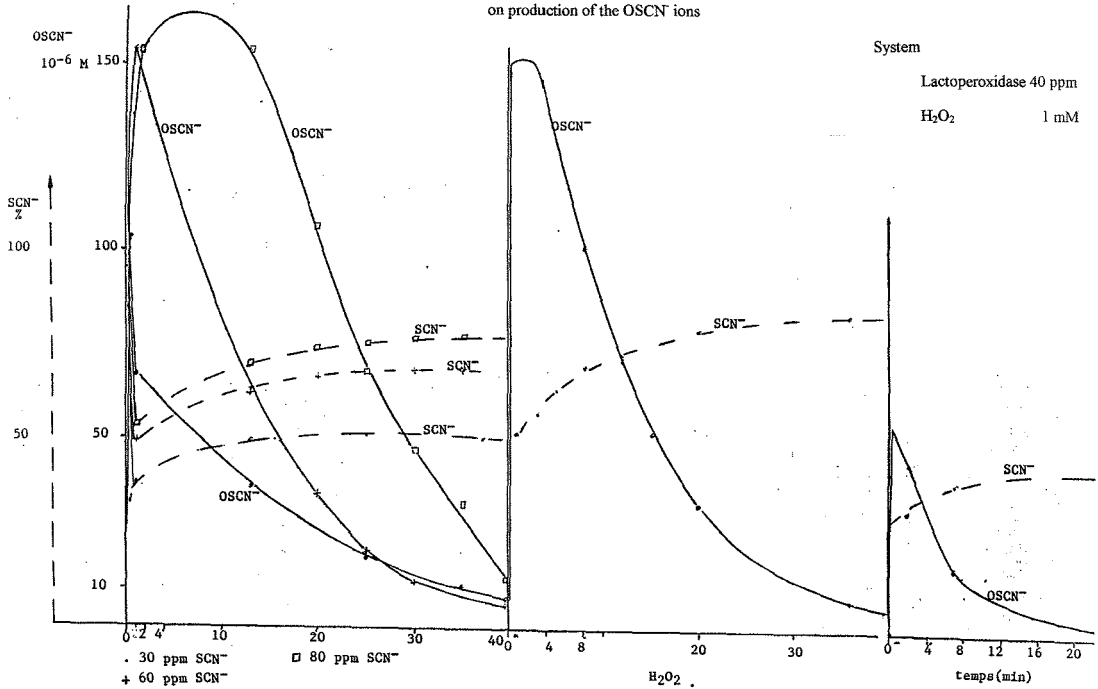
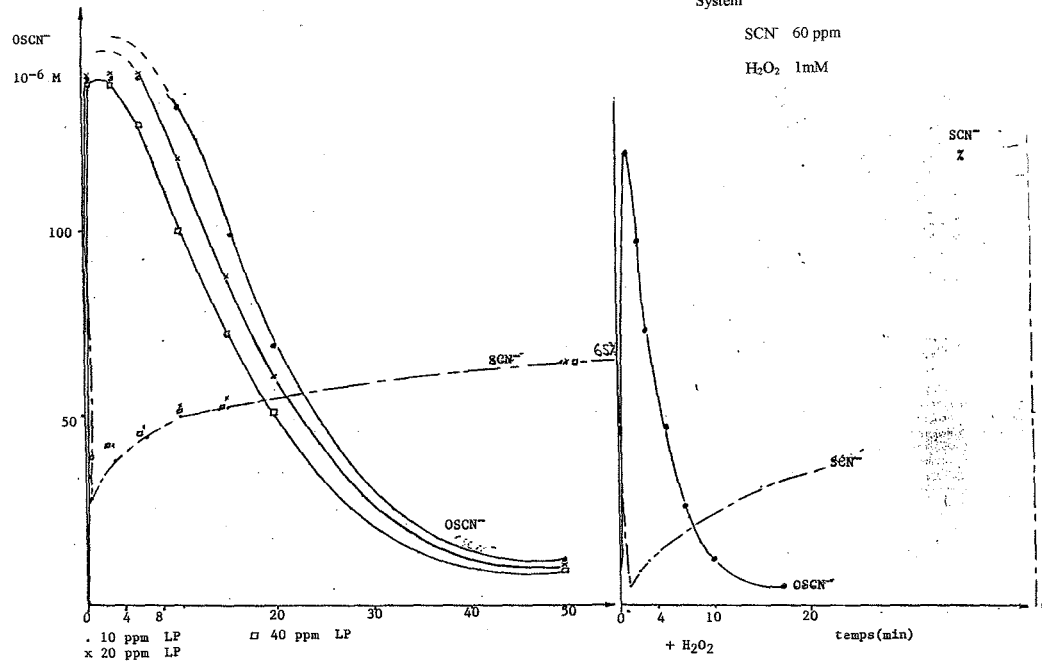


Figure 4: Influence of the Lactoperoxidase concentration on the production of the $OSCN^-$ ions



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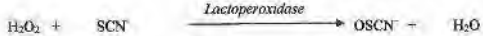
C) 2nd system



Glucose Oxidase

In the enzymatic system, the production of the hypothiocyanite ions is limited by the activity of the glucose oxidase.

The maximum of the OSCN- ions production is reached after 10 minutes. It was instantaneous in the 1st system (non-enzymatic system) due to the presence of the H_2O_2 in the beginning of the reaction

With the injection of oxygen, we can observe that more the concentration of glucose oxidase is important, more the concentration of $OSCN^-$ is important but the decomposition of $OSCN^-$ ions is much quicker since the concentration of glucose oxidase is important (figure 5).

However, under the same conditions, we observe that the decomposition of $OSCN^-$ is not done in benefit of the SCN^- ions. In fact, the SCN^- concentration decreases continuously in function of time (figure 6).

If we don't inject oxygen, we can observe a stabilization of the OSCN- ions at 50µM (figure 7) for a time exceeding 120 minutes.

SCN^-

With the injection of oxygen, we can observe that more we use a high concentration of SCN^- more the $OSCN^-$ ions production lasts for a longer time. We succeed to maintain a $OSCN^-$ concentration of 160µM after 60 minutes (figure 8). Here also, we can confirm that the decomposition of the $OSCN^-$ is transformed to another specie that SCN^- (figure 9).

Figure 5: Influence of the Glucose Oxidase on the production of the $OSCN^-$ ions

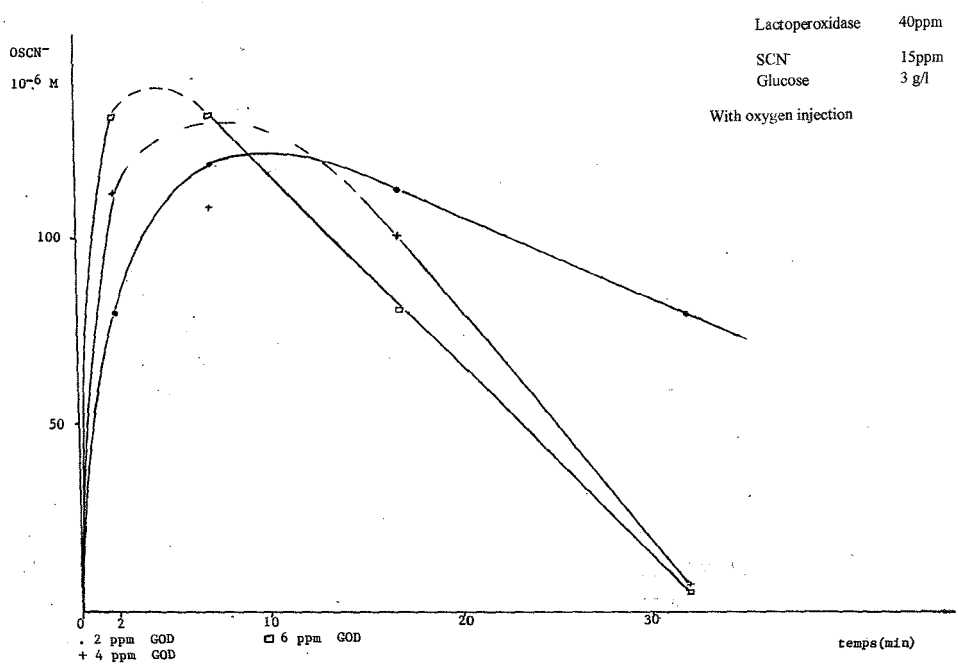


Figure 6: Influence of the Glucose Oxidase concentration on the oxidation of the SCN^- ions

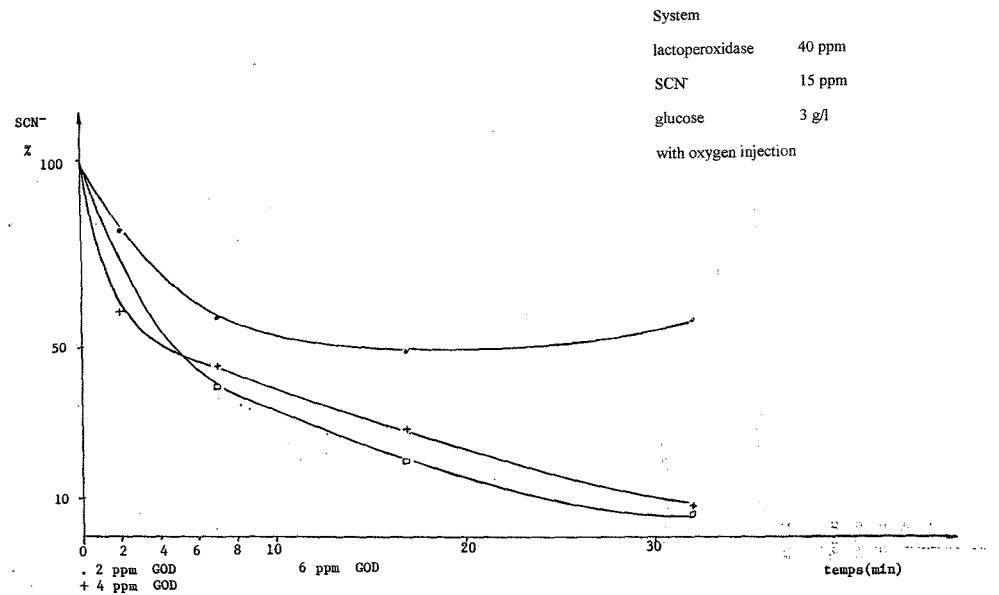


Figure 7: Influence of the Glucose Oxidase concentration on the production of the $OSCN^-$ ions

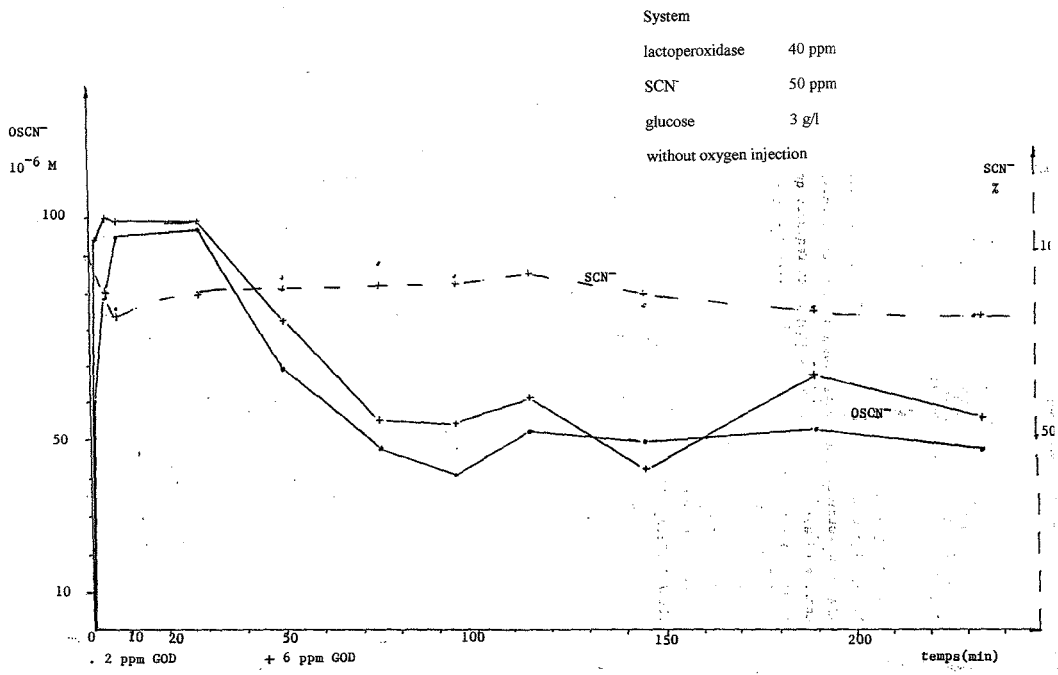


Figure 8: Influence of the SCN^- ions on the production of $OSCN^-$ ions

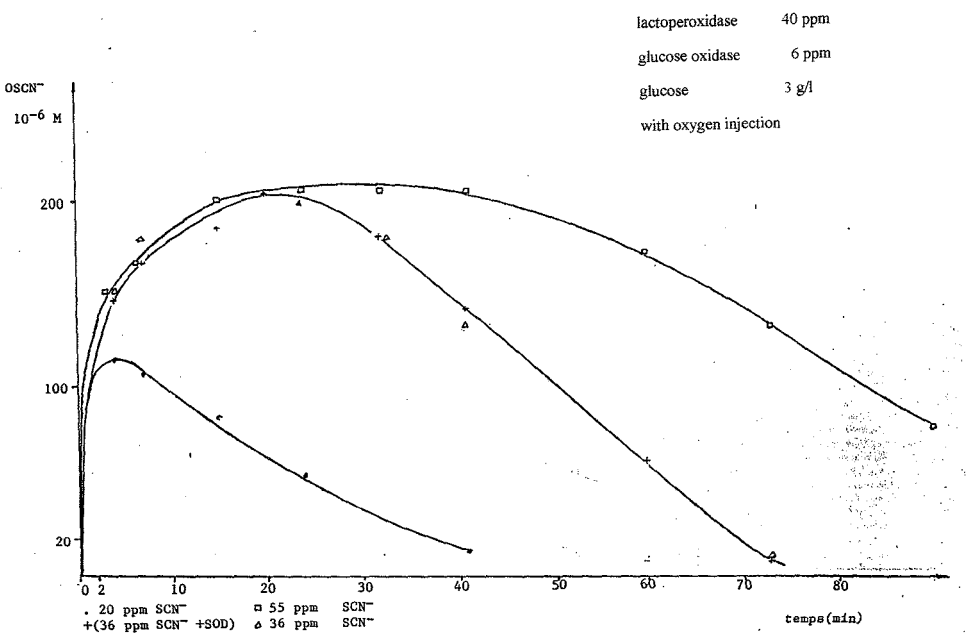
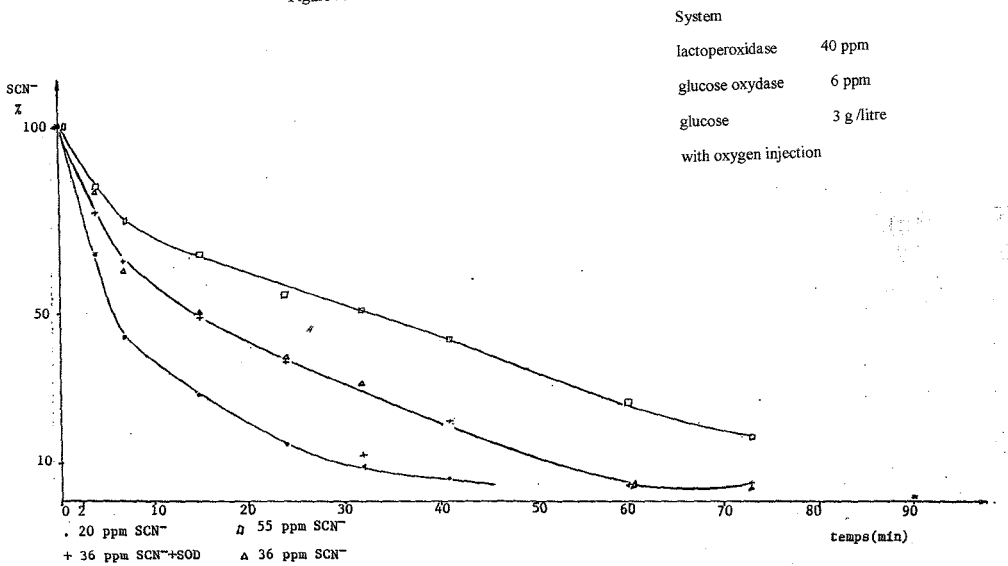


Figure 9: Influence of the concentration of the SCN^- ions



In absence of oxygen, the two experiments performed with 35 and 70 ppm SCN^- concentrations, demonstrate that the decomposition of the $OSCN^-$ ions is less rapid that in the 1st system and that with the injection of oxygen in the 2nd system (figure 10).

The conversion in $OSCN^-$ ions is only of 60% and of 85% after 180 minutes, for the initial SCN^- solutions of 35 and 70 ppm respectively. If we inject oxygen during the reaction, the $OSCN^-$ production starts again. Moreover, the SCN^- ion is no more regenerated (see at the right side of the figure 10).

Glucose

Here also, we can observe that more we use a high concentration of glucose more the $OSCN^-$ ions production last for a longer time. Since we inject oxygen, the concentration of $OSCN^-$ ions decreases rapidly (figure 11) which is confirmed in analyzing the SCN^- concentration in the same experiment (figure 12).

D) Conclusions

What is the system that we use, in both cases, the OSCN- ions production is dependant to the SCN^- ions concentrations, independent to the lactoperoxydase concentration which is considered as catalyser.

We can conclude also that if we want a longer time of the $OSCN^-$ ions production, that it is better to use a constant supply of H_2O_2 as it is the case of the 2nd system (enzymatic system) instead to use a fixed H_2O_2 concentration as it is the case of the 1st system (non-enzymatic system).

Moreover, without the bubbling of oxygen, we could increase the time of the OSCN- ions production in the 2nd system. It is clear that between the experiment using injection of oxygen and without the injection of oxygen, it was demonstrated that there is a problem of limitation of the oxygen dissolved available for the enzymatic system. It seems to appear in the case of limitation of oxygen and then a low H_2O_2 productivity, that that promotes the formation of the $OSCN^-$ species to the detriment of the more oxidized species such as O_2SCN^-, O_3SCN^-,.... The $OSCN^-$ which is formed can be decomposed progressively in SCN^-.

In the case where the dissolved oxygen concentration is no more limiting (injection of oxygen) the reaction between SCN^- and H_2O_2 will probably not stop to the step of the $OSCN^-$ ions but will continue to the more oxidized species such as O_2SCN^-, O_3SCN^-,.... ions production. These oxidized species will not be allowed to give back SCN^- but $NH4^+$, CO_2 and SO_4^{2-}.

In fact, the oxygen is also a substrate of the glucose oxidase. When we stop the injection of oxygen, we liberate the active sites of the enzyme and that lead to an increasing of the H_2O_2 production and then to an increasing of the $OSCN^-$ ions production.

Figure 10: Influence of the SCN- concentration on the production of the $OSCN^-$ ions in absence of oxygen

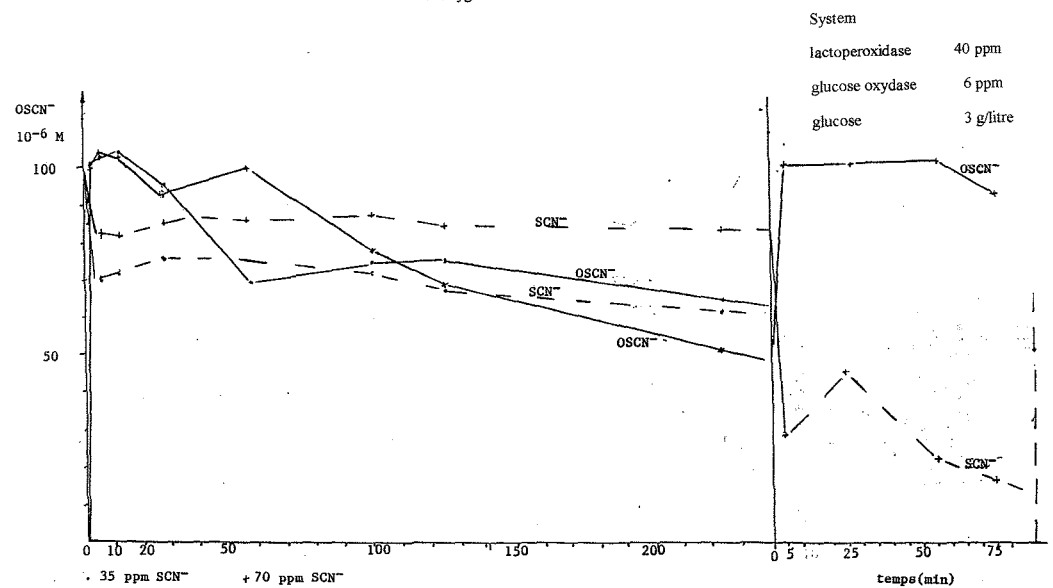


Figure 11: Influence of the oxygen on the decomposition of the $OSCN^-$ ions for different glucose concentrations

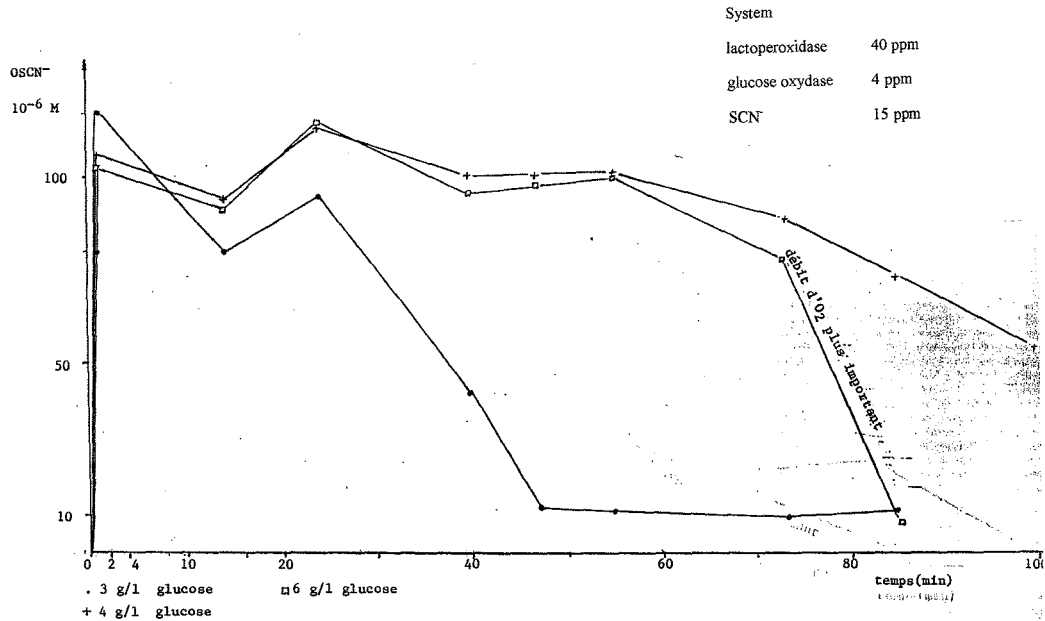
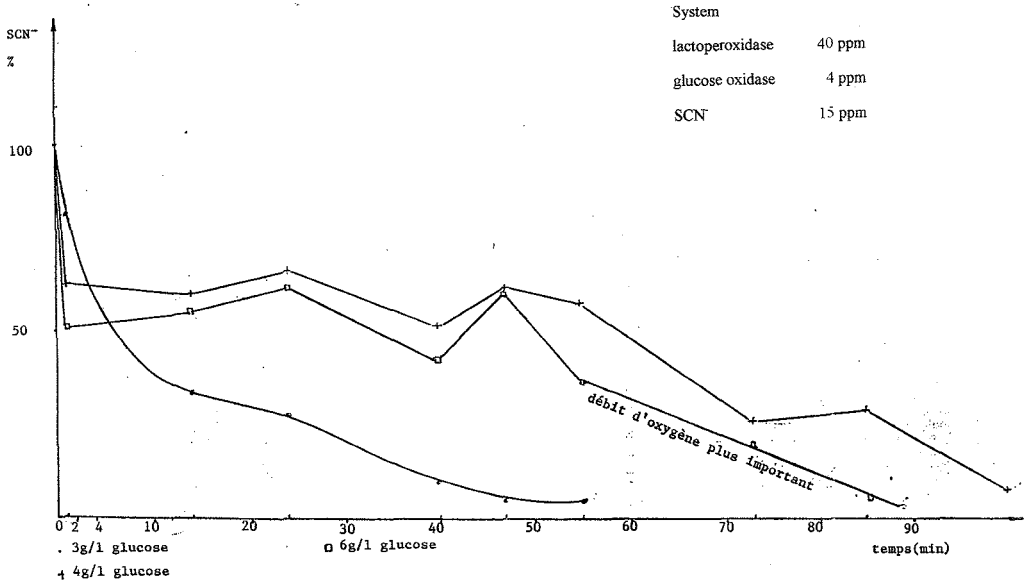


Figure 12: Influence of th oxygen on the oxidation of the SCN^- ions for different glucose concentrations



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We have also studied the influence of the anti-oxidants such as the superoxide dismutase and the rodoxan D20 on the evolution of the OSCN ions. As we can observe in the figure 13, the production of OSCN ions is not influenced by the presence of the anti-oxidants.

On the other hand, in presence of a microorganism, the regeneration of the SCN is no more observed. The decrease of the OSCN- ions concentration in the medium results to the consumption of the ions by the *Escherichia coli* (Figure 14).

Figure 13: Evolution of the $OSCN^-$ ions in presence of antioxidants

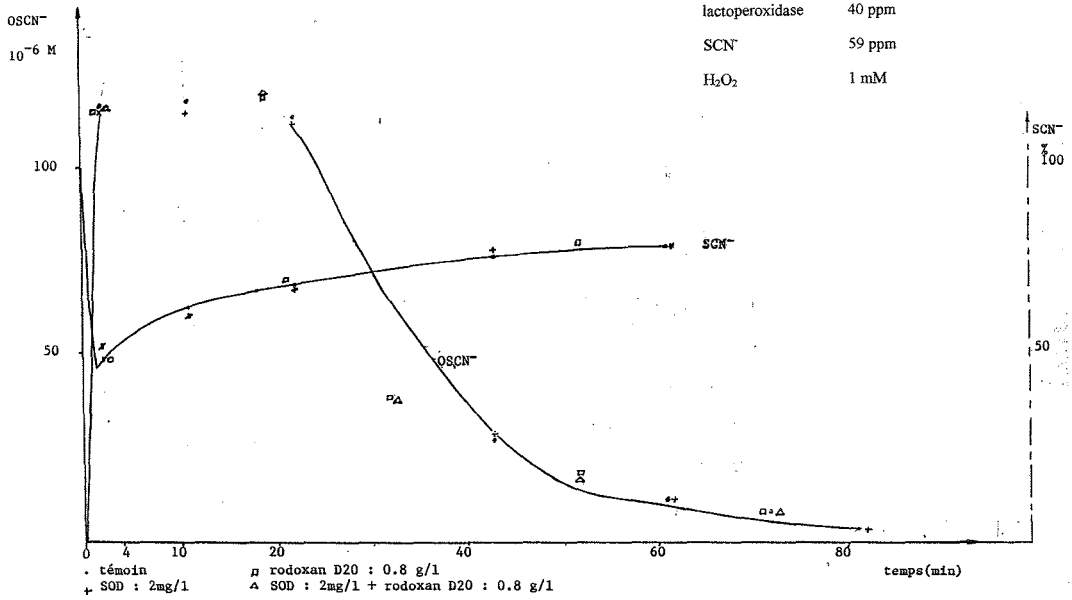
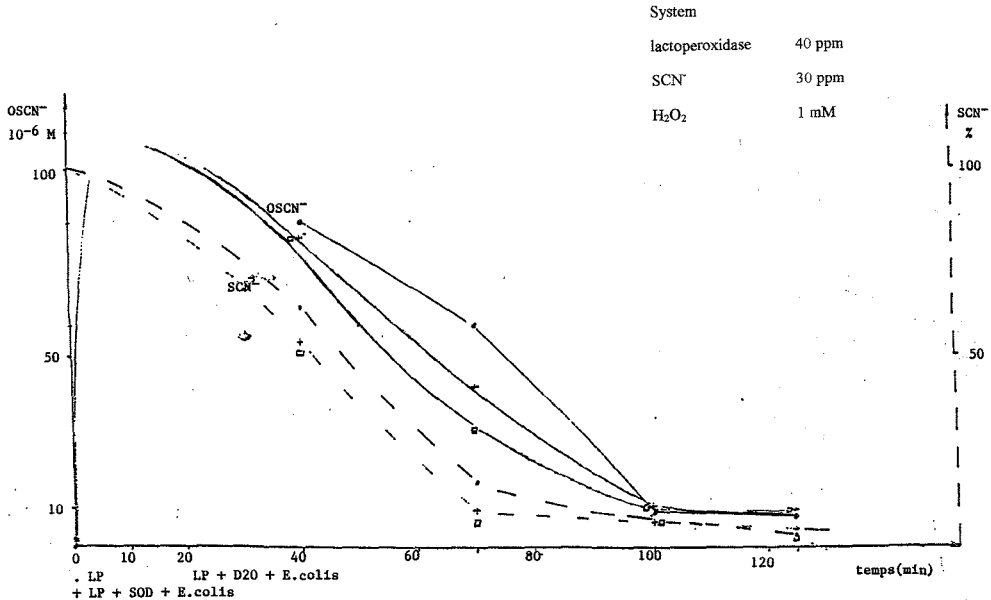


Figure 14: Evolution of the $OSCN^-$ ions in presence of Escherichia coli and antioxydants



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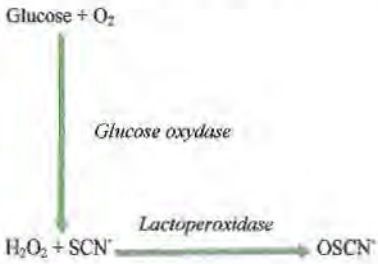
Chapter II
Determination of the optimal conditions for the $OSCN^-$ ions production

A) Introduction

The aim of this study was to determine the experimental conditions for which the $OSCN^-$ ion production is optimal.
The mechanism of the production is the following:



The parameters which have been followed are:

- The concentration of the lactoperoxidase, the glucose oxidase, the thiocyanate and the glucose
- The pH conditions
- The temperature conditions

The basic conditions are the ones which have been used in the tests performed for the cheese production: LP/SCN^-/GOD/Glucose: 25/50/2/4000 mg/liter

From these concentrations, some modifications have been done to determine exactly the best concentrations of the ingredients to get the optimal production of the $OSCN^-$ ions. Among the different formulations which have been tested, the composition LP//SCN^-/GOD/Glucose: 25/50/2/4000 mgr per liter, has shown the optimal production of the $OSCN^-$ ions and the maintenance of a plateau of the concentration.

For this concentration, we have studied the production of $OSCN^-$ in function of the pH and in function of the temperature. The maximal production of $OSCN^-$ has been obtained at pH7 and at a temperature of 20°C.

According to these conditions, in order to test the reproduction of the $OSCN^-$ production, 13 tests have been performed whose 10 tests during 1 hour (10) and 3 tests during between 5 to 7 hours.

The variations of the SCN^-, LP, GOD, Glucose are described in the following table.

Test performed at pH 7 and at 20°C

	Composition (mg/litre)			
	LP	**SCN^-**	**GOD**	**Glucose**
Δ SCN^-	25	10	2	4000
	25	25	2	4000
	25	100	2	4000
Δ LP	5	50	2	4000
	15	50	2	4000
	20	50	2	4000
	50	50	2	4000
	25	50	2	4000
Δ GOD/Glucose ½	25	50	1	2000
	25	50	4	8000
Δ Glucose en excès	25	50	2	20000

The $OSCN^-$ production at pH 7 and for the composition LP/SCN^-/GOD/Glucose: 25/50/2/4000 mg/litre has been studied in function of the temperature: 12, 20, 30, 37 and 50°C.

B) Methods of analyses

The activity of the lactoperoxidase and the glucose oxidase have been measured at it is described in the Annex 1 and 2 respectively

It is the same for the dosage of thiocyanate (SCN^-) ions and of the hypothiocyanite ($OSCN^-$) ions in the Annex 3 and 4 respectively.

C) Results

For each of these tests, the $OSCN^-$ concentration in µM have been presented in function of the time (minutes)

On the other hand, the initial speed of the $OSCN^-$ production (µ moles $OSCN^-$/min), the maximal concentration in $OSCN^-$ and the concentration measured after 60 minutes of reaction (µM) have been reported in graphic, in function of the modified parameters.

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Conditions

20°C – pH6 = $OSCN^-$ production in function of the time
25/50/2/2000 Graphic 1: 4 times the same assay
Graphic 2: average curve (value +/- σ

20°C – ΔpH = $OSCN^-$ production in function of the time
25/50/2/2000 Graphic 3 and 4

20°C – pH7 = $OSCN^-$ production in function of the time
25/50/2/4000 Graphic 5: 13 times the same assay
Graphic 6: average curve (value +/- σ)
Graphic 7: $OSCN^-$ production during 5 to 7 hours (3 assays)

20°C – pH7 = $OSCN^-$ production in function of the time
25/ΔSCN/2/4000 Graphic 8: influence of the SCN^- concentration
Initial speed, $OSCN^-$ max and $OSCN^-$ at 60 minutes in function of the SCN^- concentration: Graphic 9

20°C – pH7 = $OSCN^-$ production in function of the time
ΔLP/50/2/4000 Graphic 10: influence of the LP concentration
Initial speed, $OSCN^-$ max and $OSCN^-$ at 60 minutes in function of the LP concentration: Graphic 11

20°C – pH7 = $OSCN^-$ production in function of the time
25/50/ΔGOD/ΔGlucose Graphic 12: influence of the GOD/Glucose concentration
(GOD/Glucose = ½
Initial speed, $OSCN^-$ max and $OSCN^-$ at 60 minutes in function of
The ratio GOD/Glucose LP concentration: Graphic 13

20°C – pH7 = $OSCN^-$ production in function of the time
25/50/2/ΔGlucose Graphic 14: influence of the concentration in glucose

(Excess Glucose) Initial speed, $OSCN^-$ max and $OSCN^-$ at 60 minutes in function of the glucose concentration: Graphic 15

20°C – pH7 = $OSCN^-$ production in function of the time
25/50/2/4000 Graphic 16 : Influence of the temperature
Initial speed, $OSCN^-$ max and $OSCN^-$ at 60 minutes in function of the ration of the temperature: Graphic 17

20°C – pH6 = $OSCN^-$ production in function of the time
25/50/4/4000 Graphic 18: Composition for the cheese production
Concentration maximal: 200 µM in 5 minutes, follows by the decomposition to 100 µM in 60 minutes

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20°C – pH6 = OSCN production in function of the time
250/500/40/40000 Graphic 19: Concentrated Lactoperoxidase system ingredients: 10 times
Immediate production very high – quick decomposition – plateau at 60µM

20°C – pH6 = OSCN production in function of the time
25/50/2/4000 Graphic 20: influence of another H_2O_2 source: urea peroxyde
Immediate production very high – quick decomposition – plateau at a weak concentration (30µM at 60 minutes)

20°C – pH7 = SCN consumption in function of the time
25/50/2/4000 Graphic 21:

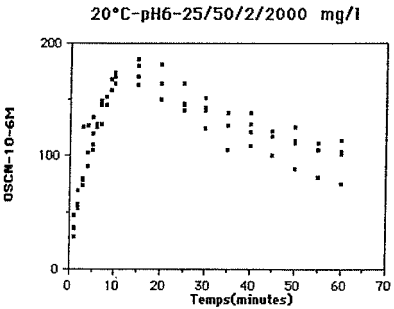
D) Remarks:

Stability of the lactoperoxidase: 100% of stability for all the tests performed (whatever the conditions of the lactoperoxidase system, the pH and the temperature)

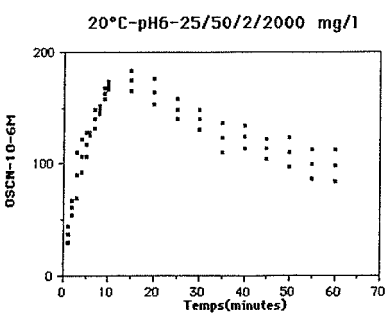
Concentration of glucose: not change in glucose concentrations, always sufficient concentration

Concentration in SCN: for the basic conditions such as 20°C, pH7, the composition 25/50/2/4000 mg/litre, a curve of consumption in function of time is represented in the graphic 21.

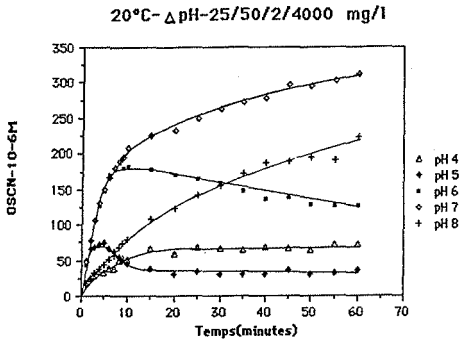
Graphique 1 (4 x)



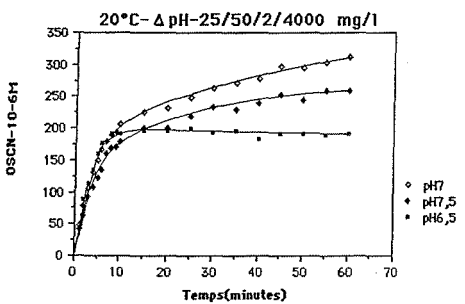
Graphique 2 ($\overline{m}$)



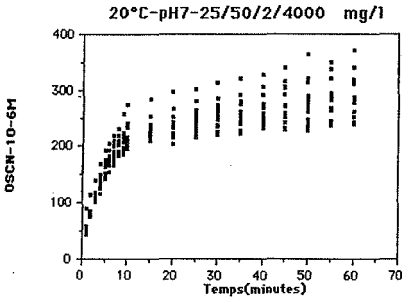
Graphique 3



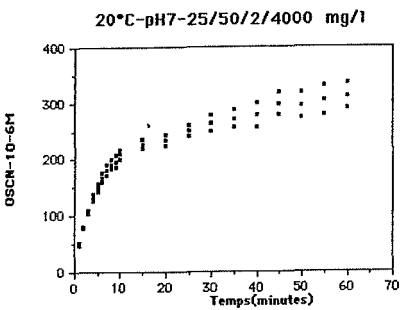
Graphique 4



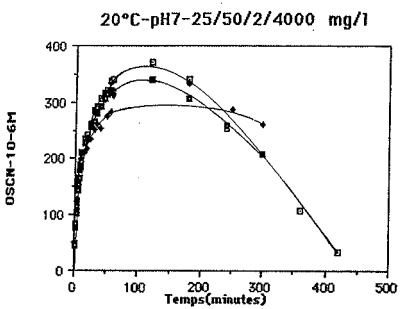
Graphique 5 (13 x)



Graphique 6 ($\overline{m}$)



Graphique 7 (long terme)



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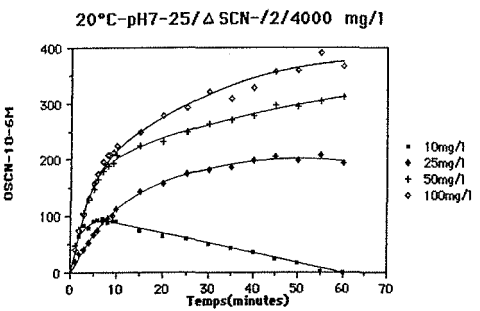

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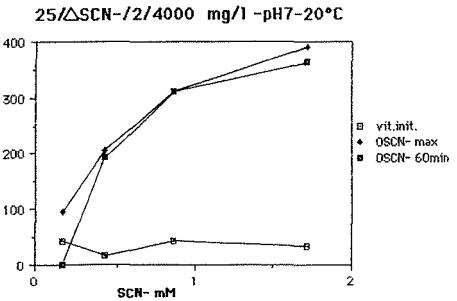
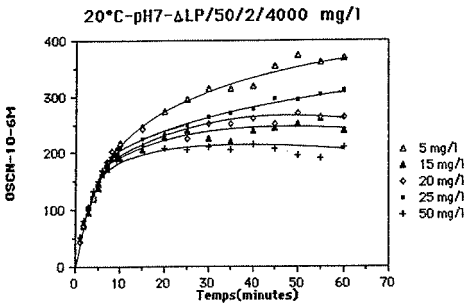


Graphique 10



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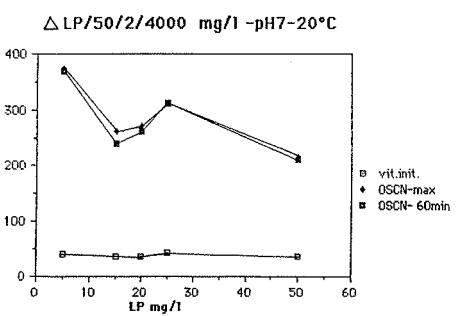

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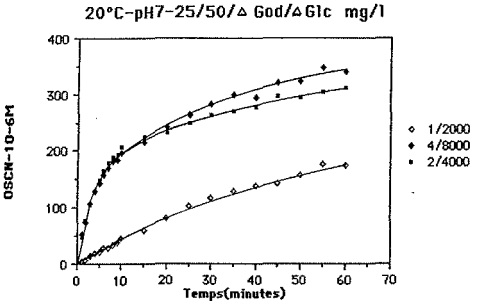

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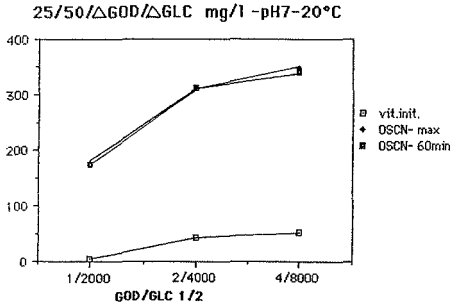
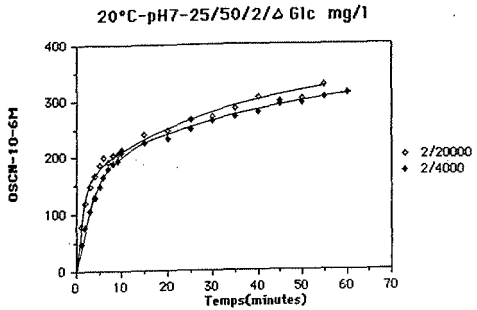


Graphique 14



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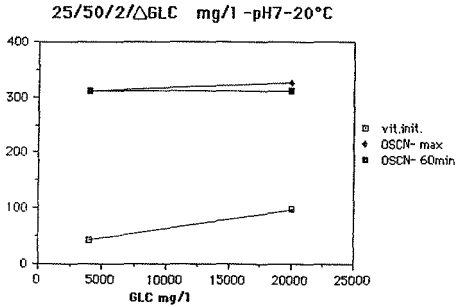

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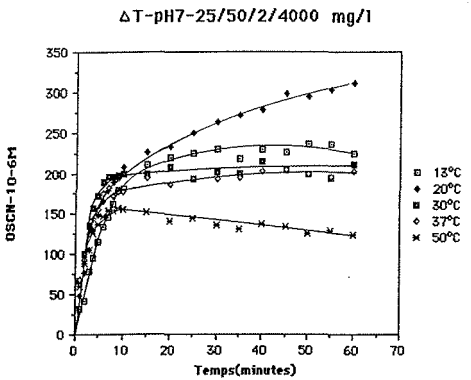
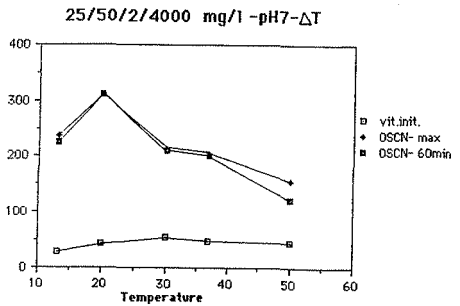
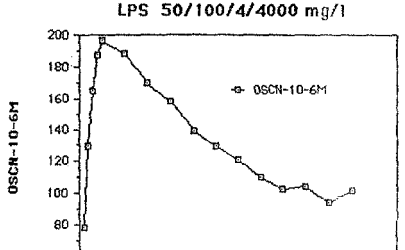


Graphique 17



1) $OSCN^-$ production at 20°C and pH 6, for the conditions LP/SCN^-/GOD/Glucose: 25/50/4/4000 mg/litre

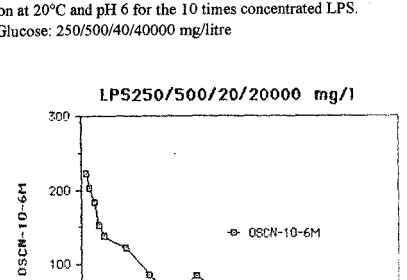
Graphic 18



Maximal concentration (200 µM) in 5minutes and after decomposition to 100µM in 60 minutes.

2) $OSCN^-$ production at 20°C and pH 6 for the 10 times concentrated LPS. LP/SCN^-/GOD/Glucose: 250/500/40/40000 mg/litre

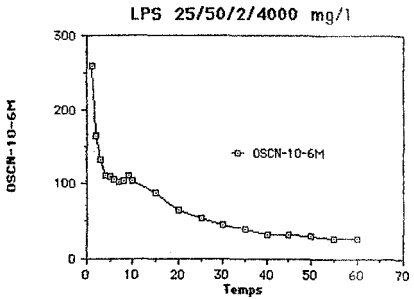
Graphic 19



Immediate production very high – quick decomposition – plateau of concentration at 60µM

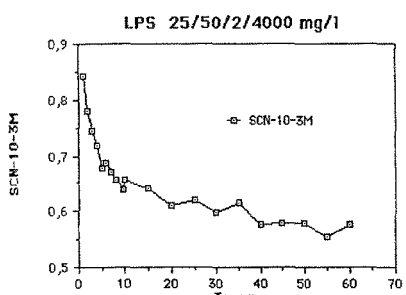
(rem: for the 100 times LPS, the $OSCN^-$ production is too high and it was difficult to measure).

3) $OSCN^-$ production at 20°C in presence of 2mM urea peroxide (2mM H_2O_2)
Graphic 20



Immediate production very high – quick decomposition – plateau of concentration very weak at 30µM

Graphic 21



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ANNEX 1

A. LACTOPEROXIDASE

DETERMINATION OF THE LACTOPEROXIDASE ACTIVITY

A.1. Principle

H_2O_2 + 2 ABTS ——LP——► 2 ABTS + 2 H_2O

A.2. Materials and reagents

- Spectrophotometer at 412 nm with a thermostat at 37°C
- 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)) : ABTS
- Sodium citrate buffer 50 mM pH 5.5
- Hydrogen peroxide at 30 %

A.3. Preparation of the reagents

- Incubation of the buffer *A* : sodium citrate buffer 50 mM pH 5.5 + ABTS 1 mM (50 mg ABTS in the buffer, qsp 100 ml)
- solution H_2O_2 0.1 M *B*: H_2O_2 at 30 % diluted 100 x (250 µl H_2O_2 in the H_2O, qsp 25 ml)
- Prepare newly these solutions, to be kept in a cold place (fridge)

A.4. Preparation of the enzyme

- 35 mg of LP in 10 ml sodium citrate buffer 50 mM pH 5 ([LP]= 3.5 mg/ml) keep the solution 1 h in the fridge
- Dilute 1000 x reconstituted enzyme *C* : 50 µl d'enzyme in H_2O, qsp 50 ml) To be prepared just before the measurements

A.5. Method

- Pipet 2 ml of *A* in a cuvette
- Incubate in a bath at 37 °C during 10 min
- Add 100 µl of *C* in the cuvette, mix in turning inside out the cuvette
- Add 2 µl of *B* in the cuvette, mix in turning inside out the cuvette
- Measure the O.D. at 412 nm at 1 min and 2 min

Control: 2 ml of solution *A* + 100 µl of *C* + 2 µl of water

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A.6. Activity calculation

$$\frac{\text{O.D 412 nm (2'-1') x dilution}}{\text{total volume x enzyme volume x enzyme concentration}} = \text{Units/mg}$$

That means:

$$\frac{\text{O.D. 412 nm (2' – 1') x 1000}}{\text{2.1 x 0.1 x 3.5}} = \text{units/mg}$$

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ANNEX 2

A. GLUCOSE OXIDASE (GOD)

DETERMINATION OF THE GLUCOSE OXYDASE ACTIVITY

A.1. Principle

Glucose + O_2 ——GOD——▶ H_2O_2 + acide gluconique

H_2O_2 + 2 ABTS ——LP——▶ 2 ABTS + 2 H_2O

A.2. Materials and reagents

- Spectrophotometer at 412 nm with a thermostat at 37°C
- 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)) : ABTS
- Sodium citrate buffer 50 mM pH 5,5
- Non-hydrate Glucose D+ : 1 gr /5ml H2O

A.3. Preparation of the reagents

- Buffer ABTS A : 10 mg ABTS qsp 20 ml of citrate buffer (Prepare just before to start the measurements, to keep in the fridge)

A.4. Preparation of the enzymes

- 20 mg of GOD qsp 100 ml in citrate buffer ([GOD]= 0,2 mg/ml).
- Lactoperoxidase solution: 25 mg LP qsp 10 ml in citrate buffer (let some time to get a good dissolution).
- Just before the measurement, dilute the GOD 50 X (1ml/50ml in a citrate buffer).

A.5. Method

- Pipet 2 ml of A in a cuvette.
- Incubate in a bath at 37°C during 10 min.
- Add 100 µl of glucose, add 10 µl of LP, add 100 µl of GOD enzyme. Mix in turning inside out the cuvette.
- Measure the O.D. at 412 nm at 3 min. and 5 min

Control: 2 ml of A + 100 µl glucose + 110 µl citrate buffer; measure the O.D. at 3 min and at 5 min.

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A.6. Activity calculation

$$\frac{\text{O.D. 412 nm (5'-3') / 2 x dilution factor}}{\text{Total volume x enzyme volume x enzyme concentration}} = \text{units/ mg}$$

That means:

$$\frac{\text{(O.D. 412 nm (5' - 3')/2 x 50)}}{\text{(2,21 x 0,1 x 0,2)}} = \text{units/mg}$$

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ANNEX 3

A. THIOCYANATE (SCN^-)

Measurement of the thiocyanate (SCN^-):
Method of Thomas& Edwin

09_267-316AN

A.1. Principle

The thiocyanate (SCN^-) reacts with $FeCl_3$ in acid solution.

$$Fe^{3+} + SCN^- \longrightarrow FeSCN^{2+}$$

The product of this reaction ($FeSCN^{2+}$) absorb at 450mm.
Following a range of absorbance measurements of precise known concentrations of SCN^- solutions, it is possible to establish a standard straight line to determine the unknown SCN^- concentration in the samples.

A.2. Materials

- Range of sodium thiocyanate concentrations (NaSCN)
- Iron (III) chloride hexahydrate ($FeCl_3$)
- Hydrochloric acid (HCl) to ajust the pH
- Microfiltrated water

A.3. Method

Preparation of the reagent: (20mM FeCl3 in 1M HCl) :

- To dissolve 0.324g of $FeCl_3$ in 50 ml distillated water
- Add 12.35 ml of HCl 30%
- Add with distillated water qsp 100mL

A.4. Preparation of the standard solutions

- Standard solutions
  2mM NaSCN: 16,2mg NaSCN diluted in 100ml distillated water
  1mM NaSCN: 8,1mg NaSCN diluted in 100 ml distillated water
  0.5mM NaSCN:4,05 mg NaSCN diluted in 100 ml distillated water
  0.2mM NaSCN:1,62 mg NaSCN diluted in 100ml distillated water
  0.1mM NaSCN:0,81 mg NaSCN diluted in 100 ml distillated water

A.5. Measurements

In the cuvette for the spectrophotometer, add :

Control: 2ml of the reagent in 1 ml distillated water
Standard solutions : 2ml of the reagent in 1 ml of each standard solution
Sample solutions : 2 ml of the reagent in 1 ml of the sample solution

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The absorbance is read in the minute using the spectrophotometer at 450 nm.

A.6. Results

To obtain a ΔOD, we have to subtract the control absorbance value from the samples absorbance values.

To establish a linear regression straight line (y=ax+b) for the ΔOD values in function of the known concentrations of the SCN^- (calibration).

To deduct from the ΔOD of the tested solution, the measurements of the SCN^- concentration.

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A. HYPOTHIOCYANITE ($OSCN^-$)

A.1. Principle

The hypothiocyanite ($OSCN^-$) ion is measured by a colorimetric method

The adding of sulfhydryl component TNB [5-thio-2-nitrobenzoic acid] which absorbed at 412 nm in a $OSCN^-$ solution induces an oxydo-reduction reaction producing DTNB (5,5' dithiobis (2-nitrobenzoic acid)) (incolor)

The follow-up of this bleaching allows us to obtain a $\Delta O.D$ proportional to the d'$OSCN^-$ concentration in the solution.

It is the oxidation of TNB (yellow color) into DTNB (incolor) which allows possible the follow-up and the quantitative determination of $OSCN^-$ possible by absorbance measurement.

$$2\ \text{TNB} \xrightarrow{OSCN^-} \text{DTNB}$$

A.2. Materials

- Tris base
- Sodium Borohydride
- DTNB (5,5' dithiobis (2-nitrobenzoic acid))
- HCl
- Microfiltrated water

A.3, Method

Preparation of Tris 0.5M pH7

Dissolve 60.55g Tris in 1 l distillated water. Ajust the pH to 7 with HCl.

Preparation of the reagent:

Dissolve 40mg of DTNB with 20mg Sodium borohydrate in 100mL Tris 0.5M pH7.

Rq : Protect from the light and keep at 4°C during 1 day maximum

A.4. Measurements :

In a cuvette of the spectrophotometer

- For the control: 0.200ml of reagent in 2.8 ml distillated
- For the solution: 0.200ml of reagent in 2.7ml of distillated water + 0.1ml of the solution which has to be tested.

The absorbance is read in the minute using 412 nm as wavelenght in the spectrophotometer

A.5. Results

$$C_{OSCN^-} = \frac{\Delta OD \times V}{\varepsilon \times 2 \times V_{mixed} \times l}$$

$\Delta OD = OD_{max} - OD_{min}$ = Control absorbance – Sample absorbance

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V_{added} = Volume$_{solution}$ added in the cuvette (L)

ε= Molar coefficient of absorption of the TNB (Lxmol-1xcm-1)

C_{OSCN^-}= Concentration $OSCN^-$ of the sample tested (mol/L)

l= lenght of the cuvette (cm)

2= Stœchiometric correction

Application:

$$[OSCN^-](M) = \frac{\Delta DO \times 3}{13600 \times 2 \times 0.1}$$

ΔOD= ODmax -ODmin= Control absorbance – Sample Absorbance

3= Volume (ml)

0.1= Volume of the sample (mL)

13600= Molar extension coefficient of TNB in mol/l/cm

2= Stœchiometric correction

Remark: To obtain a result in mM, we have to multiply by a factor 1.000

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Annex 10

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INSTITUT PASTEUR DE LILLE

Toxicology Laboratory

Professor Daniel MARZIN

ACUTE ORAL TOXICITY STUDY

IN MICE

ON THE SUBSTANCE

Hypothiocyanate Generating Mixture

(Glucose + Glucose oxidase
+ Lactoperoxidase + Sodium thiocyanate)

FOR : SYNFINA OLEOFINA
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Cedex Tel. 20.87.79.14 - Telex 820 187 F - Fax 20.87.79.06

STUDY	:	**ACUTE ORAL TOXICITY STUDY IN MICE**
SUBSTANCE	:	**Hypothiocyanate Generating Mixture (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)**
SPONSOR	:	**SYNFINA OLEOFINA**
SPONSOR CONTACT PERSON	:	**Mrs. D. DEFRISE**
ADDRESS	:	Rue Jacques de Lalaing, 4 B-1040 BRUSSELS - BELGIUM
TELEPHONE	:	19 (32) 22.33.33.88
TEST FACILITY	:	INSTITUT PASTEUR DE LILLE Laboratoire de Toxicologie Génétique B.P. 245 59019 LILLE CEDEX

INSTITUT PASTEUR DE LILLE CONTACT PERSON:

Study Director	:	Professor **D. MARZIN** Pharmacologist-Toxicologist Expert	.....	.....
Scientist	:	**H. VO PHI** Doctor of Pharmacy	.....	.....
Technicians	:	**M. MANGEZ**	.....	.....
	:	**T. CHASSAT**	.....	.....
	:	**D. LAGACHE**	.....	.....
Quality Assurance Unit	:	**M.P. DEHOUCK** Master of Sciences	.....	.....
			Date	Signature

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Report:	Archive Room No.1 Archive Box: No.1

The complete report contains 27 pages

LILLE, July 11, 1989

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ACUTE ORAL TOXICITY STUDY IN MICE

SUMMARY

SPONSOR : SYNFINA OLEOFINA

STUDIED SUBSTANCE : **Hypothiocyanate Generating Mixture** (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)

BATCH NUMBERS : Thiocyanate: 70 80 58 61; Glucose oxidase: 87 34 50 78; Lactoperoxidase: 04064P

TEST FACILITY : INSTITUT PASTEUR DE LILLE

STUDY CONDUCTED IN COMPLIANCE WITH GOOD LABORATORY PRACTICE

PROCEDURE

- SPECIES and STRAIN	:	OF1 Mouse
- SEX	:	Males and females
- NUMBER PER DOSE	:	10 males and 10 females
- ROUTE OF ADMINISTRATION	:	Oral
- FORM OF ADMINISTRATION	:	102.24 mg/kg (solution), 15.975 g/kg (suspension)
- VOLUME OF ADMINISTRATION	:	25 ml/kg
- OBSERVATION PERIOD	:	15 days

RESULTS

Acute oral toxicity study in mice of **Hypothiocyanate Generating Mixture** was conducted with 102.24 mg/kg and 15.975 g/kg doses that correspond respectively to the dose generating optimum amounts of hypothiocyanate and to the maximum administrable doses. No treatment-related toxicity was recorded at the lower administered dose. One mortality was observed at the maximum administrable dose, gastric lesions were observed at this dose.

ACUTE TOXICITY STUDY IN MICE

****

1. INTRODUCTION

1.1 OBJECTIVE

To determine the toxic symptoms and lethal doses of the substance to be studied after a single administration in mice.

1.2 PRINCIPLE

Groups of male and female animals receive a single dose of the substance to be studied at various dose levels. The symptoms, changes in body weight, the number and date of deaths are recorded at different times after the administration. Dead or sacrificed animals are subjected to necropsy examination at the end of the observation period.

1.3 RATIONALE FOR SELECTING THE TEST SYSTEM

The mouse is an animal typically used as a rodent species for determining acute toxicity: it is an animal recommended by most OECD and EEC registering regulatory bodies as they have a well-known responsiveness, hence there is a large database.

2. GENERAL PROTOCOL

2.1. TEST SYSTEM

2.1.1. ANIMALS

a) Species/strain: OF1 Mouse

b) Source: IFFA CREDO (Saint-Germain-sur-l'Abresles)

c) Weight: Approximately 17-24 g

d) Sex: Males and females

e) Number of animals per dose : 10 males and 10 females

f) Acclimatization period: 5 days minimum

g) Distribution and identification of animals in groups: The animals are distributed at random and are identified by marking the coat with picric acid.

h) Fasting: The animals are fasted for about 18 hours prior to treatment.

2.1.2. STABULATION

a) Animal housing

Temperature 21 ± 2°C
Humidity 60 ± 20 %
Air exchange rate: 12 changes per hour
Lighting: 12 hours of light(from 8 a.m. to 8 p.m.)/12 hours of darkness

b) Cages

- Polypropylene, stainless steel cover

- 25 x 45 x 15 cm

- Number: 5 per cage

c) Bedding: Dust-free, sterilized woodchip bedding.

d) Feed: UAR 113 sterilized diet for rats/mice.

e) Drink: pH 3.5 water (acidified with hydrochloric acid).

2.2. ADMINISTRATION

- Route: ORAL
- Excipient: DISTILLED WATER (pH = 7.12 at 15°C)

2.3. DETERMINATION OF DOSES TO BE ADMINISTERED

The study will determine the acute toxicity of 2 doses of the combination (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate: 4000/2/25/50) :

- 25 ml/kg of a solution at 4.072 g/l: this solution represents the concentration generating an optimal amount of the hypothiocyanate ion, therefore it is the best conditions for determining the acute toxicity of the generated ion.

- 25 ml/kg for a suspension of the combination at the highest concentration that can be administered: this solution is intended to assess the toxicity of an accidental overdose of food by the preparation.

2.4. EXAMINATIONS

The following examinations were performed:

- The behavior, symptoms and mortality were recorded:

  . during 15 minutes after the treatment, then

  . 1 hour, 2 hours and 6 hours each day after the treatment for 14 days.

If necessary (expected or observed late mortality), the observation time was extended.

- An individual weighing was done at 0, 2, 5, 10 and 14 days after treatment.

- The animals were sacrificed after the treatment by asphyxiation with CO_2.

- A necropsy was performed on dead or sacrificed animals on the 15th day with:

  . A macroscopic examination of the thoracic and abdominal organs in dead or sacrificed animals on the 15th day.

The lesions were recorded for each animal.

2.4. CALCULATION

The following calculations were performed (see methods in Appendices).

- LD50 by the following methods

  . PROBIT
  . LITCHFIELD and WILCOXON
  . ARC SINUS
  . or DRAGSTED and LANG

- Comparison of the LD50 between sexes for the same species (if possible) by the LITCHFIELD and WILCOXON method.

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(3) **DRAGSTEDT C.A., LANG V.F.** (1928)
Respiratory stimulant in acute cocaïne poisoning in rabbits.
J. of Pharmacol.and Exptl. Ther., 32, 215-222.

(4) **OECD** - The OECD Guidelines for the Testing of Chemicals. "Acute oral toxicity" No. **401,** May 12, 1981, pp. 1-7.

(5) **EEC -** Commission Directive of April 25, 1984 "Acute toxicity - oral administration". Official Journal of the European Communities, September 19, 1984, p L251/96 - L251/98.

3. SPECIFIC PROTOCOL

SPONSOR	:	SYNFINA OLEOFINA
SUBSTANCE	:	**Hypothiocyanate Generating Mixture** (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)
BATCH NUMBERS	:	Thiocyanate: 70 80 58 61; Glucose oxidase: 87 34 50 78; Lactoperoxidase: 04064P
I.P.L. REFERENCES	:	Thiocyanate: 89/12; Glucose oxidase: 89/11; Lactoperoxidase: 89/10; Glucose: 89/13
QUANTITY	:	Thiocyanate: 5 g; Glucose oxidase: 100 mg; Lactoperoxidase: 1 g; Glucose: 100 g

The substance, where the characteristics are indicated on the identification sheet (attached in the Appendix), was studied according to the general protocol, with the following exceptions or specificities:

To take into account the characteristics of the supplied substances, the composition for 1 liter of preparation was as follows:

	LOW DOSE mg/l	HIGH DOSES g/l
Glucose	4000.00	625
Lactoperoxidase (1)	18.72	2.925
Glucose oxidase	2.00	0.3215
Sodium thiocyanate	68.9	10.75
Doses administered in mg/kg under 25 ml/kg	**102.24**	**15.975**

(1) In order to take account of the specific activity of 801 units/mg instead of 600 units per mg.

VEHICLE USED : DISTILLED WATER (pH = 7.12 at 15°C)

ROUTE OF ADMINISTRATION : ORAL

VOLUME OF ADMINISTRATION : 25 ml/kg

STUDY INITIATION DATE : June 1st, 1989

STUDY COMPLETION DATE : June 16th, 1989

4. RESULTS

4.1. 102.24 mg/kg DOSE

4.1. MORTALITY

No animals died during the observation period. The oral dose of 102.24 mg/kg in mice is less than or equal to the LD0 in this preparation.

4.2. SYMPTOMS

No abnormal symptoms were noted during the observation period.

4.3. CHANGES IN BODY WEIGHT (see tables 1, 2 and figure)

Changes in animal body weight slowed down on the 5th day of the observation period and then were normal for the remaining time of the observation period.

4.4. MACROSCOPIC EXAMINATIONS AT NECROPSY (see tables)

Necropsies performed at the time of the animals' sacrifice demonstrated the non-treatment-related lesions: lung hepatization, but also desquamation of the fundic stomach wall and in one animal, redness of the fundic stomach wall. These gastric lesions can be attributed to the treatment.

4.1. 15.975g/kg DOSE

4.1. MORTALITY

The oral dose of 15.975 g/kg in mice caused the death of 4 out 10 males while no female animals died. Under these conditions, the thiocyanate generating mixture LD0 is less than the 15.975 mg/kg dose in males and higher than this dose in females.

4.2. SYMPTOMS

No abnormal symptoms were noted during the observation period in female animals while in male animals, sedation in 2 animals on D1 and in 1 animal on D2 with an inhibition of the righting reflex in one animal on D1. No abnormal symptoms were noted during the remaining time of the observation period.

4.3. CHANGES IN BODY WEIGHT (see tables 3, 4 and figure)

Changes in animal body weight slowed down on the 5th day of the observation period and then were normal for the remaining time of the observation period.

4.4. MACROSCOPIC EXAMINATIONS AT NECROPSY (see tables)

Necropsies performed at the time of the animals' sacrifice demonstrated the non-treatment-related lesions: lung hepatization, but also hemorrhages or hemorrhagic points on the stomach. These gastric lesions can be attributed to the treatment.

5. CONCLUSION

Acute oral toxicity study in mice of **Hypothiocyanate Generating Mixture** was conducted with 102.24 mg/kg and 15.975 g/kg doses that correspond respectively to the dose generating optimum amounts of hypothiocyanate and to the maximum administrable doses. At the lowest dose no mortality was recorded, however signs of gastric toxicity were noted. At the highest dose, 4 out of 10 male animals died and no mortality was recorded in female animals.
The recorded mortality is due to the excessive release of hydrogen peroxide, provoking hemorrhagic gastric lesions, which caused the death of the animals.

TABLE No.1

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE: **SCNO- generating mixture** SPECIES **MOUSE**

SPONSOR: SYNFINA OLEOFINA SEX MALES

TREATMENT DATE June 1st, 1989 VOLUME 25 ml/kg

SACRIFICE DATE June 16th, 1989 EXCIPIENT distilled water PH = 7
12 - 15°C

DOSE: **LOW DOSE: 102.24 mg/kg** ROUTE **ORAL**

		BODY WEIGHT IN g			
ANIMAL No.	D0	D2	D5	D10	D15
1	22	29	29	31	32
2	22	30	30	34	36
3	21	28	29	32	34
4	23	29	29	33	34
5	22	29	29	33	35
6	20	27	27	29	31
7	22	28.5	29	31	33
8	24	33	33	36	37
9	21	27	27	28	30
10	21	'27	27	29	31
Number	10	9	10	10	10
AVERAGE	**21.8**	**28.9**	**28.9**	**31.6**	**33.3**
Standard	1.1	1.8	1.8	2.5	2.3

TABLE No. 2

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE: **SCNO- generating mixture**

SPONSOR: SYNFINA OLEOFINA

TREATMENT DATE June 1st, 1989

SACRIFICE DATE June 16th, 1989

DOSE: **LOW DOSE: 102.24 mg/kg**

SPECIES **MOUSE**

SEX FEMALES

VOLUME 25 ml/kg

EXCIPIENT distilled water PH = 7
12 - 15°C

ROUTE **ORAL**

		BODY WEIGHT IN g			
ANIMAL No.	D0	D2	D5	D10	D15
1	19	24	23	25	25
2	19	22.5	21	22	24
3	19	21	21	22	24
4	22	27	26	28	28
5	21	25	24	26	29
6	21	24	24	25	26
7	21	24	24	25	26
8	21	26.5	26	29	29
9	17	25	26	26	26
10	20	21	20	23	23
Number	10	10	10	10	10
AVERAGE	**20**	**24**	**23.5**	**25.1**	**26**
Standard	1.5	2	2.2	2.3	2.1

TABLE No. 3

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE: **SCNO- generating mixture**

SPONSOR: SYNFINA OLEOFINA

TREATMENT DATE June 1st, 1989

SACRIFICE DATE June 16th, 1989

DOSE: **HIGH DOSE:** 15.975 g/kg

SPECIES **MOUSE**

SEX MALES

VOLUME 25 ml/kg

EXCIPIENT distilled water PH = 7
12 - 15°C

ROUTE **ORAL**

		BODY WEIGHT IN g			
ANIMAL No.	D0	D2	D5	D10	D15
1	23	death	death	death	death
2	20	17	death	death	death
3	21	27	20	death	death
4	22	27	28	30	31
5	22	death	death	death	death
6	22	28	28	30	33
7	22	29	29	32	35
8	24	31	29	32	33
9	22	28	31	35	37
10	22	28	29	31	33
Number	10	8	7	6	6
AVERAGE	**22**	**26.9**	**27.7**	**31.7**	**33.7**
Standard Deviation	1.1	4.2	3.5	1.9	2.1

TABLE No. 4

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE: **SCNO- generating mixture**

SPONSOR: SYNFINA OLEOFINA

TREATMENT DATE June 1st, 1989

SACRIFICE DATE June 16th, 1989

DOSE: **HIGH DOSE:** 15.975 g/kg

SPECIES **MOUSE**

SEX FEMALES

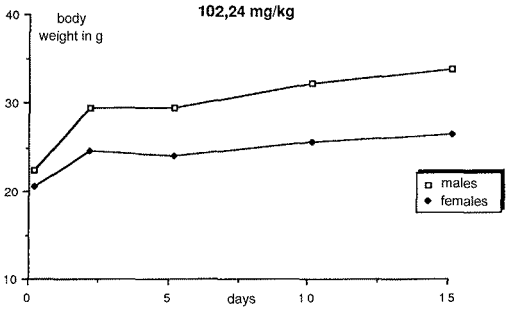
VOLUME 25 ml/kg

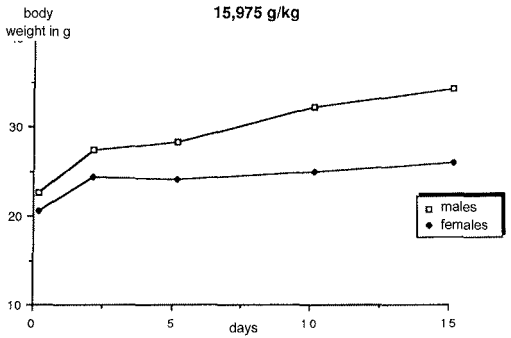
EXCIPIENT distilled water PH = 7
12 - 15°C

ROUTE **ORAL**

		BODY WEIGHT IN g			
ANIMAL No.	D0	D2	D5	D10	D15
1	21	22	22	23	25
2	21	26	26	26	28
3	18	20	20	23	23
4	23	24.5	25	26	27
5	18	24	23	25	27
6	19	25	25	26	26
7	21	25	25	24	25
8	17	21	21	22	22
9	22	28	28	28	29
10	20	21	20	20	22
Number	10	10	10	10	10
AVERAGE	**20**	**23.7**	**23.5**	**24.3**	**25.4**
Standard	1.9	2.6	2.7	2.4	2.5

CHANGES IN BODY WEIGHT





DOSE: 102.24 mg/kg	SPECIES: mouse	SUBSTANCE:			SCNO generating mixture			treatment		date		1 / 6 / 8 9
SEX: MALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation	*	*	*	*	*	*	*	*	*	*	10
	redness								*			1
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

DOSE: 102.24 mg/kg	SPECIES: mouse	SUBSTANCE:			SCNO generating mixture			treatment		date		1 / 6 / 8 9
SEX: FEMALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration								*	*	*	3
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	redness						*					1
	perforation											
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization											
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

DOSE: 15.975 mg/kg	SPECIES: mouse	SUBSTANCE:			SCNO generating mixture			treatment		date		1 / 6 / 86
SEX: MALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		2.6	5.6	6.6	16.6	2.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism			A	C								
nose	blood											
eyes	blood											
liver	discoloration	*										1
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air	*				*						2
	substance	*				*						2
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage					*						1
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	discoloration					*						1
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization				*		*	*	*	*	*	6
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
hemorrhagic points on the stomach										*	*	2

DOSE: 15.975 g/kg	SPECIES: mouse	SUBSTANCE:		SCNO generating mixture			treatment		date		1 / 6 / 86
SEX: FEMALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling					*						1
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation				*	*	*	*	*	*	*	7
	redness											
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
congested and red duodenum				*								1

APPENDIX 1

LD50 CALCULATION METHOD

There are many methods to calculate the LD50, each method has its limitations and advantages.

Where there are more than 2 doses that caused a mortality different to that of 0 and 100%, three methods of calculation were used.

1. **PROBIT METHOD (BLISS 1935)** (1)

This method linearizes the dose-response curve by a log-probit transformation. This is the most rigorous method if we accept the normality of the effect assumption as a function of the logarithm of the dose. The method used here fits the line after several successive estimates.

2. **LITCHFIELD AND WILCOXON METHOD** (2)

This is a simplified analysis based on the previously described method, its result is less accurate (greater confidence interval), but it is frequently used to compare 2 LD50s using a statistical calculation.

3. **ARCSINE METHOD**

This method linearizes the dose-response curve by a simpler transformation than the PROBIT method; it only uses doses relating to responses between 10 and 90%.

For the 3 methods, we checked the validity of the fitting made: the calculated Chi2(X^2) value must be compared to the threshold given by the table for n-2 degrees of freedom (n = number of used doses).

In the case where there are less than 2 doses caused a mortality different to that of 0 and 100%, the DRAGSTED and LANG method (3) was used, which is a calculation method using the summed mortality.

REFERENCES

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(4) **OECD** - The OECD Guidelines for the Testing of Chemicals. "Acute oral toxicity" No. **401**, May 12, 1981, pp. 1-7.

(5) **EEC** - Commission Directive of April 25, 1984 "Acute toxicity - oral administration". Official Journal of the European Communities, September 19, 1984, p L251/96 - L251/98.

QUALITY ASSURANCE UNIT

CERTIFICATE

******

STUDY TITLE	ACUTE ORAL TOXICITY STUDY IN MICE
SPONSOR:	SYNFINA OLEOFINA
STUDY SITE	INSTITUT PASTEUR DE LILLE Laboratoire de Toxicologie B.P. 245 59019 LILLE CEDEX

This trial was conducted in compliance with Good Laboratory Practice (GLP US Food and Drug Administration Federal Register, Part II, December 22, 1978, Part 58, Title 21 and GLP, Instruction No. 1065 of May 31, 1983, Bulletin of the Ministry for Social Affairs and National Solidarity, p. 1–16), the OECD Principles of Good Laboratory Practice (GLP) (Guidelines C(81)30 (final), June 1, 1981) and in accordance with the INSTITUT PASTEUR DE LILLE procedures (SOP).

For the short-term studies, the Quality Assurance Unit inspects such studies at least once every 3 months.

INSPECTION	CONDUCTED BY	DATE
PROTOCOL No. 89007	Study Director Quality Assurance Unit Sponsor	February 21, 1989 Wednesday, February 22, 1989 Friday, March 03, 1989
REPORT No. 89044	Study Director Quality Assurance Unit	11/09/89 11/09/89

QUALITY ASSURANCE UNIT

M.P. DEHOUCK

STUDY DIRECTOR

Professor D. MARZIN

High dose, maximum ingestible dose, mouse♀

SUBSTANCE:	DOSE: 15.975 g/kg	SPECIES: mouse	ROUTE		
Hypothiocyanate Generating Mixture	VOLUME: 25 ml/kg	DATE: 1.06.89	(PO)	IP	IV
	EXCIPIENT: H_2O	TIME:	IM	PO	
	PH: 7				

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFI-CATION	D0	D2	D5	D10	D15	NECROPSY
	DATE		1.6.89	3.6	6.6	12.6.89	16.6	
	INITIALS		MM/TC	TC	MM/TC	MM	MM/TC	
A	♀	R	21	22	22	23	25	
		T	21	26	26	26	28	
		D	18	20	20	23	23	
		Q	23	24.5	25	26	27	
		TQ	18	24	23	25	27	
B	♀	R	19	25	25	26	26	
		T	21	25	25	24	25	
		D	17	21	21	22	22	
		Q	22	28	28	28	29	
		TQ	20	21	20	20	22	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture		DOSE: mg/kg VOLUME 25 ml/kg EXCIPIENTH$_2$O				SPECIES mouse SEX M (F) NUMBER			DATE TIME: INITIALS				ROUTE PO IP IV IM PC OTHER		
SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, ,	N	N	N		N	N	N	N	N					*0*
RIGHTING REFLEX	+ or - N ,+, ++														
HYPERESTHESIA	N, +, ++														
HYPOESTHESIA	N,+ , + +														
TITUBATIONS	N,+ , + +														
JUMPS	N,+ , + +														
CRIES	N ,+, ++														
TREMORS	N ,+, ++														
CLONIC CONV.	N ,+, ++														
TONIC CONV.	N ,+, ++														
FLACCID ANIMAL	N ,+														
STIFFNESS	N ,+														
AGGRESSIVENES	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N, N ,+, ++														
PILOERECTION															
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,color														
URINE VOLUME	N ,+, -														
DIARRHEA	N ,+ ,+ +														
FECES COLOR	N ,color														
MIOSIS	N,+ , + +														
MYDRIASIS	N,+ , + +														
PTOSIS	N ,+, ++														
IRREG.BREATHIN	N,+ , + +														
WHEEZING	N,+ , + +														
SHALLOW BREATHING	N,+,++														
ABDOMINAL CRAMPS	N ,+														
SWOLLEN ABDOMEN	N ,+, ++														
CYANOSIS	N ,+, ++														
REDNESS	N,+ , + +														
MOUTH BLOOD	N,+,++														
NOSE BLOOD	N ,+, ++														
EYES BLOOD	N ,+, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: high	SPECIES: mouse	SUBSTANCE		HCNO Generating Mixture			Date/initials					
SEX: female	ROUTE: oral	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling					x						1
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	desquamation				x	x	x	x	x	x	x	7
	hemorrhage											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	x	x	x	x	x	x	x	x	x	x	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations congested and red duodenum				x								1

High dose, maximum ingestible dose, mouse♂

SUBSTANCE	DOSE: 15.975 g/kg	SPECIES: mouse	ROUTE		
Hypothiocyanate Generating Mixture	VOLUME: 25 ml/kg	DATE: 1.06.89	(PO)	IP	IV
	EXCIPIENT: H_2O	TIME:	IM	PO	
	pH: 7				

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFI-CATION	D0	D2	D5	D10	D15	NECROPSY
	DATE		1.6.89	3.6.89	6.6.89	12.6.89	16.6	
	INITIALS		MM/TC	TC	TC/MM	MM	MM/TC	
A	♂	R	23	† D1				Stomach filled with air + substance Nothing to report Discolored liver
		T	motor activity D1 ↓ 20	17	†D4			Autolysis due to death
		D	21	27	reproductive tract infection 20	† D5		Devoured animal
		Q	22	27	28	30	31	
		TQ	22	† D1				Stomach filled with air + substance Hemorrhage of the fundus inner wall
B	♂	R	22	28	28	30	33	
		T	22	29	29	32	35	
		D	24	31	29	32	33	
		Q	22	28	31	35	37	
		TQ	22	28	29	31	33	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE	DOSE: mg/kg	SPECIES mouse	DATE	ROUTE
Hypothiocyanate Generating Mixture	VOLUME 25 ml/kg	SEX (M) F	TIME:	PO IP IV
	EXCIPIENT H_2O pH: 7	NUMBER	INITIALS	IM PC
				OTHER

SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, ,	N				↓2	↓1	N	N	N	N	N	N		N
RIGHTING REFLEX	+ or - N ,+, ++					Teta-nized mouse	Teta-nized mouse								
HYPERESTHESIA	N, +, ++														
HYPOESTHESIA	N ,+ , ++														
TITUBATIONS	N ,+, ++														
JUMPS	N ,+, ++														
CRIES	N ,+, ++														
TREMORS	N ,+, ++														
CLONIC CONV.	N ,+, ++														
TONIC CONV.	N ,+, ++														
FLACCID ANIMAL	N ,+														
STIFFNESS	N ,+														
AGGRESSIVENES	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N, N ,+, ++														
PILOERECTION															
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,col.														
URINE VOLUME	N ,+, -														
DIARRHEA	N ,+ ,+ +														
FECES COLOR	N ,col.														
MIOSIS	N ,+, ++														
MYDRIASIS	N ,+, ++														
PTOSIS	N ,+, ++														
IRREG.BREATHIN	N ,+, ++														
WHEEZING	N ,+, ++														
SHALL.BREATHIN	N ,+, ++														
ABDOMINAL CRAMPS	N ,+														
SWOLLEN ABDOMEN	N ,+, ++														
CYANOSIS	N ,+, ++														
REDNESS	N ,+, ++														
MOUTH BLOOD	N ,+, ++														
NOSE BLOOD	N ,+, ++														
EYES BLOOD	N ,+, ++														
MORTALITY						2	2	3	3	4	4	4	4		4

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

High dose, maximum ingestible dose, mouse♂

DOSE: high	SPECIES: mouse	SUBSTAN SNCO Generating Mixture					Date/initials					
SEX: male	ROUTE:	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		2.6.89	5.6.89	6.6.89	Nothin	2.6.89	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism			A	C								
nose	blood											
eyes	blood											
liver	discoloration	X										1
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air	X				X						2
	Substance	X				X						2
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	ulcers											
	hemorrhage					X						1
	perforation											
	scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	discolored					X						1
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization				X		X	X	X	X	X	6
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
further comments hemorrhagic points										X	X	2

High dose, maximum ingestible dose, mouse♀

SUBSTANCE	DOSE	SPECIES mouse	ROUTE		
Hypothiocyanate Generating Mixture	4.088 g/l or 0.102225 g/kg	DATE 1.06.89	IG	IP	IV
	VOLUME 25 ml/kg	TIME:	IM	PO	
	EXCIPIENT: H_2O				
	PH 7 (12–15°)				

BODY WEIGHT AND DATE OF DEATH

SEX	IDENTIFI-CATION	D0	D2	D5	D10	D15	NECROPSY
DATE		1.6.89	3.6.89	6.6.9	12.6.89	18.6	
INITIALS		TC/MM	TC	TC/MM	MM	MM/TC	
A ♀	R	19	24	23	25	25	
	T	19	22.5	21	22	24	
	D	19	21	21	22	24	
	Q	22	27	26	28	28	
	TQ	21	25	24	26	29	
♀	R	21	24	24	25	26	
	T	21	24	24	25	26	
	D	21	26,5	26	29	29	
	Q	17	25	26	26	26	
	TQ	20	21	20	23	23	
AVERAGE							
STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture	DOSE: mg/kg VOLUME 25 ml/kg EXCIPIENT H_2O pH 7	SPECIES mouse SEX M (F) NUMBER	DATE TIME INITIALS	ROUTE PO IP IV IM PC OTHER

SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	8 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N,	N	N	N	N	N	N	N	N	N	N	N	N		N
RIGHTING REFLEX	+ or - N, +, ++														
HYPERESTHESIA	N, +, ++														
HYPOLSTHESIA	N, +, ++														
TITUBATIONS	N, +, ++														
JUMPS	N, +, ++														
CRIES	N, +, ++														
TREMORS	N, +, ++														
CLONIC CONVULSIONS	N, +, ++														
TONIC CONVULSIONS	N, +, ++														
PLACIDO ANIMAL	N +														
STIFFNESS	N, +														
AGGRESSIVENESS	N, + ++														
CATALEPSY	N, +, ++														
STEREOTYPIES	N, +, ++														
FEVER	N, N, +, ++														
PILOERECTION															
WEEPING	N, +, +, +														
SALIVATION	N, +, ++														
PERSPIRATION	N, +, ++														
URINE COLOR	N, 000														
URINE VOLUME	N, +, -														
DIARRHEA	N, +, + +														
FECES COLOR	N, 000														
MIOSIS	N, +, + +														
MYDRIASIS	N, +, + +														
PTOSIS	N, +, + +														
IRREGULAR	N, +, + +														
WHEEZING	N, +, + +														
SHALLOW BREATHING	N, + + +														
ABDOMINAL CRAMPS	N, +														
SWOLLEN ABDOMEN	N, +, ++														
CYANOSIS	N, +, ++														
REDNESS	N, +, ++														
MOUTH BLOOD	N, +, ++														
NOSE BLOOD	N, +, ++														
EYES BLOOD	N, +, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: low	SPECIES: mouse	SUBSTANCE SCNO generating mixture						Date/initials				
SEX: ♀	ROUTE:	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration								x	x	X	3
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	Scar tissue											
fundic stomach	ulcers											
	hemorrhage											
	perforation											
kidneys	Scar tissue											
	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization											
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations	Redness of the fundic stomach wall						X					1

Low dose, maximum ingestible dose, mouse♂

SUBSTANCE	DOSE: 4.089 g/l or 0.102225 g/kg	SPECIES: mouse	ROUTE		
Hypothiocyanate Generating Mixture	VOLUME: 25 ml/kg	DATE: 9.06.89	PO	IP	IV
	EXCIPIENT: H_2O	TIME:	IM	PO	
	pH: 7 (12-15 °)				

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFI -CATION	D0	D2	D5	D10	D15	NECROPSY
	DATE		1.6.89	3.6.89	6.6.89	12.6.89	16.6	
	INITIALS		TC/MM	TC	TC/MM	MM	MM/TC	
A	♂	R	22	29	29	31	32*	
		T	22	30	30	34	36*	
		D	21	28	29	32	34*	
		Q	23	29	29	33	34	
		TQ	22	29	29	33	35*	
B	♂	R	20	27	27	29	31	
		T	22	28.5	29	31	33	
		D	24	33	33	36	37	
		Q	21	27	27	28	30	
		TQ	21	27	27	29	31	
	AVERAGE							
	STANDARD DEVIATION							

- Bitten testicles (left) D15

SUBSTANCE Hypothiocyanate Generating Mixture		DOSE: VOLUME EXCIPIENT H_2O	mg/kg 25 ml/kg	SPECIES mouse SEX (M) F NUMBER 10		DATE TIME: INITIALS						ROUTE (PO) IP IV IM PC OTHER		
SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d	14d
SPONTANEOUS MOTOR ACTIVITY	N, -	N	N	N		N	N	N	N	N	N	N	N	N
RIGHTING REFLEX	+ or - N ,+, ++													
HYPERESTHESIA	N, +, ++													
HYPOESTHESIA	N,+ , + +													
TITUBATIONS	N,+ , + +													
JUMPS	N,+ , + +													
CRIES	N ,+, ++													
TREMORS	N ,+, ++													
CLONIC CONVULSIONS	N ,+, ++													
TONIC CONVULSIONS	N ,+, ++													
FLACCID ANIMAL	N ,+													
STIFFNESS	N ,+													
AGGRESSIVENESS	N ,+, ++													
CATALEPSY	N ,+, ++													
STEREOTYPIES	N ,+, ++													
FEVER	N, N ,+, ++													
PILOERECTION														
WEEPING	N ,+ ,+ +													
SALIVATION	N ,+, ++													
PERSPIRATION	N ,+, ++													
URINE COLOR	N ,col.													
URINE VOLUME	N ,+, -													
DIARRHEA	N ,+ ,+ +													
FECES COLOR	N ,col.													
MIOSIS	N,+ , + +													
MYDRIASIS	N,+ , + +													
PTOSIS	N ,+, ++													
IRREGULAR	N,+ , + +													
WHEEZING	N,+ , + +													
SHALLOW BREATHING	N,+, ++													
ABDOMINAL CRAMPS	N ,+													
SWOLLEN ABDOMEN	N ,+, ++													
CYANOSIS	N ,+, ++													
REDNESS	N,+ , + +													
MOUTH BLOOD	N,+, ++,													
NOSE BLOOD	N ,+ , +													
EYES BLOOD	N ,+, ++													
MORTALITY														

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: low	SPECIES: mouse	SUBSTANCE SCNO generating mixture						Date/initials				
SEX: male	ROUTE:	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	16.6	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	Scar tissue											
fundic stomach	desquamation	x	x	x	x	x	x	x	x	x	x	10
	hemorrhage											
	perforation											
	Scar tissue											
kidneys	discolored											
	marbling											
	spots											
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	x	x	x	x	x	x	x	x	x	x	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations	Redness of the fundic stomach wall								x			1

IPL – R
89044

STUDY : **ACUTE ORAL TOXICITY STUDY IN MICE**

SUBSTANCE : Hypothiocyanate Generating Mixture (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)

SPONSOR : SYNFINA OLEOFINA

STUDY DIRECTOR : Professor D. MARZIN 20/7/89

Date Signature

The attached draft report is sent to you as a copy for any corrections before final publication

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☐ Edited without corrections

☐ Corrected according to the attached comments

☐ - After correction, the final version of the report may be published

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FOR THE SPONSOR:

Date Signature

Taradon
laboratory

APPENDIX C

Study report of Acute toxicity test in rats of an orally administrated $OSCN^-$ generating solution

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INSTITUT PASTEUR DE LILLE

TOXICOLOGY LABORATORY

ACUTE ORAL TOXICITY STUDY

IN RATS

ON THE SUBSTANCE

Hypothiocyanate Generating Mixture

(Glucose + Glucose oxidase
Lactoperoxidase + Sodium thiocyanate)

FOR: SYNFINA OLEOFINA
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STUDY	:	**ACUTE ORAL TOXICITY STUDY IN RATS**
SUBSTANCE	:	**Hypothiocyanate Generating Mixture (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)**
SPONSOR	:	**SYNFINA OLEOFINA**
SPONSOR CONTACT PERSON	:	**Mrs. D. DEFRISE**
ADDRESS	:	Rue Jacques de Lalaing, 4 B-1040 BRUSSELS - BELGIUM
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TEST FACILITY	:	INSTITUT PASTEUR DE LILLE Laboratoire de Toxicologie Genetique B.P. 245 59019 LILLE CEDEX

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Technicians	:	**M. MANGEZ**	.....	.....
	:	**T. CHASSAT**	.....	.....
	:	**D. LAGACHE**	.....	.....
Quality Assurance Unit	:	**M.P. DEHOUCK** Master of Sciences	.....	.....
			Date	Signature

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LILLE, April 7, 1989

CONTENTS

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ACUTE ORAL TOXICITY STUDY IN RATS

SUMMARY

SPONSOR : SYNFINA OLEOFINA

STUDIED SUBSTANCE : **Hypothiocyanate Generating Mixture** (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)

BATCH NUMBERS : Thiocyanate : 70 80 58 61; Glucose oxidase: 87 34 50 78; Lactoperoxidase: 04064P

TEST FACILITY : INSTITUT PASTEUR DE LILLE

STUDY CONDUCTED IN COMPLIANCE WITH GOOD LABORATORY PRACTICE

PROCEDURE

- SPECIES and STRAIN	OFA Rat
- SEX	Males and females
- NUMBER PER DOSE	10 males and 10 females
- ROUTE OF ADMINISTRATION	Oral
- FORM OF ADMINISTRATION	40.8962 mg/kg (solution), 8.052 g/kg (suspension)
- VOLUME OF ADMINISTRATION	10 ml/kg
- OBSERVATION PERIOD	15 days

RESULTS

Acute oral toxicity study in rats of **Hypothiocyanate Generating Mixture** was conducted with 40.8962 mg/kg and 8.52 g/kg doses that correspond respectively to the dose generating optimum amounts of hypothiocyanate and to the maximum administrable doses, no treatment-related toxicity was recorded at these two doses.

ACUTE TOXICITY STUDY IN RATS

* * * *

1 INTRODUCTION

1.1 OBJECTIVE

To determine the toxic symptoms and lethal doses of the substance to be studied after a single administration in rats.

1.2 PRINCIPLE

Groups of male and female animals receive a single dose of the substance to be studied at various dose levels. The symptoms, changes in body weight, the number and date of deaths are recorded at different times after the administration. Dead or sacrificed animals are subjected to necropsy examination at the end of the observation period. If possible the 50% lethal dose or LD50 is determined.

1.3 RATIONALE FOR SELECTING THE TEST SYSTEM

The rat is an animal typically used as a rodent species for determining acute toxicity: it is an animal recommended by most OECD and EEC registering regulatory bodies as they have a well-known responsiveness, hence there is a large database.

2 GENERAL PROTOCOL

2.1. TEST SYSTEM

2.1.1. ANIMALS

a) Species/strain: OFA Rat

b) Source: IFFA CREDO (Saint-Germain-sur-l'Abresles)

c) Weight: Approximately 100-120 g

d) Sex: Males and females

e) Number of animals per dose : 10 males and 10 females

f) Acclimatization period: 5 days minimum

g) Distribution and identification of animals in groups: The animals are distributed at random and are identified by marking the coat with picric acid.

h) Fasting: The animals are fasted for about 18 hours prior to treatment.

2.1.2. STABULATION

a) Animal housing

Temperature 21 ± 2°C
Humidity 60 ± 20 %
Air exchange rate: 12 changes per hour
Lighting: 12 hours of light(from 8 a.m. to 8 p.m.)/12 hours of darkness

b) Cages

- Polypropylene, stainless steel cover
- 25 x 45 x 15 cm
- Number: 5 per cage

c) Bedding: Dust-free, sterilized woodchip bedding.

d) Feed: UAR 113 sterilized diet for rats/mice.

e) Drink: pH 3.5 water (acidified with hydrochloric acid).

2.2. ADMINISTRATION

- Route: Specified in specific protocol
- Excipient: Specified in specific protocol

2.3. DETERMINATION OF DOSES TO BE ADMINISTERED

The study will determine the acute toxicity of 2 doses of the combination (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate: 4000/2/25/50) :

- 10 ml/kg of a solution at 4.072 g/l: this solution represents the concentration generating an optimal amount of the hypothiocyanate ion, therefore it is the best conditions for determining the acute toxicity of the generated ion.

- 10 ml/kg for a suspension of the combination at the highest concentration that can be administered: this solution is intended to assess the toxicity of an accidental overdose of food by the preparation.

In case of mortality at this dose, the LD50 or maximum tolerated dose will be determined.

2.4. EXAMINATIONS

The following examinations were performed:

- The behavior, symptoms and mortality were recorded:

  . during 15 minutes after the treatment, then

  . 1 hour, 2 hours and 6 hours each day after the treatment for 14 days.

If necessary (expected or observed late mortality), the observation time was extended.

- An individual weighing was done at 0, 2, 5, 10 and 14 days after treatment.

- The animals were sacrificed after the treatment by asphyxiation with CO_2.

- A necropsy was performed on dead or sacrificed animals on the 15th day with:

  . A macroscopic examination of the thoracic and abdominal organs in dead or sacrificed animals on the 15th day.

The lesions were recorded for each animal.

2.5. CALCULATION

The following calculations were performed (see methods in Appendices).

- LD50 by the following methods

  . PROBIT
  . LITCHFIELD and WILCOXON
  . ARC SINUS
  . or DRAGSTED and LANG

- Comparison of the LD50 between sexes for the same species (if possible) by the LITCHFIELD and WILCOXON method.

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3. SPECIFIC PROTOCOL

SPONSOR	:	SYNFINA OLEOFINA
SUBSTANCE	:	**Hypothiocyanate Generating Mixture** (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)
BATCH NUMBERS	:	Thiocyanate : 70 80 58 61; Glucose oxidase: 87 34 50 78; Lactoperoxidase: 04064P
I.P.L. REFERENCES	:	Thiocyanate: 89/12; Glucose oxidase: 89/11; Lactoperoxidase: 89/10; Glucose: 89/13
QUANTITY	:	Thiocyanate: 5 g; Glucose oxidase: 100 mg; Lactoperoxidase: 1 g; Glucose: 100 g

The substance, where the characteristics are indicated on the identification sheet (attached in the Appendix), was studied according to the general protocol, with the following exceptions or specificities:

To take into account the characteristics of the supplied substances, the composition for 1 liter of preparation was as follows:

	LOW DOSE mg/l	HIGH DOSES g/l
Glucose	4000.00	833.33
Lactoperoxidase (1)	18.72	3.9
Glucose oxidase	2.00	0.416
Sodium thiocyanate	68.9	14.354
Doses in mg/kg under 10 ml/kg	40.9	8.52

(1) In order to take account of the specific activity of 801 units/mg instead of 600 units per mg.

VEHICLE USED : DISTILLED WATER (pH = 12 at 15°C)

ROUTE OF ADMINISTRATION : ORAL

VOLUME OF ADMINISTRATION : 10 ml/kg

STUDY INITIATION DATE : March 9, 1989

STUDY COMPLETION DATE : March 24,1989

4. RESULTS

4.1. 40.8962 mg/kg DOSE

4.1. MORTALITY

No animals died during the observation period. The oral dose of 40.8962 mg/kg in rats is less than or equal to the LD0 in this preparation.

4.2. SYMPTOMS

No abnormal symptoms were noted during the observation period.

4.3. CHANGES IN BODY WEIGHT (see tables 1 and 2)

Changes in animal body weight were normal throughout the observation period.

4.4. MACROSCOPIC EXAMINATIONS AT NECROPSY (See tables)

Necropsies performed at the time of the animals' sacrifice demonstrated the non-treatment-related lesions such as kidney discoloration or lung hepatization.

4.1. 8.52 g/kg DOSE

4.1. MORTALITY

No animals died during the observation period. The oral dose of 8.52 g/kg in rats is less than or equal to the LD0 in this preparation.

4.2. SYMPTOMS

No abnormal symptoms were noted during the observation period.

4.3. CHANGES IN BODY WEIGHT (see tables 3, 4 and figure)

Changes in animal body weight were normal throughout the observation period.

4.4. MACROSCOPIC EXAMINATIONS AT NECROPSY (See tables)

Necropsies performed at the time of the animals' sacrifice demonstrated the non-treatment-related lesions such as kidney discoloration or lung hepatization.

5. CONCLUSION

Acute oral toxicity study in rats of **Hypothiocyanate Generating Mixture** was conducted with 40.8962 mg/kg and 8.52 g/kg doses that correspond respectively to the dose generating optimum amounts of hypothiocyanate and to the maximum administrable doses, no treatment-related toxicity was recorded at these two doses.

TABLE No.1

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE **HYPOTHIOCYANATE GENERATING MIXTURE**		SPECIES	**RAT**
SPONSOR: SYNFINA-OLEOFINA		SEX	MALES
TREATMENT DATE	March 9, 1989	VOLUME	10 ml/kg
SACRIFICE DATE	March 24, 1989	EXCIPIENT water	Distilled
DOSE:	**40.8962 mg/kg**	ROUTE	**ORAL**

	BODY WEIGHT in g				
ANIMAL No.	D0	D2	D5	D10	D15
1	160	190	210	260	290
2	170	195	215	265	300
3	165	195	220	275	305
4	165	195	215	270	305
5	175	205	235	285	325
6	170	205	230	280	310
7	170	195	220	280	320
8	155	180	205	250	280
9	160	190	220	270	300
10	160	190	220	270	300
Number	10	10	10	10	10
AVERAGE	**165**	**194**	**219**	**270.5**	**303.5**
Standard Deviation	6.2	7.4	8.8	10.4	13.1

TABLE No. 2

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE **HYPOTHIOCYANATE GENERATING MIXTURE**		SPECIES	**RAT**
SPONSOR: SYNFINA-OLEOFINA		SEX	FEMALES
TREATMENT DATE	March 9, 1989	VOLUME	10 ml/kg
SACRIFICE DATE	March 24, 1989	EXCIPIENT water	Distilled
DOSE:	**40.8962 mg/kg**	ROUTE	**ORAL**

	BODY WEIGHT in g				
ANIMAL No.	D0	D2	D5	D10	D15
1	155	175	180	210	220
2	150	165	180	200	205
3	135	155	170	185	195
4	145	165	175	195	210
5	150	170	180	210	220
6	155	170	190	210	230
7	145	165	180	200	215
8	145	160	175	205	220
9	150	170	190	205	225
10	150	165	180	200	210
Number	10	10	10	10	10
AVERAGE	**148**	**166**	**180**	**202**	**215**
Standard Deviation	5.9	5.7	6.2	7.9	10.3

TABLE No. 3

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE **HYPOTHIOCYANATE GENERATING MIXTURE**		SPECIES	**RAT**
SPONSOR: SYNFINA-OLEOFINA		SEX	MALES
TREATMENT DATE:	March 9, 1989	VOLUME	10 ml/kg
SACRIFICE DATE:	March 24, 1989	EXCIPIENT water	Distilled
DOSE:	**8.52 mg/kg**	ROUTE	**ORAL**

		BODY WEIGHT in g			
ANIMAL No.	D0	D2	D5	D10	D15
1	175	200	230	280	310
2	170	205	235	295	330
3	170	200	220	280	315
4	165	190	220	275	320
5	170	200	230	275	305
6	170	200	230	290	335
7	160	185	205	255	285
8	170	195	220	270	305
9	165	190	210	270	300
10	165	190	215	265	295
Number	10	10	10	10	10
AVERAGE	**168**	**195.5**	**221.5**	**275.5**	**310**
Standard Deviation	4.2	6.4	9.7	11.7	15.5

TABLE No. 4

CHANGES IN BODY WEIGHT
(individual values)

SUBSTANCE: **HYPOTHIOCYANATE GENERATING MIXTURE**		SPECIES	**RAT**
SPONSOR: SYNFINA-OLEOFINA		SEX	FEMALES
TREATMENT DATE	March 9, 1989	VOLUME	10 ml/kg
SACRIFICE DATE:	March 24, 1989	EXCIPIENT	Distilled water
DOSE:	**8.52 mg/kg**	ROUTE	**ORAL**

		BODY WEIGHT in g			
ANIMAL No.	D0	D2	D5	D10	D15
1	160	175	180	200	215
2	160	180	190	225	240
3	150	170	180	205	215
4	150	170	175	210	225
5	135	155	170	195	205
6	140	165	175	195	205
7	150	165	165	190	205
8	140	160	170	200	215
9	140	160	165	195	205
10	145	165	175	195	210
Number	10	10	10	10	10
AVERAGE	**147**	**166.5**	**174.5**	**201**	**214**
Standard Deviation	8.6	7.5	7.6	10.2	11.3

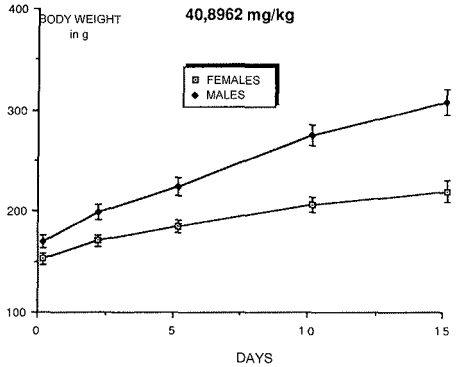
DOSE: 8.52 g/kg	SPECIES: RAT	SUBSTANCE :		SCNO generating mixture				treatment		date		9-03
SEX: MALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	Blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling		*	*	*	*	*	*	*	*	*	9
	spots (dark)	*	*		*	*	*	*				6
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

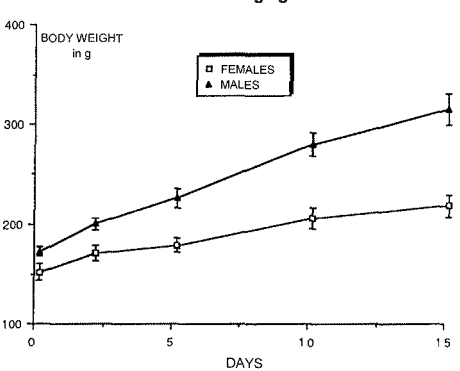
DOSE: 8.52 g/kg	SPECIES: RAT	SUBSTANCE:		SCNO generating mixture				treatment		date		9-03
SEX: FEMALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling		*	*								2
	spots (dark)				*	*		*	*	*	*	6
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

DOSE: 40.8962 mg/kg	SPECIES: RAT	SUBSTANCE:		SCNO generating mixture				treatment		date		9-03
SEX: MALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling	*	*	*	*	*	*	*	*	*	*	10
	spots (dark)			*								1
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

DOSE: 40.8962 mg/kg	SPECIES: RAT	SUBSTANCE :		SCNO generating mixture				treatment		date		9-03
SEX: FEMALE	ROUTE: ORAL	1	2	3	4	5	6	7	8	9	10	TOTAL
date of death or sacrifice		24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	10
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	erosion											
	desquamation											
	redness											
	scar tissue											
kidneys	discolored											
	marbling											
	spots (dark)			*	*		*	*	*	*	*	7
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	*	*	*	*	*	*	*	*	*	*	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

HYPOTHIOCYANATE GENERATING MIXTURE





INFORMATION TO BE PROVIDED ON THE STUDIED SUBSTANCE

SPONSOR : **SYNFINA OLEOFINA**

- Substance name or code number:

- Chemical name (if any): ...GLUCOSE (DEXTROSE) .....
- Batch number: .....
- Purity: 99.5%
- Expiry date: ...UNLIMITED ..... ☐ unknown
- Supplied Quantity: ..... 100 g

- Storage conditions:
  - * temperature ambient ..... ☐ unknown
  - * light .....
  - * humidity .....
  - * air .....
  - * other .....

– Solubility/stability (if known):

SOLVENT	Solubility g/l		Stability		
			(1)	T°	duration
water	1g/	1ml	.....	.....	.....
ethanol	1g/	60ml	.....	.....	.....
DMSO	.....	.....	.....	.....	.....
acetone	.....	.....	.....	.....	.....
other	.....	.....	.....	.....	.....

(1): yes/no/unknown

- Known incompatibility with:
  - glass : ..... yes/no/unknown
  - plastics: ..... yes/no/unknown

– Physical properties:

- Appearance: ..... white powder....
- melting point ..156..... °C ☐ unknown
- boiling point ..... °C ☐ unknown
- vapor pressure at 20°C.....mm Hg ☐ unknown

– Precautions to be taken during handling:

the substance is	yes	no	not known
irritant	.....	...x...	.....
allergenic	.....	...x...	.....
teratogenic	.....	...x...	.....
mutagenic	.....	...x...	.....
carcinogenic	.....	...x...	.....
other	.....	...x...	.....

Special Precautions .....
.....
.....
.....

antidote .....

– Destruction method:
  - incineration ...x... minimum temperature ..... °C
  - chemical .....
  - other ..... ☐ unknown

INFORMATION TO BE PROVIDED ON THE STUDIED SUBSTANCE

SPONSOR : **SYNFINA OLEOFINA**

- Substance name or code number: ...GLUCOSE OXIDASE (E.C. 1.1.3.4)
- Chemical name (if any): ...Beta-D-glucose:oxygen 1-oxido-reductase.....
- Batch number: 87 34 50 78 .....
- Purity: ..... %
- Expiry date ..... ☐ unknown
- Supplied Quantity:..... 0.1 g
- Storage conditions:
  - * temperature -18°C..... ☐ unknown
  - * light away from light and in a dry place
  - * humidity away from light and in a dry place
  - * air away from light and in a dry place
  - * other.....
- Solubility/stability (if known):

SOLVENT	Solubility g/l	Stability		
		(1)	T°	duration
water	.....	.....	.....	.....
ethanol	.....	.....	.....	.....
DMSO	.....	.....	.....	.....
acetone	.....	.....	.....	.....
other	.....	.....	.....	.....

(1): yes/no/unknown

- Known incompatibility with:
  - glass :..... yes/no/unknown
  - plastics: ..... yes/no/unknown
- Physical properties:
  - appearance ...white powder.....
  - melting point .....°C ☐ unknown
  - boiling point .....°C ☐ unknown
  - vapor pressure at 20°C.....mm Hg ☐ unknown
- Precautions to be taken during handling:

the substance is	yes	no	not known
irritant	.....	.....	x
allergenic	.....	.....	x
teratogenic	.....	.....	x
mutagenic	.....	.....	x
carcinogenic	.....	.....	x
other	.....	.....	x

Special Precautions .....

antidote .....

- Destruction method:
  - incineration x minimum temperature ..... °C
  - chemical .....
  - other ..... ☐ unknown

INFORMATION TO BE PROVIDED ON THE STUDIED SUBSTANCE

SPONSOR : **SYNFINA OLEOFINA**

- Substance name or code number: ...LACTOPEROXIDASE (EC 1.11.1.7)
- Chemical name (if any): ...hydrogen-peroxide oxidoreductase .....
- Batch number: ...04064P.....
- Purity: ...+/- 72 ..... %
- Expiry date ...06/90 ..... ☐ unknown
- Supplied Quantity:..... 1 g *at 200 units ABTS/mg*

- Storage conditions:
  - * temperature ...-18° C ..... ☐ unknown
  - * light away from light and in a dry place
  - * humidity away from light and in a dry place
  - * air away from light and in a dry place
  - * other.....

- Solubility/stability (if known):

SOLVENT	Solubility g/l	Stability		
		(1)	T°	duration
water	50	oui	20°C	24h
ethanol	.....	.....	.....	.....
DMSO	.....	.....	.....	.....
acetone	.....	.....	.....	.....
other	.....	.....	.....	.....

(1): yes/no/unknown

- Known incompatibility with:
  - glass : ...adsorption..... yes/no/unknown
  - plastics: .....idem..... yes/no/unknown *(to check)*

- Physical properties:
  - appearance ...brown powder.....
  - melting point .....°C ☐ x ☐ unknown
  - boiling point .....°C ☐ x ☐ unknown
  - vapor pressure at 20°C.....mm Hg ☐ x ☐ unknown

- Precautions to be taken during handling:

the substance is	yes	no	not known
irritant	.....	.....	.....
allergenic	.....	.....	.....
teratogenic	.....	.....	.....
mutagenic	.....	.....	.....
carcinogenic	.....	.....	.....
other	.....	.....	.....

Special Precautions dustiness of the powder.....

- Destruction method:
  - incineration ...x... minimum temperature ..... °C
  - chemical extreme pH.....
  - other ..... ☐ unknown

INFORMATION TO BE PROVIDED ON THE STUDIED SUBSTANCE

SPONSOR : **SYNFINA OLEOFINA**

- Substance name or code number: SODIUM THIOCYANATE
- Chemical name (if any): (CAS No. 540-72-7).....
- Batch number: 70 80 5861 .....
- Purity: > 99 ..... %
- Expiry date ..... [x] unknown
- Supplied Quantity:..... 5 g
- Storage conditions:
  - * temperature ambient..... [ ] unknown
  - * light .....
  - * humidity .....
  - * air .....
  - * other .....
- Solubility/stability (if known):

SOLVENT	Solubility g/l	Stability		
		(1)	T°	duration
water	1,390	yes	20°	
ethanol		yes		
acetone		yes		
DMSO				
other				

(1): yes/no/unknown

- Known incompatibility with:
  - glass : ..... yes/no/unknown
  - plastics: ..... yes/no/unknown
- Physical properties:
  - appearance ....white crystals .....
  - melting point ..... 287°C [ ] unknown
  - boiling point ..... °C [x] unknown
  - vapor pressure at 20 °C .....mm Hg [x] unknown
- Precautions to be taken during handling: harmful by inhalation/skin contact/ingestion

the substance is	yes	no	not known
irritant			
allergenic			
teratogenic			
mutagenic			
carcinogenic			
other			

Special Precautions
contact with acids releases toxic gases

- Destruction method:
  - incineration ..... minimum temperature ..... °C
  - chemical x.....
  - other .... [ ] ..... unknown

APPENDIX 1

* * * *

LD50 CALCULATION METHOD

There are many methods to calculate the LD50, each method has its limitations and advantages.

Where there are more than 2 doses that caused a mortality different to that of 0 and 100%, three methods of calculation were used.

1. **PROBIT METHOD (BLISS 1935)** (1)

This method linearizes the dose-response curve by a log-probit transformation. This is the most rigorous method if we accept the normality of the effect assumption as a function of the logarithm of the dose. The method used here fits the line after several successive estimates.

2. **LITCHFIELD AND WILCOXON METHOD** (2)

This is a simplified analysis based on the previously described method, its result is less accurate (greater confidence interval), but it is frequently used to compare 2 LD50s using a statistical calculation.

3. **ARCSINE METHOD**

This method linearizes the dose-response curve by a simpler transformation than the PROBIT method; it only uses doses relating to responses between 10 and 90 %.

For the 3 methods, we checked the validity of the fitting made: the calculated Chi2(X^2) value must be compared to the threshold given by the table for n-2 degrees of freedom (n = number of used doses).

In the case where there are less than 2 doses caused a mortality different to that of 0 and 100%, the DRAGSTED and LANG method (3) was used, which is a calculation method using the summed mortality.

R/89023

SUBSTANCE: Hypothiocyanate Generating Mixture (6 + 60D + Lact + ThioNa)	DOSE: 8.52 g/kg [ ] Max g/kg VOLUME: 10 ml/kg EXCIPIENT: H_2O mg pH 7 (12–15°)	SPECIES: RAT DATE: March 9, 1989 TIME: 13:45 > A 15:56 > B	ROUTE PO IP IV IM PO

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFI-CATION	D0 13:45	D2	D5	D10	D15	NECROPSY
	DATE		9.3.89	11.3.89	14.3.89	20.3.89	24.3.89	
	INITIALS		TC	DL	MM/TC	MM/TC		
A	♂	R	175	200	230	280	310	
		T	170	205	235	295	330	
		D	170	200	220	280	315	
		Q	165	190	220	275	320	
		TQ	170	200	230	275	305	
B	♂	R	15:56 170	200	230	290	335	
		T	160	185	205	255	285	
		D	170	195	220	270	305	
		Q	165	190	210	270	300	
		TQ	165	190	215	265	295	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture	DOSE: 8520.0 mg/kg VOLUME 10 ml/kg EXCIPIENT H_2O L	SPECIES RAT SEX **M** F NUMBER 10	DATE March 9, 1989 TIME 13:45 -> A 15:56 -> B INITIALS	ROUTE **PO** IP IV IM PC OTHER

SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, -	N	N			N	N	N	N	N	N	N	N		*N*
RIGHTING REFLEX	+ or - N,+, ++	+	+			+	+								
HYPERESTHESIA	N,+, ++	N	N			N	N								
HYPOESTHESIA	N,+, ++														
TITUBATIONS	N,+, ++														
JUMPS	N,+, ++														
CRIES	N, +, ++														
TREMORS	N, +, ++														
CLONIC	N ,+, ++														
TONIC	N ,+, ++														
FLACCID ANIMAL	N ,+														
STIFFNESS	N ,+														
AGGRESSIVENESS	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N,														
PILOERECTION	N, +, ++														
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,col.														
URINE VOLUME	N ,+, -														
DIARRHEA	N ,+ ,++														
FECES COLOR	N ,col.														
MIOSIS	N,+ , ++														
MYDRIASIS	N,+ , ++														
PTOSIS	N,+, ++														
IRREGULAR	N,+ , ++														
WHEEZING	N,+ , ++														
SHALLOW	N, +, ++														
ABDOMINAL CRAMPS	N ,+														
SWOLLEN ABDOMEN	N ,+, ++														
CYANOSIS	N ,+, ++														
REDNESS	N,+ , + +														
MOUTH BLOOD	N,+, ++														
NOSE BLOOD	N ,+ , ++														
EYES BLOOD	N ,+, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: ♂[] g/kg	SPECIES RAT	SUBSTANCE Hypothiocyanate generating mixture							date/initials		MM/TC
SEX: ♂	ROUTE PO	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	24/3/89	
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling		x	x	x	x	x	x	x	x	X	9
	spots dark	x	x		x	x	x	x				6
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	x	x	x	x	x	x	x	x	x	x	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

SUBSTANCE: Hypothiocyanate Generating Mixture (G + GOD + Lact + ThioNa)	DOSE: 8.52 g/kg [ ] Max g/kg VOLUME: 10 ml/kg EXCIPIENT: H2O mg PH 7 (12–15°C)	SPECIES: RAT DATE: March 9, 1989 TIME: 13:45 > A 15:56 > B	ROUTE: PO, IP, IV, IM, PO

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFICATION	D0 13:45	D2	D5	D10	D15	NECROPSY
A	DATE		9.3.89	11.3.89	14.3.89	20.3.89	24.3.89	
	INITIALS		MM	DL	MM/TC	MM/TC	MM/TC	
	♀	R	160	175	180	200	215	
		T	160	180	190	225	240	
		D	150	170	180	205	215	
		Q	150	170	175	210	225	
B		TQ	135	155	170	195	205	
	♀	R	3:46 PM 140	165	175	195	205	
		T	150	165	165	190	205	
		D	140	160	170	200	215	
		Q	140	160	165	195	205	
		TQ	145	165	175	195	210	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture		DOSE: 8520.0 mg/kg [ ] mg/kg VOLUME 10 ml/kg EXCIPIENT H_2O !				SPECIES RAT SEX M F NUMBER 10			DATE March 9, 1989 TIME 13:45 > A 15:56 > B INITIALS TC/MM/DL				ROUTE PO IP IV IM PC OTHER		
SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, -	N	N			N	N	N	N	N	N	N	N		N
RIGHTING REFLEX	+ or - N ,+, ++	+	+			+	+								
HYPERESTHESIA	N,+,++	N	N												
HYPOESTHESIA	N,+ , + +														
TITUBATIONS	N,+ , + +														
JUMPS	N,+ , + +														
CRIES	N ,+, ++														
TREMORS	N ,+, ++														
CLON.CONVULSIONS	N ,+, ++														
TON.CONVULSIONS	N ,+, ++														
FLACCID ANIMAL	N ,+														
STIFFNESS	N ,+														
AGGRESSIVENESS	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N. N ,+, ++														
PILOERECTION															
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,col.														
URINE VOLUME	N ,+, -														
DIARRHEA	N ,+ ,+ +														
FECES COLOR	N ,col.														
MIOSIS	N,+ , + +														
MYDRIASIS	N,+ , + +														
PTOSIS	N ,+, ++														
IRREGULAR	N,+ , + +														
WHEEZING	N,+ , + +														
SHALLOW	N,+,++														
ABDOMINAL CRAMPS	N,+														
SWOLLEN ABDOMEN	N,+, ++														
CYANOSIS	N,+, ++														
REDNESS	N,+, ++														
MOUTH BLOOD	N,+, ++														
NOSE BLOOD	N,+, ++														
EYES BLOOD	N,+, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: 8520.0 mg/kg	SPECIES RAT	SUBSTANCE Hypothiocyanate Generating Mixture							date/initials 24.3.89			
SEX: ♀	ROUTE PO	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		Nothing to report	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	0
autolysis/cannibalism		24.3										
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling		X	X								2
	spots				X	X		X	X	X	X	6
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	X	X	X	X	X	X	X	X	X	X	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

maximum ingestible dose, rat ♂

SUBSTANCE: Hypothiocyanate Generating Mixture (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)	DOSE: 4.08962 g/l or 40.8962 mg/kg *c TC March 9, 1989 VOLUME: 10 ml/kg EXCIPIENT: H_2O; pH 7 (12–15°)	SPECIES: RAT DATE: March 9, 1989 TIME:	ROUTE: PO, IP, IV, IM, PO

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFICATION	D0 14:00	D2	D5	D10	D15	NECROPSY
	DATE		9.3.89	11.3.89	14.3.89	20.3.89	24.3.89	
	INITIALS		TC	DL	MM/TC	MM/TC	TC/DL	
A	♂	R	160	190	210	260	290	
		T	170	195	215	265	300	
		D	165	195	220	275	305	
		Q	165	195	215	270	305	
		TQ	175	205	235	285	325	
B	♂	R	170	205	230	280	310	
		T	170	195	220	280	320	
		D	155	180	205	250	280	
		Q	160	190	220	270	300	
		TQ	160	190	220	270	300	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture		DOSE: 4089.62 mg/kg VOLUME 10 ml/kg EXCIPIENT H_2O I				SPECIES RAT SEX **M** F NUMBER 10			DATE March 9, 1989 TIME 14:00 INITIALS TC/MM				ROUTE **PO** IP IV IM PC OTHER		
SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, -	N	N	N		N	N	N	N	N	N	N	N		*N*
RIGHTING REFLEX	+ or - N,+, ++	+	+	+		+	+								
HYPERESTHESIA	N,+, ++	N	N	N		N	N								
HYPOESTHESIA	N,+ , ++														
TITUBATIONS	N,+ , ++														
JUMPS	N,+ , ++														
CRIES	N,+, ++														
TREMORS	N,+, ++														
CLON.CONVULSIONS	N,+, ++														
TON.CONVULSIONS	N,+, ++														
FLACCID ANIMAL	N, +														
STIFFNESS	N,+														
AGGRESSIVENESS	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N, , N ,+, ++														
PILOERECTION															
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,col.														
URINE VOLUME	N ,+, -														
DIARRHEA	N, +, ++														
FECES COLOR	N ,col.														
MIOSIS	N,+ , + +														
MYDRIASIS	N,+ , + +														
PTOSIS	N,+, ++														
IRREGULAR	N,+ , + +														
WHEEZING	N,+ , + +														
SHALLOW	N, +, ++														
ABDOMINAL CRAMPS	N, +														
SWOLLEN ABDOMEN	N, +, ++														
CYANOSIS	N, +, ++														
REDNESS	N, +, ++														
MOUTH BLOOD	N, +, ++														
NOSE BLOOD	N, +, ++														
EYES BLOOD	N, +, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE	SPECIES RAT	SUBSTANCE	Hypothiocyanate Generating Mixture				date/initials		MM/TC/DL	March 24, 1989	
SEX ♂	ROUTE PO	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	24.3	
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling	X	X	X	X	X	X	X	X	X	X	10
	spots dark			X								1
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	X	X	X	X	X	X	X	X	X	X	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

SUBSTANCE: Hypothiocyanate Generating Mixture (Glucose + Glucose oxidase + Lactoperoxidase + Sodium thiocyanate)	DOSE: 4.08962 g/l or 40.8962 mg/kg "c TC March 9, 1989 VOLUME: 10 ml/kg EXCIPIENT: H_2O; pH 7 (12–15°)	SPECIES: RAT DATE: March 9, 1989 TIME:	ROUTE: PO, IP, IV, IM, PO

BODY WEIGHT AND DATE OF DEATH

	SEX	IDENTIFICATION	D0 14:00	D2	D5	D10	D15	NECROPSY
	DATE		9.3.89	11.3.89	14.3.89	20.3.89	24.3.89	
	INITIALS		TC	DL	MM/TC	MM/TC	TC/DL	
A	♀	R	155	175	180	210	220	
		T	150	165	180	200	205	
		D	135	155	170	185	195	
		Q	145	165	175	195	210	
		TQ	150	170	180	210	220	
B	♀	R	155	170	190	210	230	
		T	145	165	180	200	215	
		D	145	160	175	205	220	
		Q	150	170	190	205	225	
		TQ	150	165	180	200	210	
	AVERAGE							
	STANDARD DEVIATION							

SUBSTANCE Hypothiocyanate Generating Mixture		DOSE: 4089.62 mg/kg VOLUME 10 ml/kg EXCIPIENT H_2O I				SPECIES RAT SEX M F NUMBER 10			DATE March 9, 1989 TIME 14:00 INITIALS TC/MM/DL				ROUTE PO IP IV IM PC OTHER		
SYMPTOMS	RESPONSE TYPE	15'	1 h	2 h	6 h	1d	2d	3d	4d	5d	6d	7d	10d		14d
SPONTANEOUS MOTOR ACTIVITY	N, -	N	N	N		N	N	N	N	N	N	N	N		N
RIGHTING REFLEX	+ or - N,+, ++	+	+	+		+	+								
HYPERESTHESIA	N,+, ++						N								
HYPOESTHESIA	N,+ , ++														
TITUBATIONS	N,+ , ++														
JUMPS	N,+ , ++														
CRIES	N,+, ++														
TREMORS	N,+, ++														
CLON.CONVULSIONS	N,+, ++														
TON.CONVULSIONS	N,+, ++														
FLACCID ANIMAL	N, +														
STIFFNESS	N,+														
AGGRESSIVENESS	N ,+, ++														
CATALEPSY	N ,+, ++														
STEREOTYPIES	N ,+, ++														
FEVER	N, , N ,+, ++														
PILOERECTION															
WEEPING	N ,+ ,+ +														
SALIVATION	N ,+, ++														
PERSPIRATION	N ,+, ++														
URINE COLOR	N ,col.														
URINE VOLUME	N ,+, -														
DIARRHEA	N, +, ++														
FECES COLOR	N ,col.														
MIOSIS	N,+ , + +														
MYDRIASIS	N,+ , + +														
PTOSIS	N,+, ++														
IRREGULAR	N,+ , + +														
WHEEZING	N,+ , + +														
SHALLOW	N, +, ++														
ABDOMINAL CRAMPS	N, +														
SWOLLEN ABDOMEN	N, +, ++														
CYANOSIS	N, +, ++														
REDNESS	N, +, ++														
MOUTH BLOOD	N, +, ++														
NOSE BLOOD	N, +, ++														
EYES BLOOD	N, +, ++														
MORTALITY															

Note in each case the number of animals involved and the type of response

OTHER COMMENTS

DOSE: 4.08962 g/kg	**SPECIES** RAT	**SUBSTANCE** Hypothiocyanate Generating Mixture **date/initials** March 24, 1989 **MM/TC**										
SEX: ♀	**ROUTE** PO	1R	1T	1D	1Q	1TQ	2R	2T	2D	2Q	2TQ	TOTAL
date of death or sacrifice		24.3	24.3.8	24.3.8	24.3	24.3	24.3	24.3	24.3	24.3	24.3	
autolysis/cannibalism												
nose	blood											
eyes	blood											
liver	discoloration											
	spots (color)											
	hemorrhagic area											
	hypertrophy											
	marbling											
small intestine	blood											
	ulcers											
	congestion											
	air											
	substance											
caecum	blood											
	atrophy											
	hypertrophy											
colon	congestion											
	blood											
whole stomach	hypervascularization											
	air											
	substance											
posterior stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
fundic stomach	ulcers											
	hemorrhage											
	perforation											
	scar tissue											
kidneys	discolored											
	marbling											
	spots dark			X	X		X	X	X	X	X	7
	appearance on cutting											
adrenal glands	atrophy											
	hypertrophy											
	blood											
spleen	atrophy											
	hypertrophy											
	dark color											
pancreas	atrophy											
	blood											
bladder	blood											
	deposits, crystals....											
abdominal cavity	liquid											
	blood											
	substance											
testicle or ovary												
epididymis or uterus												
lungs	hemorrhage											
	hepatization	X	X	X	X	X	X	X	X	X	X	10
heart	ischemia											
	hypertrophy											
thymus	blood (petechiae)											
	atrophy											
	hypertrophy											
other observations												

Annex 11

IPL - R
89079

INSTITUT PASTEUR DE LILLE

Toxicology Laboratory

Professor Daniel MARZIN

SUB-ACUTE ORAL TOXICITY STUDY

OF

HYPOTHIOCYANATE

GENERATING MIXTURE

For : **SYNFINA OLEOFINA S.A.**
Rue Jacques de Lalaing, 4
1040 BRUXELLES
Belgium

1, rue du Professeur Calmette - B.P. 245 - 59019 Lille Cedex
Tel. 20.87.79.14 - Telex 820 187 F - Fax 20.87.79.06

STUDY	:	**SUB-ACUTE ORAL TOXICITY STUDY IN RATS**
SUBSTANCE	:	**HYPOTHIOCYANATE GENERATING MIXTURE**
SPONSOR	:	**SYNFINA OLEOFINA S.A.**
SPONSOR'S CONTACT PERSON:	:	**Mrs. D. DEFRISE**
ADDRESS	:	Rue Jacques de Lalaing, 4 B-1040 BRUXELLES Belgium
TELEPHONE	:	19 (2) 223 91 11
TEST FACILITY	:	INSTITUT PASTEUR DE LILLE Laboratoire de Toxicologie Genetique B.P. 245 - 59019 LILLE CEDEX

INSTITUT PASTEUR DE LILLE CONTACT PERSON:

Study Director	:	Professor **D. MARZIN** Pharmacologist-Toxicologist Expert	~~January~~ 19, ~~1990~~	
Scientist	:	**H. VO PHI** Doctor of Pharmacy	.....	.....
Technicians	:	**M. MANGEZ**	.....	.....
	:	**T. CHASSAT**	.....	.....
	:	**D. LAGACHE**	.....	.....
Quality Assurance Unit Master of Sciences	:	**M.P. DEHOUCK**	.....	.....
			Date	Signature

ARCHIVING:

Raw data:
Archive Room No.1
Archive Box: SYNFINA OLEOFINA No. 1

Report:
Archive Room No.1
Archive Box: SYNFINA OLEOFINA No. 1

The complete report contains 798 pages

LILLE, January 19, 1990

CONTENTS

* * *

QUALITY ASSURANCE UNIT
CERTIFICATE

* * * *

STUDY TITLE : **SUB-ACUTE ORAL TOXICITY STUDY IN RATS OF HYPOTHIOCYANATE GENERATING MIXTURE**

SPONSOR : **SYNFINA OLEOFINA S.A.**

STUDY SITE : INSTITUT PASTEUR DE LILLE
Laboratoire de Toxicologie Génétique
B.P. 245
59019 LILLE CEDEX

This trial was conducted in compliance with **Good Laboratory Practice** (GLP US Food and Drug Administration Federal Register, Part II, December 22, 1978, Part 58, Title 21 and GLP, Instruction No. 1065 of May 31, 1983, Bulletin of the Ministry for Social Affairs and National Solidarity, p. 1–16), the OECD Principles of Good Laboratory Practice (GLP) (Guidelines C(81)30 (final), June 1, 1981) and in accordance with the INSTITUT PASTEUR DE LILLE procedures (SOP).

INSPECTION	CONDUCTED BY	DATE
PROTOCOL **No. 89079**	Study Director	March 13, 1989
	Quality Assurance Unit	March 13, 1989
		March 21, 1989
ADDENDUM TO PROTOCOL **No. 89079**	Study Director	April 17, 1989
	Quality Assurance Unit	April 17, 1989
		April 18, 1989
REPORT **No. 89079**	Study Director	
	Quality Assurance	

QUALITY ASSURANCE UNIT **STUDY DIRECTOR**

M.P. DEHOUCK Professor D. MARZIN

QUALITY ASSURANCE UNIT

INSPECTION DATES

* * * *

STUDY : SUB-ACUTE ORAL TOXICITY STUDY IN RATS

SUBSTANCE : HYPOTHIOCYANATE GENERATING MIXTURE

SPONSOR : SYNFINA OLEOFINA

INSPECTION DATE	TYPE OF INSPECTION	REPORT DATE
13/03/89	PROTOCOL	13/03/89
22/05/89	PREPARATION OF SOLUTIONS	8/08/89
9/05/89	TREATMENT OF ANIMALS	8/08/89
9/05/89	OBSERVATION OF ANIMALS	8/08/89
9/05/89	WEIGHING ANIMALS	8/08/89
9/05/89	WEIGHING FEED	8/08/89
8/08/89	FINAL REPORT	8/08/89

TEST FACILITY AND PERSONNEL RESPONSIBLE FOR CONDUCTING THE STUDIES

D. MARZIN (1):	Professor of Toxicology Head of Laboratory Study Director	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature
H. VO PHI (2)	Assistant Doctor of Pharmacy	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature

EXAMINATIONS AND STAGES OF THE STUDY:

- Stabulation-treatment	**D. MARZIN** Professor of Toxicology	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature
- Hematological examinations	**A.S. LERMOYER** Doctor of Pharmacy	Biochemical and Metabolic Analysis Unit of the INSTITUT PASTEUR DE LILLE Date Signature
- Biochemical examinations	**A.S. LERMOYER** Doctor of Pharmacy	Biochemical and Metabolic Analysis Unit of the INSTITUT PASTEUR DE LILLE Date Signature
- Urine tests	**D. MARZIN** Professor of Toxicology	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature
- Histology: * Necropsies	**D. MARZIN** Professor of Toxicology	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature
* Histological examinations	**G. SIOU** Doctor of Science Pharmacologist-Toxicologist Expert	CERTI-VERSAILLES (see report in Appendix)
- Writing the report and processing the results	**D. MARZIN** Professor of Toxicology	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE Date Signature

(1) Authorization no. for experimenting on live animals: 0522
(2) Authorization no. for experimenting on live animals: 0523

SUMMARY

EXPERIMENTAL PROTOCOL:

Test facility	:	INSTITUT PASTEUR DE LILLE
Substance		HYPOTHIOCYANATE Generating Mixture
Studied species	:	OFA Sprague-Dawley rat Males and females source IFFA CREDO – L'Arbresle (France)
Number per group and per sex	:	10
Doses administered	:	Group 1 : Control Group 2 : low dose Group 3 : medium dose Group 4 : high dose
Route of administration	:	Oral
Form of administration	:	Solution
Duration of treatment	:	12 weeks
Treatment frequency	:	7 days/week
First day of treatment	:	25 to 27 April 1989
Last days of treatment and date of sacrifice	:	16 to 19 July 1989
Objectives	:	To determine the systemic toxicity in male and female rats by oral administration for 12 weeks.

RESULTS:

Mortality	:	No treatment-related mortality.
Clinical signs	:	No treatment-related toxic clinical signs.
Changes in body weight	:	Greater weight increase in treated male animals
Feed consumption	:	Increased feed consumption in treated male animals
Feed consumption/weight increase ratio	:	No significant change.
Ophthalmic examinations	:	No treatment-related effects.
Hematological examinations	:	Increased leukocyte levels in male animals at 6 weeks, not observed at 12 weeks.
Biochemical examinations	:	Only a slight increase in triglycerides in treated male animals was observed at 6 weeks, but not observed at 12 weeks.
Urine tests	:	No treatment-related effects.
Examinations at necropsy	:	No treatment-related lesions.
Organ weight	:	Slightly increased liver weights in treated males in proportion to the dose.
Anatomopathological examinations	:	Increased vacuolization of hepatocytes with slight lipid excess in male animals and particularly in males treated with a high dose.

CONCLUSION:

The administration of the HYPOTHIOCYANATE Generating Mixture causes minor changes in treated male animals that appear to result from an increased feed consumption. These changes were not observed in female animals.

CHRONIC 12-WEEK ORAL TOXICITY STUDY IN RATS OF THE HYPOTHIOCYANATE GENERATING MIXTURE (GLUCOSE + GLUCOSE OXIDASE + LACTOPEROXIDASE + SODIUM THIOCYANATE)

* * * *

A/ GENERAL PROTOCOL

1. PURPOSE OF THE STUDY

The purpose of this study conducted at the INSTITUT PASTEUR DE LILLE, is to determine possible toxic effects of the studied substance that was repeatedly administered orally to rats for a period of 12 weeks.

2. PRINCIPLE OF THE STUDY

Young male and female rats receive the studied substance by gavage for 12 weeks. During the treatment period, the animals undergo clinical and behavioral examinations. In the middle of the study (6 weeks), animals undergo hematological and biochemical examinations. At the end of this period, the animals are sacrificed and subjected to necropsy. Hematological, biochemical and anatomopathological examinations are performed.

3. PROCEDURE

3.1 Test system

3.1.1 *Animals*

- Species : rat
- Strain : OFA Sprague-Dawley
- Source : IFFA CREDO (Saint-Germain-sur-l'Arbresle)

a) Reason for selecting the animal species

Animals requested by the Sponsor and registration authorities (OECD No. 407 of May 12, 1981), are a species traditionally used in the sub-acute and chronic toxicity studies due to their known responsiveness to toxic substances.

b) Weight/age

At the beginning of treatment, the animals have a body weight of about 140 g for males and 120 g for females and aged 6 to 7 weeks.

c) Acclimatization

Minimum 7 days before starting the treatment. The animals are subjected to a clinical examination to verify their general condition. Additional animals are subjected to acclimatization and can be used to replace any animal showing signs of poor general condition. Additional animals can be used to replace dead animals during the first week of treatment.

d) Identification

Animals are identified by marking the coat (with picric acid) and ear enabling to identify the number in a cage and the treatment group, respectively.

3.1.2. *Animal housing conditions*

Temperature	:	21±2°C
Humidity	:	60±20 %
Air exchange rate	:	12 changes per hour
Cages	:	25 x 45 x 12 cm polypropylene
Number per cage	:	3 or 4
Bedding	:	Dust-free, sterilized woodchip bedding
Feed	:	UAR 113
Drink	:	Acidified water pH 3.5

Bedding, drink and cages are changed weekly. The cages are not randomized, but are arranged in order, vertically on the racks. Every week, the cages are moved on the rack to avoid observing an effect related to the position of the cage on the rack.

3.2 Treatment

ADMINISTERED DOSES	GROUP A Control	Excipient: Glucose solution, 4 g/l	10 males and 10 females
	GROUP B Low dose	(1)	10 males and 10 females
	GROUP C Medium dose	(1)	10 males and 10 females
	GROUP D High dose	(1)	10 males and 10 females
ADMINISTRATION OF SUBSTANCE	ROUTE	Oral using a esophageal feeding tube	
	VOLUME	10 ml/kg (2)	
	FORM (excipient)	Aqueous solution or suspension in carboxymethyl cellulose	
	FREQUEN	7 days/7, 1 time a day	

(1) In principle, doses (in mg/kg) will be the **following:**

	Group B **Low dose**	Group C **Medium dose**	Group D **High dose**
Glucose	40	40	40
Glucose oxidase	0.002	0.006	0.02
Lactoperoxidase	0.025	0.075	0.25
Sodium thiocyanate	0.05	0.15	0.5

All animals receive the same amount of glucose.

The administered doses can be modified according to preliminary tests on the hypothiocyanate production.

(2) The volume administered is adjusted according to the latest recorded body weight.

3.2.1. *Rationale for selecting the route of administration*

The studied substance is a technological additive for use in cheese making, any residue would be ingested by consumers that is why the oral route was chosen.

3.2.2. *Rationale for selecting the doses*

In all cases, we will consider the maximum conditions of use. The mixture can be used in two ways:

1. The powdered substance is used as a basis for the preparation of ice cream, pastry ... (=mix) in this case, commercially, it can hardly exceed 1%, or 10 g of LPS/kg of powder. However, these 10 g of LPS may contain different amounts of LP, SCN, GOD and glucose, depending on the formulation selected and dosing of the mix.

e.g.: 500 g of mix for 1.5 kg of cream (500 g of mix + 1 l of water or milk) formulation selected 25/50/2/2 (final concentration for 1 kg of finished product)

for 5 g of LPS

(LP 37.5 mg
(SCN 75 mg
(GOD 3 mg
(Gluc 3 g
(and medium up to 5 g

This represents 3.1155 g of mixture for about 1.5 l or 2.077 g/l.
Therefore a subject consuming 100 g of this cream per day consumes 207.7 mg of the mixture per day or for a subject who weighs about 50 kg: **4 mg/kg**

2 - The mixture is directly introduced into a liquid product (for example milk for yogurt) in this case the composition is:

- glucose 4000 mg
- glucose oxidase 2 mg
- lactoperoxidase 25 mg
- sodium thiocyanate 50 mg per liter

or 4077 mg/l of mixture.

A subject consuming 250 g of the product per day consumes 1000 mg/day, for a subject who weighs 50 kg: **20 mg/kg.**
The maximum ingested amount is between 4 and 20 mg/kg/day. The study of the hypothiocyanate production shows that the composition of the mixture described in item 2 is optimal for maximum production. Higher concentration produces hydrogen peroxide in an amount that destroys the lactoperoxidase.

So the system is diverted to another type of production without producing hypothiocyanate. This is why the highest studied dose is 40.72 mg/kg of the mixture, which corresponds to twice the maximum concentrations ingested by humans during consumption. Furthermore, ingestion is usually not performed immediately after mixing the hypothiocyanate generating substances. These two parameters provide a significant safety factor for extrapolation to humans. The lowest dose is the dose corresponding to the maximum ingested dose in humans as defined in item 1 above. The medium dose being an intermediate concentration between these 2 doses.

3.3 Preparation of solutions

The solutions are prepared extemporaneously, the concentration of prepared solutions is not checked given the instability of the preparation.

The solutions used for the treatment are prepared by mixing the previously prepared solutions. Given the instability of the preparation, glucose and sodium thiocyanate solutions are kept at 4°C in solution form while the glucose oxidase and lactoperoxidase solutions in 0.9% NaCl are kept as aliquoted frozen solutions to maintain the stability of enzyme activity. Solutions are administered between 10 and 20 minutes after mixing them.

3.3.1 *Preparation of stock solutions*

For the preparation, the following solutions are prepared in water.

1 - Filter-sterilized aqueous solution of sodium thiocyanate (two-fold concentrated), 139.6 mg/l, stored at 4°C

2 - Filter-sterilized aqueous solution of glucose (two-fold concentrated), 8 g/l, stored at 4°C in 0.9% NaCl

3 - Filter-sterilized aqueous solution of glucose oxidase (hundred-fold concentrated), 200 mg/l, stored at –18°C in 0.9% NaCl as 1 ml aliquots

4 - Filter-sterilized solution of lactoperoxidase in 0.9% NaCl (hundred-fold concentrated), 1.872 g/l, stored at –18°C as 1 ml aliquots

The concentrations are adjusted according to the activities of 2 enzymes and the degree of hydration of the sodium thiocyanate

3.3.2. *Preparation of treatment solutions*

The following mixtures are extemporaneously prepared under the following conditions:

	GROUP A	GROUP B	GROUP C	GROUP D
Solution 1	0	2.5	7.5 ml	25 ml
Solution 2	25 ml	25 ml	25 ml	25 ml
Solution 3		50 µl	1500	500 µl
Solution 4		50 µl	150 µl	500 µl
Distilled water	25 ml	22.5 ml	17.2 ml	0

3.4 Examinations

All animals are subjected to clinical, hematological, ophthalmic, blood examinations and urine tests, which are described in Table No. 1.

TABLE No.1

TYPE OF EXAMINATION		METHOD	FREQUENCY OF
3.4.1 Clinical examinations	Changes in body weight Increased weight	Weighing	Once per week
	Feed consumption Drink consumption	By measuring unconsumed feed and water	Once per week
	GENERAL EXAMINATION: (1) appearance, behavior, excreta, skin, fur, respiratory system, circulatory system, motor system and nervous system, mortality	Using a behavior grid	daily
3.4.2 Hematological examinations	erythrocyte count, leukocyte count, hematocrit, hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume	Contrav AL 801	6 and 13 weeks
	Differential leukocyte count	Preparation of blood smears on slides, staining	6 and 13 weeks
3.4.3 Ophthalmic examinations		Heinz ophthalmoscope	D0 then at 1, 2, 4, 6, 8 and 12 weeks
3.4.4. Blood biochemistry	Glucose, urea, creatinine, cholesterol, triglycerides, total protein, alkaline phosphatase, ASAT-ALAT aminotransferases, bilirubin	Hitachi automatic analyzer	6 and 13 weeks
3.4.5. Biochemistry	Urine 24-hour volume, appearance, analysis for: glucose, bilirubin, blood proteins, ketone bodies Determining pH Density	Test strips Densimeter	6 and 13 weeks

(1) Any animal showing a general condition indicating a probable quick death is sacrificed. Any dead or sacrificed animal during the study is subjected to macroscopic examination. The tissue samples specified in the protocol are stored unless autolysis or cannibalism occurs.

3.4.6 *Anatomopathological examinations*

a) Sacrifice

At the end of treatment, after about 18 hours of a clear liquid diet, all animals are anesthetized by intraperitoneal injection of Imalgene then sacrificed by exsanguination. The animals are weighed prior to sacrifice.

b) Anatomopathological examinations			
	Type of examinati	Animals concerned	ORGANS
Macroscopic examinations	General Weighing	All	Thoracic and abdominal Liver, kidney, heart, spleen, adrenal glands, gonads, thyroids with parathyroids, thymus, uterus or seminal glands, prostate
Macroscopic examinations (1)	Histology	All	All the weighed organs + lungs, stomach, duodenum, ileum, colon, pancreas, bladder, submaxillary glands, mesenteric lymph nodes

(1) : The removed organs are fixed in Bouin solution except for the portion of liver and kidneys, which are fixed in neutralized formalin for analyzing fat content.

3.5 Statistical analysis

All the results are analyzed statistically by performing the following calculations:

- Average
- Standard Deviation
- Student's t-test

This test is used to find out if the difference between the average values obtained in the control and treated groups is statistically significant at the threshold of 5% by comparing the value obtained from the Student's t-test with the one from Student's t-table for (n1 + n2-2) degrees of freedom (n1 and n2 are the size of each control and treated group.)

For all groups, a regression analysis is determined with a correlation coefficient calculation, of the dose-dependent response calculated on the total of the individual values. The regression line is drawn.

4. GOOD LABORATORY PRACTICE

4.1. Archives

The following documents and specimens will be retained:

- In the archives of the INSTITUT PASTEUR DE LILLE

  - Protocols and any amendments
  - Original data
  - Correspondence
  - Final reports and any amendments
  - Sample of the studied substance
  - Hematological slides

- In the archives of CERTI (Versailles)

  - Tissue samples in the preservative
  - Blocks
  - Histological slides

4.2. Test facility

TOXICOLOGY LABORATORY CONTACT PERSON

D. MARZIN	-	Professor of Toxicology Head of Toxicology Unit Study Director	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE
H. VO PHI	-	Doctor of Pharmacy Assistant of the Toxicology Laboratory	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE

EXAMINATIONS

- Stabulation-treatment D. MARZIN	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE
- Ophthalmic examinations D. MARZIN	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE
- Hematological examinations A.S. LERMOYER Pharmacist	Biochemical and Metabolic Analysis Department of the INSTITUT PASTEUR DE LILLE
- Urine tests D. MARZIN	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE
- Histology	
• Necropsies D. MARZIN	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE
• Histological examinations G. SIOU Doctor of Science Pharmacologist-Toxicologist Expert	CERTI-VERSAILLES
Writing the report and processing the results D. MARZIN	Toxicology Laboratory of the INSTITUT PASTEUR DE LILLE

B/ **SPECIFIC PROTOCOL:**

SPONSOR	:	**SYNFINA OLEOFINA**	
SUBSTANCE	:	**HYPOTHIOCYANATE Generating Mixture**	
BATCH NUMBER	:	1415/04064 P	
APPEARANCE	:	Light yellow liquid	
QUANTITY	:	Lactoperoxidase	0.019 g
		Glucose oxidase	0.200 g
		Glucose	1200.000 g
		Sodium thiocyanate	0.019 g
I.P.L. NUMBER	:	Lactoperoxidase	89/22
		Glucose oxidase	89/21
		Glucose	89/20
		Sodium thiocyanate	89/23

This substance was studied according to the general protocol, with the following exceptions or specificities:

ROUTE	:	ORAL
EXCIPIENT	:	Phosphate buffer 5 mM pH 7
VOLUME OF ADMINISTRATION	:	10 ml/kg

STUDY INITIATION DATE:	:	April 25, 1989
STUDY COMPLETION DATE	:	July 19, 1989

B/ RESULTS

1. MORTALITY

No animals died during the study.

2. BEHAVIOR - CLINICAL EXAMINATIONS

For the duration of the treatment, the animals showed normal behavior and no treatment-related effects were noted.

Clinical examinations observed sporadic epistaxis, an effect that does not appear to be linked to treatment as it appeared in control and treated animals, but rather it is a result of injuries inflicted between animals, or lesions caused by blood sampling from retro-orbital sinus at week 6. A few cases of chromodacryorrhea also not related to treatment were noted. These observations are related to a spontaneous benign pathology.

Daily clinical examinations did not find any other changes in animal behavior. It is therefore apparent that no treatment-related effects on behavior were noted.

3. CHANGES IN BODY WEIGHT

Treated male animals showed a slightly higher body weight increase compared to the control animals. The increase was particularly significant in male animals treated with the medium dose starting from the second week and throughout the duration of the treatment period, the body weight of male animals treated in this group remained statistically significantly higher.

At the higher dose, the body weight of the animals was also higher than that of the control animals, but the difference was not significant.

The analysis of linear regression did not demonstrate the dose-response relationship in female animals. A slightly lower body weight of females treated with the medium dose was only recorded at 10 weeks of treatment. For the rest of the treatment period, no difference in body weight was observed. No statistically significant changes were observed with other doses.

The body weight increase was higher than that of the control animals in male animals at the following time points:

- 5, 8 and 9 weeks at the high dose.
- 2 and 9 weeks at the medium dose.
- 10 and 12 weeks at the low dose.

It therefore appears that male animals have a statistically significant higher body weight increase. However, analysis of the regression line did not reveal a dose-response relationship at any time point. In female animals, however, only the animals treated with the low dose showed a significant decrease in body weight gain at 6 weeks. To the extent that there is no dose-response relationship and this increase was only observed at a time and dose that corresponds to the week when the animals were fasted in order to carry out biological tests at the intermediate time point. Therefore it appears that this change has no toxicological significance.

4. FEED CONSUMPTION

In treated male animals, feed consumption was slightly higher to a statistically significant degree than that of control animals:

- At the low dose, weeks 4, 5 and 11.
- At the medium dose, the observed increase was not statistically significant.
- At the high dose, weeks 4, 5, 9 and 11.

It therefore appears that in male animals, the treatment caused increased feed consumption.

Analysis of the dose-response relationship (simple regression analysis) showed a significant dose-response relationship at 2, 4, 5, 9 and 11 weeks.

In female animals, only at 4 weeks, a statistically significant increase in feed consumption was observed in animals treated with the high dose. The statistically significant dose-response relationship was demonstrated only at week 3 and 4.

5. CALORIC USE FACTORS

The caloric use factors were comparable between the treatment and control groups throughout the treatment period.

6. DRINK CONSUMPTION

In male animals, no statistically significant change in the drink consumption was noted throughout the treatment period. A decrease at 6 weeks and an increase at 10 weeks, proportional to the dose was observed. However, in the absence of statistically significant changes, this effect appears to have no toxicological significance.

In female animals, lower drink consumption was observed in animals treated with the low dose at week 1 to 7 inclusive and during week 9. In females treated with the medium dose, the decrease was statistically significant at week 1 and 10, whereas in animals treated with the high dose, no statistically significant change was observed.

With the exception of the 10 week time point, no statistically significant dose-response relationship was observed.

7. OPHTHALMIC EXAMINATIONS

Before the 6 week time point, no ocular lesion was observed. After 6 weeks, ocular lesions were observed in both control and treated animals, these lesions are in fact attributable to traumatic injuries unrelated to treatment and subsequent to blood sampling from retro-orbital sinus at week 6.

8. HEMATOLOGICAL EXAMINATIONS

8.1. 6 WEEK TIME POINT

In male animals, an increase in the number of leukocytes at the highest 2 doses with a dose-response relationship (r= 0.451; p = 0.0035) was observed.

No such change was noted in female animals.

Moreover, in male animals, a slight increase in erythrocytes at the medium dose and hematocrit at the highest 2 doses with a decreased mean corpuscular hemoglobin concentration at the highest 2 doses was observed, including a decreased MCH at the medium dose. Furthermore, a decrease in the number of monocytes in the low dose was noted.

However, these changes remained within physiological limits and have no toxicological significance.

In female animals, decreased level of monocytes was observed at the 2 highest doses, however, the dose-response relationship was not statistically significant (r = 0.273; p = 0.0884). This change remained within physiological limits and has no toxicological significance.

8.2. 12 WEEK TIME POINT

In male animals, an increased level of leukocytes observed at 6 weeks was not found. In females, the decrease in monocytes was statistically significant at the 3 studied doses, but without demonstrating a dose-response relationship. In male animals however, the increased percentage of monocytes was noted, but only at the intermediate dose.

Finally, the slight, but statistically significant increase in MCH in males treated with medium dose should be noted.

These changes, however, remain within the physiological range and cannot be attributed to treatment.

9. BIOCHEMICAL EXAMINATION OF BLOOD

9.1. 6 WEEK TIME POINT

In male animals, a statistically significant decrease in aminotransferase (AST) was observed at the low dose without changes at the 2 higher doses. Furthermore, a decrease in the aminotransferase (ALT) level was observed at the low and medium doses. In animals treated with the high dose, no changes were observed. Such decreases in plasma enzyme activity have no toxicological significance.

However, a statistically significant increase of the plasma triglyceride levels in male animals proportional to the dose ($r = 0.363$; $p = 0.0212$) appears to be treatment-related.

9.2. 12 WEEK TIME POINT

In male animals decreased aminotransferase (AST) level was noted in animals treated with the medium dose. This change has no toxicological significance.

No other statistically significant change was observed at this time point in treated males animals. Note that the increase in plasma triglycerides observed at 6 weeks did not appear to be statistically significant at 12 weeks and no dose-response relationship was observed. In female animals, only a statistically significant decrease in creatinine levels was noted. This change has no toxicological significance.

10. URINE TESTS

At 2 observation points, no change in quality or quantity was observed.

11. ANATOMOPATHOLOGICAL EXAMINATIONS

11.1. MACROSCOPIC EXAMINATIONS

In male animals the following lesions were observed:

- Male No. 23 treated with the low dose: one of the thyroids was below normal size (6.3 mg), while the other had a distinctly normal size (12.3 mg).
- Male No. 27 treated with the low dose: presented a significant folding of the stomach smooth muscle.
- Male No. 30 treated with low dose presented a thymic atrophy.
- Male No. 44 treated with medium dose presented an atrophy of the left adrenal (12.9 mg), the other adrenal (26.9 mg) also weighed slightly below normal.
- Males 47 and 48 treated with the medium dose: a desquamation of the fundic stomach wall was noted.
- Male No. 49 treated with the medium dose presented an atrophy of the left thyroid.
- Male No. 61 treated with the high dose presented a testicular hematoma.
- Male No. 65 treated with high dose presented a desquamation of the fundic stomach wall.

In female animals the following lesions were observed:

- Female No. 11, control group: hemorrhage in the ovary.
- Female No. 15, control group: atrophy of thyroids.
- Female No. 17, control group: atrophy of thyroids.
- Female No. 9, control group: significant desquamation of the fundus with erosion.
- Female No. 31 treated with the low dose: presented an atrophy of the right thyroid.
- Female No. 35 treated with the low dose: an atrophy of the right thyroid, signs of an old hemorrhage in the fundic stomach wall.
- Female No. 37 treated with the low dose: an atrophy of the right thyroid, stomach wall desquamation.
- Female No. 40 treated with the low dose: congested uterus.
- Female No. 53 treated with the medium dose: adrenal glands adherent to the upper pole of the kidney (difficult to obtain a specimen).
- Male No. 54 treated with the medium dose: desquamation of the fundic stomach wall.

The summary of lesions observed is as follows:

LESIONS	CONTROL		LOW DOSE		MEDIUM DOSE		HIGH DOSE	
	♂	♀	♂	♀	♂	♀	♂	♀
GASTRIC LESIONS	0	1	2 (1)	2 (2)	2 (1)	0	1	1
THYROID ANOMALIES	0	2	1 (2)	1 (2)	1 (2)	0	0	0
ADRENAL GLANDS ANOMALY	0	0	0	0	1 (2)	1 (2)	0	0
TESTICULAR HEMATOMA	0	-	0	-	0	-	1 (2)	-
HEMORRHAGE/ OVARY	-	1	-	0	-	0	-	0
CONGESTED UTERUS	-	0	-	1 (2)	-	0	-	0

(1) $x2 = 0.556$ N.S.
(2) $x2 = 0$ N.S.

It therefore appears that none of these lesions are related to treatment since no increase in the incidence of lesions was noted.

Moreover, if on the one hand we consider the gastric lesions, which appear to be the most frequent, no dose-response relationship appears, but on the other hand, considering all data, 8 animals out of 60 have gastric lesions compared with 1 out of 2 for control animals, the statistical comparison ($x2 = 0.376$) shows no significant difference. Even taking the worst case, that is to say, only male animals, 0/10 in control animals and 5/30 in the treated animals, the difference is not statistically significant there either ($x2 = 0.686$: N.S.). The observed gastric lesions are not related to the treatment and due solely to spontaneous changes of the mucosal structure.

11.2. ORGAN WEIGHT

In male animals, a statistically significant increase in body weight at the 2 highest doses was observed. However, the dose-response relationship was not statistically significant.

A statistically significant decrease in thymus weight was observed at the lowest dose, this change was not observed in relative value, so it has no toxicological significance. In contrast, an increase in liver weight was noted at 3 doses in absolute value and at the highest dose in relative value; an increase proportional to the dose was noted both in absolute value ($r = 0.413$; $p = 0.0089$) and in relative value ($r = 0.318$; $p = 0.00484$) and can therefore be attributed to treatment.

An increased spleen weight at the 2 highest doses with a dose-response relationship ($r = 0.332$; $p = 0.0366$) was observed, but this increase is not found in absolute values, it is only due to the increased body weight.

In female animals, only a decrease in thymus weight was noted in animals treated with the low dose, however, this change is not observed in relative value.

In relative value, only a small, but statistically significant, increase of heart weight at average dose was observed in female animals.

Therefore changes observed in female animals have no toxicological significance.

11.3. HISTOPATHOLOGICAL EXAMINATIONS

The histopathological findings are summarized in the report provided by Dr. G. SIOU.

ANATOMOPATHOLOGICAL REPORT

* * * *

C. E. R. T. I.

Histopathology Laboratory

59, *avenue* de Paris - 78000 VERSAILLES - (1) 39.53.49.76

STUDY No. 1385

INSTITUT PASTEUR DE LILLE
October 19, 1989

REPORT ON

HISTOPATHOLOGICAL EXAMINATIONS

12-WEEK ORAL TOXICITY STUDY IN RATS OF

THE HYPOTHIOCYANATE (SCNO) GENERATING MIXTURE

TABLE OF CONTENTS

ADMINISTRATIVE INFORMATION

Study laboratory:	Histopathology Laboratory CERTI 59 avenue de Paris - 78000 VERSAILLES
Work performed for:	INSTITUT PASTEUR DE LILLE 1 rue du Pr Calmette - 59019 LILLE
Studied substance:	Hypothiocyanate (SCNO) Generating Mixture
Type of study:	Histopathological examinations 12-week oral toxicity in rats
Study number:	1385
Specimens reception:	July 26, 1989
Received liquids:	70° alcohol - Baker Formaldehyde
End of examinations (verification):	October 17, 1989
Date of report:	October 19, 1989
Number of pages of the report:	100
Director of the Laboratory:	Gilbert SIOU
Histopathologist	Magali GUFFROY
Technicians:	Gilles MERCIER Martine PELLETIER Nathalie PIPON

Magali GUFFROY
Veterinarian

Gilbert SIOU
Doctor of Science
Certified Toxicologist Expert

CERTI – 1385

GOOD LABORATORY PRACTICE IN

HISTOPATHOLOGY LABORATORY

The following information is included in this report in accordance to the Good Laboratory Practice (instructions of May 31, 1983):

. Specimens reception date.
. Names of technicians participated in work.
. Brief description of used techniques.
. Equipment used.
. Procedures for identifying the specimens, the registers used to track specimens from their receipt to preparing samples for histological examination.
. Individual diagnostic sheets, dated and initialed.
. Archiving.

In addition, the following checks were performed:
– Concordance between different specimens identification registers stated in paragraph 11, of the Materials and Methods Chapter.
– Concordance between text, observations in the various tables of this report and the individual observations.

October 19, 1989

Marie-Thérèse LAMBERTI
Responsible for GLP

I. MATERIALS AND METHODS

The histological examination of the specimens was performed on sections stained with aniline blue according to Masson's trichrome staining protocol. In addition, the following histochemical methods were implemented:

- Perls' Prussian blue for detecting the presence of ferric iron and foci of hemosiderosis.

- Staining with fuchsin paraldehyde for identification of cytoplasmic granulation in B-cells of islets of Langerhans and elastic fibers.

- Staining with the Sudan Red for visualization of lipids.

1. Experiment:

. 12-week oral toxicity of Hypothiocyanate (SCNO) Generating Mixture.

2. Animal species:

. Rat (males and females)

3. Histological references:

. Correspondence between histological specimen numbers and animals number.

Group 1: control - males			Group 2: control - females		
CE 15788	...	1	CE 15798	...	11
CE 15789	...	2	CE 15799	...	12
CE 15790	...	3	CE 15800	...	13
CE 15791	...	4	CE 15801	...	14
CE 15792	...	5	CE 15802	...	15
CE 15793	...	6	CE 15803	...	16
CE 15794	...	7	CE 15804	...	17
CE 15795	...	8	CE 15805	...	18
CE 15796	...	9	CE 15806	...	19
CE 15797	...	10	CE 15807	...	20

Group 3: high dose - males			Group 4: high dose - females		
CE 15808	...	61	CE 15818	...	71
CE 15809	...	62	CE 15819	...	72
CE 15810	...	63	CE 15820	...	73
CE 15811	...	64	CE 15821	...	74
CE 15812	...	65	CE 15822	...	75
CE 15813	...	66	CE 15823	...	76
CE 15814	...	67	CE 15824	...	77
CE 15815	...	68	CE 15825	...	78
CE 15816	...	69	CE 15826	...	79
CE 15817	...	70	CE 15827	...	80

Group 5: medium dose - males			Group 6: medium dose - females		
CE 15828	...	41	CE 15798	...	51
CE 15829	...	42	CE 15799	...	52
CE 15850	...	43	CE 15800	...	53
CE 15831	...	44	CE 15801	...	54
CE 15832	...	45	CE 15802	...	55
CE 15833	...	46	CE 15803	...	56
CE 15834	...	47	CE 15804	...	57
CE 15835	...	48	CE 15805	...	58
CE 15836	...	49	CE 15806	...	59
CE 15837	...	50	CE 15807	...	60

Group 7: low dose - males			Group 8: low dose - females		
CE 15848	...	21	CE 15858	...	31
CE 15849	...	22	CE 15859	...	32
CE 15850	...	23	CE 15860	...	33
CE 15851	...	24	CE 15861	...	34
CE 15852	...	25	CE 15862	...	35
CE 15853	...	26	CE 15863	...	36
CE 15854	...	27	CE 15864	...	37
CE 15855	...	28	CE 15865	...	38
CE 15856	...	29	CE 15866	...	39
CE 15857	...	30	CE 15867	...	40

4. Removed organs:

The number of specimens provided from the same organ is show in brackets. In the case of paired organs, this figure indicates whether one part or both parts of the organ were removed. It is possible that for some animals, not all the anticipated organs were removed or that some were lost or damaged during preparation. The exact number of specimens examined for each animal is shown in the individual data.

Digestive system
- liver (1)
- stomach (1)
- small intestine (2)
- colon (1)
- salivary glands (2)
- pancreas (1)

Urinary system
- kidneys (2)
- bladder (1)

Hematolymphoid system
- thymus (1)
- spleen (1)
- lymph nodes (1)

Endocrine system
- thyroid (2)
- adrenal glands (2)

Reproductive system
- testicles (2)
- seminal vesicles (2)
- prostate (1)
- ovaries (2)
- uterine horns (2)

Cardiovascular system
- heart (1)

Respiratory system
- lungs (1)

Information concerning certain specimens

Small intestine: a specimen from the duodenum, a specimen from the ileum.

5. Fixatives:

- Bouin solution for all specimens except liver.
- Baker Formaldehyde for liver and analyzing fat content in liver and kidney specimens.

6. Fixation:

- By immersion

7. Embedding

Dehydration was performed with an Autotechnicon and samples are embedded in paraffin (Histomed).

8. Sections

a) Paraffin
3 or 4 micron-thick sections were prepared with a Leitz or Jung microtome equipped with disposable blades.

b) Freezing
20 or 25 micron-thick sections were prepared with a freezing microtome Leitz Kryomat.

9. Specimens subjected to examination

All removed organs were subjected to histological examination. The organs and animals subjected to various examinations are given in Table I at the end of this chapter.

Planes of section

Stomach: cutting through the squamous and/or glandular mucosa.

Small intestine: cross section.

Colon: cross section.

Kidneys: longitudinal section for one kidney, cross section for the other kidney.

Spleen: cross section.

Thyroid: longitudinal section.

Testicles: longitudinal section.

Uterine horns: cross section.

Heart: longitudinal or cross section.

No particular orientation for other organs.

10. Performed examinations

When some preparations required comparison by several observers, it is recorded, via a camera mounted on the Leitz Orthoplan microscope with a videotape system (Grundig VCR U-Matit Sony camera and color monitor).

a) Histological examinations

Masson's trichrome staining protocol (Goldner modification, 1938)

Ferric trioxyhematein of Hansen
Acid fuchsin and xylidine ponceau
Aniline blue

The staining was performed using an automatic slide stainer (Varistain 24, Shandon), the samples were mounted in a Hypermount between slide and cover slip using an automatic cover slipping machine (Autoslip, Shandon).

b) Histochemical examinations on paraffin sections

All histochemical reactions were performed on sections from the same paraffin blocks as sections subjected to trichrome staining. Samples were stained and mounted using the same devices as for trichrome staining.

- Perls' Prussian blue

Detecting the presence of ferric iron with potassium ferrocyanide in presence of hydrochloric acid. The Perls method used here is a close variant of the Gomori method (1951). The sections of the control animal tissues and treated animal tissues were stained at the same time. Where the number of slides exceeds the capacity of the device sample rack (40), the slides with tissue samples from animals of the same sex are always processed together. One control slide, with the sample which is known to be rich in iron (spleen) is used as a positive control. This slide is subjected to the Perls' Prussian blue reaction either simultaneously with other slides or before reaching the capacity of the slide stainer sample rack.

An assessment of the localization significance is done by estimating it in 0, +, ++, +++ for the liver and kidneys and up to ++++ for the spleen. There is no need to emphasize the relative nature of this coding, which is only valid for the slides of the same experimental series.

– Staining with fuchsin paraldehyde

Staining with fuchsin paraldehyde developed for the staining of elastic fibers (Gomori, 1950), also used for staining many secretory products. Background staining is achieved by conventional trichrome staining.

When applied to islets of Langerhans, this stain allows to highlight, on the one hand, the B-cells whose granules are stained with fuchsin paraldehyde in dark purple and, on the other hand, other islet cells, mostly A-cells, which are stained in more or less dark pink. The frequency of B-cells, visualized by their granulation is assessed by 0, +, ++, +++ coding. The considered normal aspect is +++. The thickness of the samples obviously affects the intensity of the staining. The sections of the control animal tissues and treated animal tissues were stained at the same time. Where the number of slides exceeds the capacity of the device sample rack (40), the slides with tissue samples from animals of the same sex are always processed together.

c) Analyzing lipids on frozen sections

Detecting lipids with Sudan Red in isopropyl alcohol solution. The quality of the lysochrome solution is first verified using a section obtained from a lipid-rich organ (liver or kidney). Frozen sections examination. An assessment of the localization significance is done by estimating it in 0, +, ++ or +++.

11. Procedures for identifying the specimens

Four registers are used to track specimens from their receipt to preparing samples for histological examination:

- Register 1: date of specimens received and name of the laboratory from where they came from.
- Register 2: correspondence between the histological references (number starting with "CE") and animals number (usually all specimens of the same animal have the same number).
- Register 3: indication of the received organ specimens and paraffin-embedded tissues for each histological reference, (additional section sheets).
- Register 4: date, reference and number of blocks cut with a microtome by each technician.

12. Original observation documents

Observations made during the microscopic examination are simultaneously recorded on a dictaphone and registered in computer system. Printed documents (diagnostic sheets) are checked against the tape recording. After this verification and any corrections, these diagnostic sheets are dated and initialed. They comprise the raw data collected during observations and therefore represent original documents that are included in the examination reports in the Appendices.

13. Archiving

A copy of the report is stored in archives, in a locked cabinet in order of the study number.

Paraffin sections from histological specimens are kept in a Technicon slide storage box. Extemporaneously prepared frozen sections used for analyzing fat content are discarded after analysis. The paraffin blocks are also retained. The expected duration of storage for paraffin sections from histological specimens and paraffin blocks is five years.

Specimens not embedded in paraffin are discarded.

14. Bibliographical references

The methods used are included in following two publications:

GABE (M.).
Techniques histologiques.
Masson Edit., Paris 1968, un vol., 1113 pages.

MARTOJA (R.) et MARTOJA (M.).
Initiation aux techniques de l'histologie animale.
Masson Edit., Paris 1967, un vol., 345 pages.

For the pathology, the cited references can be found in the following publications:

BENIRSCHKE (K.), GARNER (F.M.) et JONES (T.C.).
Pathology of Laboratory Animals.
Springer-Verlag, New-York 1978, 2 vol., 2171 pages.

TURUSOV (V.S.).
Pathology of Tumours in Laboratory Animals.
Volume I : Tumours of the Rat (2 parties), 214 et 219 pages.
Volume II : Tumours of the Mouse, 669 pages.
I.A.R.C., Lyon 1979.

GREAVES (P.) et FACCINI (J.M.).
Rat Histopathology.
Elsevier Edit., Amsterdam 1984, un vol., 251 pages.

DUBREUIL (G.) et CANIVENC (R.).
Manuel théorique et pratique d'histologie.
Vigot Frères Edit., Paris 1967, 2 vol., 452 et 365 pages.

PAGES (A.) et MARTY-DOUBLE (Ch).
Histopathologie endocrinienne.
Masson Edit., Paris 1977, un vol., 294 pages.

Table I. – Histopathological examinations – HYPOTHIOCYANATE – Rat
Specimens subjected to examination

Method	Organs	Groups	Animals
Trichrome	All organs	1 to 8	All animals
Fuchsin paraldehyde + Trichrome	Lungs – Pancreas	1 to 8	All animals
Perls' Prussian blue	Liver – Spleen – Kidneys (2) Lymph node	1 to 8	All animals
Sudan Red	Liver – Kidneys (1)	1 to 8	All animals

II. – RESULTS

Individual observations are given in the Appendices. For each animal, the number of examined specimens is indicated and, when several specimens of the same organ are examined, the number of specimens from the anatomical site for each type of lesion. The computer system takes into account only the presence or absence of signs in the columns and does not calculate an exact sum. The sum given in the last column of each table is for the number of animals and not the number of specimens. In this chapter we provide a general report for each organ class. The organs are grouped into large systems and all the observations made for each organ in different groups are given in the tables shown at the end of each report relating to a system. These tables show the summations given in the last column of the tables of the individual data.

The observations for some organs include specific remarks for defining certain aspects or the presence of common observation structures.

Some paired organs may not have the same functional aspect for two different specimens; therefore the appearance of each of the two specimens is described. When the specimen does not match the specified organ, it is stated, whenever possible, the type of tissue or organ actually present in the specimen.

DIGESTIVE SYSTEM

Morphological examination of the liver paraffin sections showed lipid-like vacuolization of hepatocytes, greater in treated males than in the control males (multifocal periportal microvacuolization in males treated with the high dose, diffuse microvacuolization in males treated with the three doses). The fat content analysis performed on frozen sections characterized the lipid nature of this vacuolization.

LIVER

1' Histological examinations

a) Preliminary comments concerning the observations

The dense or relatively clear appearance of the hepatocytes cytoplasm is generally related to the speed of fixing the specimen and cannot be interpreted as toxic damage to the cell. It is known that the hepatocytes appearance varies according to the time of fixation. If the fixing is done early, before the onset of autolysis, hepatocytes have a heterogeneous cytoplasm. If the fixing is done after the onset of autolysis, hepatocytes lose their glycogen, shrink and the cytoplasm becomes very dense and homogeneous. Anyway, we must not forget that the density aspect of the cell cytoplasm is partly due to the thickness of the section, the thicker the section the more dense the cytoplasm. We classified the appearance of hepatocytes cytoplasm as following:

- Homogeneous cytoplasm, cytoplasm appearing colored in very uniform manner.
- Heterogeneous cytoplasm, the cytoplasm appears finely coagulated with more or less dense areas.
- Clear cytoplasm, almost optically empty, the hepatocyte appears to have a reduced nucleus and cytoplasmic membrane.

If all hepatocytes of the specimen have the same cytological aspect, the parenchyma is considered to be homogeneous. In the case where, following lobular zones, the hepatocytes cytoplasm differs in appearance, the parenchyma is considered to be heterogeneous.

b) Observations

Except in one male treated with the low dose, the parenchyma has a homogeneous aspect; the hepatocytes in centrilobular and perilobular zones have the same tinctorial affinity. Their cytoplasm is heterogeneous. The nuclei have normal appearance, the frequency of binucleated cells does not appear to be increased. On the other hand, in one male treated at the low dose, the parenchyma has a slightly heterogeneous appearance due to the presence of hepatocytes with a clearer cytoplasm in centrilobular zones.

Elementary lesions were observed, either in control animals or in treated animals, their distribution, frequency or significance does not suggest a treatment effect:

- Inflammatory foci, usually discrete, in mononuclear cell situated more often in the venous sinusoids.

– Generally discrete ectasia of venous sinusoids without significant blood repletion without atrophy and necrosis of hepatocytes, focused in certain areas this ectasia is likely due to the agonal hemodynamic phenomenon.

– Venous sinusoids congestion, characterized by sinusoids ectasia with significant blood repletion, without noticeable alteration of hepatocytes.

– Ectasia of lymphatic capillaries in some portal areas.

– Edema of the connective tissue in portal areas.

– Hemorrhagic necrosis, hemorrhage causing atrophy and necrosis of hepatocytes at this site.

– Very discrete fibrosis localized in the sub-capsular region.

In treated males the vacuolization of hepatocytes was observed with increased significance and frequency compared to control males. This vacuolization has two different aspects:

– Systematic multifocal hyper- and microvacuolization in all periportal regions presented in the examined specimen. This hyper- and microvacuolization results in the presence of multiple small intra-hepatocyte vacuoles; the cells are slightly deformed and their nucleus remains central.
This was observed with significantly higher frequency in males treated at the high dose; however, it remains very discrete.

– Lipid vacuolization comprising the presence of some hepatocytes, scattered in the parenchyma, where the cytoplasm contains one or more large vacuoles, which occupy practically the whole cell. The cytoplasm is reduced to a thin peripheral rim containing a flattened nucleus.
This diffuse microvacuolization was observed compared to control males, with increased frequency and significance in males treated with three doses.

It should be noted that this vacuolization of hepatocytes (systematic multifocal microvacuolization and diffuse microvacuolization) is not accompanied by necrotic lesions of hepatocytes or inflammatory reaction. It remains very discrete in all cases. So it is reasonable to think that this is probably a reversible phenomenon. Intensification of this vacuolization was not observed in treated females.

2' Histochemical examinations

– Analyzing iron

Bearing in mind that the iron deposits are mainly located in Kupffer cells (hemolytic crisis) and hepatocytes (food overload phenomenon or hemolysis). This is why we systematically analyzed these cells.

In this study discrete ferric iron deposits were noted in the hepatocytes and/or Kupffer cells in some of the animals, mainly females. There was no increase in these deposits in treated animals compared to control animals for both sexes. No hemosiderosis reaction was observed.

– Analyzing lipids

Although the hepatic lobule conventionally divided into three zones, we have arbitrarily divided it into two zones: centrilobular zone extending approximately to half of the lobule and perilobular zone representing the rest of the lobular parenchyma. For each of these zones, hepatocytes lipid content is assessed in +, ++ or +++. When lipids do not appear to be located in one of these two zones, but distributed throughout the parenchyma, they are reported as diffuse and their significance is assessed according to previously defined criteria.

In this study fat deposits in the hepatocytes in perilobular zones were observed in many animals. There was no significant difference between control and treated females. On the other hand, an increase in the significance of these deposits was noted in treated males, mainly in those treated with the high dose. This lipid excess in hepatocytes supports the observations made on trichrome-stained histological specimens.

STOMACH

a) Preliminary comments concerning the observations

The gastric mucosa in mice and in rats comprises two regions (glandular and squamous), the appearance of which may be due to the fixing conditions and the cutting angle. Generally the glandular mucosa cannot be preserved in a satisfactory manner during the sampling procedure of a toxicity study. On the one hand, the epithelium may be subjected to small mechanical traumas and partial lysis. On the other hand, the zymogen granules of gastric chief cells (gastric zymogenic cells) are largely dissolved by common fixatives, which often gives a spongy appearance to the cytoplasm of these cells. Squamous mucosa may, at certain angles of tangential cuts, take on an acanthotic and hyperkeratotic appearance.

Inflammatory cell infiltrates are frequently observed in the glandular submucosa, mainly adjacent to the limiting ridge. We mention them only when they are significant.

The type of affected mucosa (squamous or glandular) in the examined specimen is specified for each animal.

b) Observations

It appears possible to come to a conclusion about the absence of erosion lesions or ulceration in the gastric mucosa for all animals, including the absence of hyperplastic and hyperkeratotic reaction in the squamous mucosa. No congestion of submucosa or abnormal appearance of the muscle layers were noted.

Localized edema have been observed in some treated or control animals in the glandular submucosa. This does not exclude an artifact caused by aqueous impregnation at this level at the time of fixing.

The observed glandular ectasia is very discrete and does not have any toxicological significance.

Furthermore, in one male treated with the low dose, the presence of some vesicles localized in the squamous epithelium adjacent to the limiting ridge was observed.

SMALL INTESTINE – COLON

The mucosa does not have any damage that can be attributed to the treatment. Its possible localized alteration is due to the difficulty in fixing. There is no congestion or inflammatory phenomenon. The presence of lymphoid clusters corresponds to the normal localization.

In one control male, glandular ectasia with mucus retention in the colon was observed.

Luminal distension noted in one control female and one female treated with the medium dose does not have any toxicological significance.

SALIVARY GLANDS

Examined specimens have the same appearance in all control and treated animals. The serous cells have a round nucleus in the parabasal position and their cytoplasm is relatively dense. The mucous cells have a nucleus localized in the basal part of the cell and their cytoplasm is very clear and foamy. The lumen of the excretory tubes appears to be free from seromucous content.

Small foci of acinar atrophy were reported in some control and treated animals without any particular predominance among the latter.

Unifocal periductal lymphoid infiltration, accompanied by degeneration, was noted in one female treated with the high dose. This lesion doesn't have any toxicological significance.

PANCREAS

Because the trichrome method does not distinguish between different types of cells in the islets of Langerhans, we used staining with fuchsin paraldehyde to highlight the B-cells.

1° **Exocrine pancreas**

There is no variation in systematic appearance between treated animals and control animals.

Pancreatic acinar cells do not appear homogeneous across the entire examined parenchyma. In the basal part of the cell, the basophilic cytoplasm occupies a more or less significant part of the cell.

A unifocal atrophy was observed in one female treated with the low dose.

2' Endocrine pancreas

The B-cells are the most significant population in the islets of Langerhans. These cells are distributed in cords encircling the blood sinuses and occupy the central part of the islet. Cytoplasmic granulation stained with fuchsin paraldehyde appears in the cytoplasm, particularly concentrated in the portion of the cell adjacent to the capillaries. The assessment of the B-cells frequency, visualized by their granulation, is based on all islets present on the section of the examined parenchyma. This frequency varies according to the cut level of the islets. It is known that the insulin release is accompanied by B-cell degranulation.

Where the examined specimen includes only the exocrine parenchyma and therefore no examination of the islets can be performed, it is considered to be absent.

In this study no significant depletion of B-cell granulation was observed in the treated animals. So there seems to be no morphological change that can be interpreted as a functional alteration of the B-cells in the islets of Langerhans.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: DIGESTIVE

LIVER	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
HOMOGENEOUS PARENCHYMA	10	10	10	10	10	10	9	10
HETEROGENEOUS PARENCHYMA	0	0	0	0	0	0	1	0
CLEAR CENTRILOBULAR PARENCHYMA	0	0	0	0	0	0	1	0
HETEROGENEOUS HEPATOCYTES CYTOPLASM	10	10	10	10	10	10	10	10
MONONUCLEAR CELL FOCI	8	6	8	7	10	7	7	7
SINUSOIDS ECTASIA	1	0	3	0	2	2	2	0
ECTASIA OF LYMPHATIC CAPILLARIES	1	0	0	0	0	0	0	0
PORTAL AREA EDEMA	0	0	1	0	0	0	0	0
SINUSOIDS CONGESTION	0	0	1	0	0	0	0	0
LOCALIZED HEMORRHAGIC NECROSIS	0	0	0	0	0	0	1	0
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	3	2	8	2	3	1	2	2
LIPID VACUOLIZATION	1	3	4	1	5	1	6	2
LOCALIZED FIBROSIS	0	0	0	0	0	0	1	0

LIVER IRON	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
NONE	9	0	10	2	9	1	10	0
HEPATOCYTES: (+)	1	9	0	7	0	9	0	10
KUPFFER CELLS: (+)	0	8	0	5	1	3	0	6

LIVER - LIPIDS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
HEPATOCYTES NONE	5	0	1	0	3	2	1	1
PERILOBULAR ZONE: (+)	2	4	1	2	2	4	4	4
PERILOBULAR ZONE: (++)	2	4	5	6	3	0	4	4
PERILOBULAR ZONE: (+++)	1	2	3	2	2	4	1	1

STOMACH	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	9	10	10	10	10
ABSENT	0	0	0	1	0	0	0	0
NUMBER OF EXAMINED SPECIMENS	10	10	10	9	10	10	10	10
SQUAMOUS MUCOSA PRESENT	7	10	8	8	10	8	10	7
GLANDULAR MUCOSA PRESENT	10	10	10	9	10	10	10	10
INTRAEPIDERMAL VESICLES	0	0	0	0	0	0	1	0
GLANDULAR ECTASIA	1	2	2	4	2	4	1	3
GLANDULAR SUBMUCOSA EDEMA	0	4	1	0	1	0	0	2

SUMMARY OF OBSERVATIONS BY GROUP

SMALL INTESTINE	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
MUCOSAL LYMPHOID FOCI	0	0	0	0	0	1	0	0
SUBMUCOSAL LYMPHOID FOCI	0	1	0	1	1	0	0	0
LUMINAL DISTENSION	0	1	0	0	0	0	0	0

COLON	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	9	10	6	10	8	10	7	10
ABSENT	1	0	4	0	2	0	3	0
NUMBER OF EXAMINED SPECIMENS	9	10	6	10	8	10	7	10
MUCOSAL LYMPHOID INFILTRATION	0	0	0	1	0	0	0	0
SUBMUCOSAL LYMPHOID FOCI	3	0	0	1	2	2	0	1
LUMINAL DISTENSION	0	0	0	0	0	1	0	0
GLAND. ECTASIA & MUCUS RETENTION	1	0	0	0	0	0	0	0

SALIVARY GLANDS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	9	10	9	10	9	10
ABSENT	0	0	1	0	1	0	1	0
NUMBER OF EXAMINED GLANDS	10	10	9	10	9	10	9	10
MUCOUS ZONE PRESENT	10	10	9	10	9	10	9	10
SEROUS ZONE PRESENT	10	10	9	10	9	10	9	10
UNIFOCAL LYMPHOID INFILTRATION	0	0	0	1	0	0	0	0
ACINAR ATROPHY	3	2	1	2	0	2	1	1

PANCREAS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	8	10	10	10	9
ABSENT	0	0	0	2	0	0	0	1
NUMBER OF EXAMINED SPECIMENS	10	10	10	8	10	10	10	9
EX. UNIFOCAL ATROPHY	0	0	0	0	0	0	0	1
B-CELLS IL. GRANULATION: (+++)	10	10	10	8	10	10	10	7
B-CELLS IL. GRANULATION: (++)	0	0	0	0	0	0	0	1

URINARY SYSTEM

No lesions that could be interpreted as an intolerance to the treatment in the various examined organs were noted.

KIDNEYS

1* Histological examinations

The Malpighian glomeruli do not have any congestion or inflammation of flocculus. The brush border of the proximal convoluted tubules is more or less well preserved. The cytoplasm of nephrons has no sign of pre-mortem necrosis. The lumen of the nephrons appear more or less open to the normal state, tubular ectasia is often difficult to assess. Also, we took into account that when the lumens appear quite widened, the tubular epithelium may be of normal appearance or otherwise have slightly flattened cells.

Some localized mineralization was observed only in females, at the corticomedullary junction. These mineralized deposits appear in tubular lumens. They are often seen in the rat, usually in the female. Their presence may be due to excess calcium or phosphorus in the diet, and most authors attach little pathologic importance to these deposits (BENIRSCHKE et al.).

In all males, both in control and treated animals, the cytoplasm in the proximal convoluted tubules contains numerous fuchsinophile vesicles. These intracytoplasmic inclusions are often observed in the rat. Their presence cannot be related to treatment.

Elementary lesions were observed, either in control animals or in treated animals, their distribution, frequency or significance does not suggest a treatment effect:

- Discrete localized glomerulonephrosis characterized by the ectasia of the glomerular chamber and atrophy of flocculus.

- Tubulonephrosis consisting of the presence of some ectatic tubes with epithelium having signs of significant microvacuolization and with a generally anhistic and slightly basophilic content.

- Tubular cysts situated in the medullar zone.

- Localized and discrete epithelial hypertrophy, characterized by the presence of some tubules lined with a monolayer consisting of large balloons cells with hypertrophied nuclei.

- Small foci of basophilic tubules situated in the cortical region.

- Tubular cylinders in some cortical and medullary ducts.

- Infiltration of the interstitial tissue by the lymphoplasmacytic elements grouped in small foci or by collagen (fibrosis).

Very discrete uni- or multifocal lesions of mixed nephritis have also been observed, in control and in treated animals.

2* Histochemical examinations

- Analyzing iron

Ferric iron deposits appear in the epithelium of the proximal convoluted tubules upon the reabsorption of the erythrocytes hemoglobin, which was filtered in glomeruli.

In this study no ferric iron deposits were noted. No hemosiderosis reaction was observed.

- Analyzing lipids

In the normal state kidneys contain the small lipid droplets in the basal part of epithelial cells in the proximal tubules. If these droplets remain localized at the base of the cells, they are considered "normal". If they occupy a large part of the cells, they are seen as representing an "excess". In each case the assessment of the localization significance is done by +, ++, +++.

In this study no steatosis was observed, the amounts of viewable lipids in the proximal tubules vary within normal limits.

BLADDER

The paramalpighien bladder lining is regular and continuous.

The very few lesions observed (epithelial vacuolization, infiltration of mononuclear cells in the lamina propria) are discrete and do not have a toxicological significance.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: URINARY

KIDNEYS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED KIDNEYS	10	10	10	10	10	10	10	10
GLOMERULONEPHROSIS	0	0	0	0	0	1	1	0
TUBULONEPHROSIS	2	0	3	0	0	0	2	0
MEDULLARY TUBULAR CYST	1	0	0	1	0	0	0	0
EPITHELIAL HYPERTROPHY	0	0	0	0	0	0	0	1
FOCI OF BASOPHILIC TUBULES	0	3	2	0	2	2	1	0
SINGLE TUBULAR CYLINDER	2	1	1	0	1	2	0	1
MULTIPLE TUBULAR CYLINDERS	2	1	4	1	0	0	4	0
INTERSTITIAL FIBROSIS	0	0	0	1	0	0	0	0
CORTICAL LYMPHOID FOCI	0	0	0	0	0	0	0	1
MINERALIZATION	0	1	0	2	0	0	0	2
UNIFOCAL MIXED NEPHRITIS	1	0	1	0	2	0	1	0
MULTIFOCAL MIXED NEPHRITIS	0	0	0	0	0	1	0	0

KIDNEY - IRON	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
IRON - NONE	10	10	10	10	10	10	10	10

KIDNEY LIPIDS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	1	10	10	10	10	10
NUMBER OF EXAMINED KIDNEYS	10	10	10	10	10	10	10	10
NONE	6	0	9	0	6	1	6	0
PROXIMAL TUBULES: NORMAL (+)	4	5	1	6	4	4	4	9
PROXIMAL TUBULES: NORMAL (++)	0	5	0	4	0	5	0	1

BLADDER	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	9
ABSENT	0	0	0	0	0	0	0	1
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	9
EPITHELIAL VACUOLIZATION	0	0	0	0	0	0	1	0
INFILTR. OF MONONUCL. CELLS IN THE LAMINA PROPRIA	0	0	1	0	0	1	0	0

HEMATOLYMPHOID SYSTEM

No lesions that could be interpreted as an intolerance to the treatment in the various examined organs were noted.

THYMUS

a) Preliminary comments concerning the observations

The cystic cavities, more or less big lumens lined with one or two layers of squamous epithelium, are described as structures very often presented in the thymic parenchyma, which form from degenerated Hassall's corpuscles (GREAVES and FACCINI). These cavities are commonly observed in the rat's thymus. The cystic cavities with monolayer of ciliated epithelium are also commonly observed and we don't think that they have any pathological significance.

b) Observations

The stratification was respected. No atrophy or involution aspects were noted. The Hassall's corpuscles appear normal in number and volume.

SPLEEN

1* Histological examinations

a) Preliminary comments concerning the observations

In the mouse and rat, the spleen in the normal state contains many megakaryocytes and erythrocytes at all stages of maturation, particularly erythroblasts (BENIRSCHKE et al.). The megakaryocytes are distributed irregularly in the venous sinuses, sometimes isolated, sometimes grouped by two or three in the same microscopic field. Assessing their numbers is therefore very difficult and hyperplasia of these cells should be clearly identified to be sure. Furthermore, according to the section planes, the splenic parenchyma can include more or less significant areas of white pulp. This means being very careful in assessing the significance of the Malpighian corpuscles development. Bearing in mind that in the rat, the spleen is a vicarious hematopoietic organ while in the adult mouse it functions similar to bone marrow, as a normal hematopoiesis site.

b) Observations

The white pulp appears more or less developed without the hyperplasia or significant hypoplasia of lymphoid follicles being observed in various groups. Observed germinal centers reflect the antigenic stimulation of the periarteriolar lymphoid sheaths; they don't look any different in treated and control animals. The red pulp is more or less rich in red blood cells, without the significant congestion that could be related to treatment, the blood repletion of the spleen depends on the agonistic response of the animal at the time of sacrifice. In addition, we did not observe any signs of megakaryocytic hyperplasia or variation in the significance of erythroblast foci in all the treated animals compared to the control animals.

2* Histochemical examinations

– Analyzing iron

In rats the ferric iron deposits are often visible in the red pulp macrophages. These deposits are generally greater in females than in males.

In this study the spleens of control animals have more or less significant deposits of ferric iron. These deposits usually appear larger in females than in males.

No meaningful difference in the significance of ferric iron deposits was noted in treated animals and control animals for both sexes. No evidence of hemosiderosis was observed.

LYMPH NODES

1* Histological examinations

a) Specific comments concerning the observations

The appearance of the lymph node can vary depending on the section plane. Three areas can be seen in the ideal section:

- The cortex with lymphoid follicles, primary location of B lymphocytes.
- The paracortex, or deep cortex, primary location of T lymphocytes.
- The medulla with lymphoid cell cords originating from the paracortex and blood sinusoids.

The trichrome method used in this study does not distinguish between various types of lymphatic parenchyma cells. Also, we paid particular attention in observing the general appearance of the primary and secondary follicles of the cortex and paracortex, the structures where the condition varies depending on the immunological status of the node, and also in looking for signs of atrophy or hyperplasia:

- in the primary follicles, follicles with no clear center;
- in the secondary follicles, follicles with a clear center containing B lymphocytes, where the presence is observed in the humoral immune responses to the antigen;
- in the paracortex, where the hypertrophy results in a cellular immune response to the antigen.

b) Observations

The cell population that can be assessed with the type of stain used does not appear to undergo significant changes in the treated animals. The significance of the reticular cells,

stem cells, lymphocytes and their precursors does not appear to change in the treated groups. In particular, no histiocytic reaction was noted. No extramedullary hematopoiesis foci were observed.

2* Histochemical examinations

- Analyzing iron

The presence of ferric iron can indicate a hemorrhagic lesion drainage or iron-rich tissue, especially in the liver iron overload (nodes of the liver lobe).

No ferric iron deposits were noted in all the animals. No evidence of hemosiderosis was observed.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: HEMATOLYMPHOID

THYMUS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	9	10
ABSENT	0	0	0	0	0	0	1	0
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	9	10
CILIATED CYST	1	0	1	0	3	0	1	1
EPIDERMOID CYST	3	8	3	8	7	9	2	7

SPLEEN	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10

SPLEEN IRON	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
NONE	0	0	0	1	0	0	0	0
RED PULP: (+)	6	0	2	0	2	0	2	1
RED PULP: (++)	2	5	5	2	6	5	5	4
RED PULP: (+++)	2	4	3	4	2	3	3	3
RED PULP: (++++)	0	1	0	3	0	2	0	2

NODES	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	9	10	10	10	10
ABSENT	0	0	0	1	0	0	0	0
NUMBER OF EXAMINED NODES	10	10	10	9	10	10	10	10

NODE - IRON	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	9	10	10	10	10
ABSENT	0	0	0	1	0	0	0	0
NUMBER OF EXAMINED SPECIMENS	10	10	10	9	10	10	10	10
NONE	10	10	10	9	10	10	10	10

ENDOCRINE SYSTEM

No lesions related to the treatment or any morphological change that could be interpreted as a functional disturbance caused by the treatment were observed in the various examined organs.

THYROIDS

a) Preliminary comments concerning the observations

The thyroid parenchyma does not have a homogeneous appearance in the normal state. Follicles have morphological aspects according to their location and different functional states. In general, the follicles with larger diameters situated on the periphery of the organ. In the rabbit, rat and guinea pig, the peripheral zone seems to be a resting area, while the central zone appears more functional and particularly reactive to the experimental loads (DUBREUIL and CANIVENC). We classified (according to PAGES and MARTY-DOUBLE) the follicles by their type based on the height of the epithelium and the appearance of the follicular cavity:

- Secretory: cuboidal (or prismatic) epithelium with normal cavity.
- Excretory: prismatic epithelium with collapsed cavity.
- Resting: mesothelium with ectatic cavity.

Due to the polymorphism of the follicles in the same section plane our assessment is based on the general appearance of the follicles in the central zone. Furthermore, the tendency towards a hypersecretion or a tendency towards resting presented by treated animals is always compared to control animals. The quality of the fixing (in particular its speed at necropsy) may be accountable for the integrity of the follicular cells.

The epidermoid cystic cavities can be observed. These cavities, usually from the ultimobranchial body are lined with squamous keratinized epithelium. They are frequently observed in mice and rats (TURUSOV). These structures are often situated in the center of the gland. They show no evidence of progressive expansion causing the compression of the surrounding tissue, but they develop through increase in the number of keratin lamellae and significance of the debris in the lumen. Some authors suggest these cysts are epidermoid cystadenomas with squamous epithelium (TURUSOV).

The parathyroids are not examined systematically. They are examined only when presented in the thyroids section plane. Therefore the individual data includes information on the presence of parathyroids in thyroid specimens and any of their possible anomalies. The adipose tissue islets, (very large optically empty cells) can be found scattered in the parenchyma. These cells develop as the animal ages.

b) Observations

In control and treated animals the thyroids have the same aspect of follicles with secretory activity and cubo-columnar epithelium more or less higher depending on the follicles.

The follicular cyst was observed in one female treated with the medium dose. In addition, a lymphoid focus was noted in some control or treated animals. These various observations do not have any toxicological significance.

The parathyroids presented in the thyroids section plane have a normal appearance.

ADRENAL GLANDS

a) Preliminary comments concerning the observations

Small foci of large cells with slightly denser cytoplasm than the surrounding cells can be seen in some animals in the zona reticularis. A few cells of this type can also be observed in the medulla. These foci do not exert any pressure on the surrounding tissue. These cells differ from other cells in size and tinctorial affinity, being usually small in size and located in the zona reticularis. These commonly observed foci are not reported in our observations.

b) Observations

The adrenal glands appear the same in animals from different groups. In the cortex the zona glomerulosa is not distinct from the zona fasciculata, which is normal in the rat under the fixing conditions used in this study. The spongiocytes in the zona fasciculata are normally vacuolized (loaded with identical fat) and cell cords in the zona reticularis are separated by capillaries that are often saturated with erythrocytes. The medulla presented in the section plane has a normal appearance.

The cortical microcyst lined with flattened epithelium was noted in one control male.

The ectasia of the sinusoids observed in zona reticularis in female treated with the high dose is likely related to an agonal hemodynamic phenomenon.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: ENDOCRINE

THYROIDS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	9	10	10	10	10
ABSENT	0	0	0	1	0	0	0	0
NUMBER OF EXAMINED THYROIDS	10	10	10	9	10	10	10	10
CUBO-COLUMNAR EPITHELIUM	10	10	10	9	10	10	10	10
NORMAL FOLLICLES	10	10	10	9	10	10	10	10
SECRETORY ASPECT	10	10	10	9	10	10	10	10
CHROMOPHILE COLLOID	10	10	10	9	10	10	10	10
EPIDERMOID CYST	3	2	3	2	0	2	2	5
FOLLICULAR CYST	0	0	0	0	0	1	0	0
UNIFOCAL LYMPHOID FOCUS	0	1	1	1	2	1	0	0
PARATHYROID PRESENT	8	4	9	9	4	6	7	5
SPECIMEN FOR MUSCLE	0	0	1	0	0	0	0	0
SPECIMEN FOR FAT	0	0	0	0	0	0	1	0

ADRENAL GLANDS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	9	10	10	10	10
ABSENT	0	0	0	1	0	0	0	0
NUMBER OF EXAMINED ADRENAL GLANDS	10	10	10	9	10	10	10	10
MEDULLA PRESENT	10	8	10	9	10	9	10	9
CORTICAL CYST	1	0	0	0	0	0	0	0
CORTICAL SINUSOIDS ECTASIA	0	0	0	1	0	0	0	0

REPRODUCTIVE SYSTEM

No lesions related to the treatment or any morphological change that could interpreted as a functional disturbance caused by the treatment were seen in the various examined organs. The increase of lymphoid interstitial infiltration in the prostate observed in animals treated with high and medium doses was not regarded as the reaction to treatment.

TESTICLES

a) Preliminary comments concerning the observations

In mice and in rats it is possible to observe in the germinal epithelium of some seminiferous tubules the presence of one or, rarely, several large optically empty vacuoles. It was described that this vacuolization affects the Sertoli cells (BENIRSCHKE et al.). When this phenomenon is very discrete and does not affect the number of the germ cells in tubular sections in the whole specimen, the presence of these vacuoles was not taken into account in our observations. The lumen of some tubules can also contain some degenerated cells probably from non-viable sperm; as to the presence of vacuoles, if the number of these cells is minimal and the tubule appears to have a normal spermatogenesis, these cells are not taken into account.

b) Observations

It appears that two stages of spermatogenesis were normally accomplished: the spermatocytogenesis (spermatogonia, spermatocytes and spermatids present in all tubules) and the spermiogenesis (sperm in varying amounts depending on the tubular sections). The interstitial tissue has a normal appearance.

The very few sections of localized tubular degeneration noted in some animals are commonly observed in rats and cannot be due to treatment given their frequency in different groups.

SEMINAL VESICLES

No inflammation infiltration or sclerotic reaction. No difference in appearance of the mucous epithelial cells in treated and control animals. The seminal vesicles contain large tubular cavities with monolayer of columnar epithelium, filled with the secretion product. At the edge of the seminal vesicles the epithelium is arranged into numerous folds giving a serrated appearance to the lumen.

PROSTATE

The prostate is composed of glandular cavities lined with a monolayer of columnar epithelium. At the edge of the gland the lumens are usually quite small and serrated because of the many folds in the mucosa. In the central region the glandular cavities are larger and there are no mucosal folds. The interstitial connective stroma is poorly developed and we observed only a few connective fibers between the glandular cavities, which are attached to each other,

or separated by interstitial edema resulting from fixation. The treatment did not cause any change in the glandular morphology.

The zone of usually ectatic glands with atrophic epithelium was observed both in control and treated animals. This is the common observation.

The unifocal or multifocal, significant in some cases, lymphoid interstitial infiltration was observed in many control or treated animals, but with a higher frequency in animals treated with high and medium doses. However, in the absence of other findings in the various examinations we do not think that this observation could be interpreted as a sign of intolerance to the treatment.

The presence of some polymorphonuclear cells in the lumen of some glands in animal treated with the low dose was also noted.

OVARIES

The presence of corpora lutea and Graafian follicles represents normal follicular maturation. The treatment therefore had no impact on the maturation of ovarian follicles.

The luteinizing cysts where the wall comprises several layers of luteal and granular cells were noted in the rare control or treated animals.

UTERINE HORNS

a) Preliminary comments concerning the observations

In order to detect any functional histologically detectable change in uterine structures we took into consideration the following criteria:

- The lumen classified as narrow, uneven thickness or dilated.
- Height of the endometrial epithelium.
- The aspect of the cytological activity (presence of mitotic figures) of epithelial cells.

Height of the endometrial epithelium was measured with an ocular micrometer having the degree of magnification x 63. For each uterine horn the epithelium was measured in two diametrically opposed points. Based on its height the epithelia were classified into four categories:

+ $\leq$ 10 marks on the micrometer scale ($\leq$ 20 µm)
++ 11 to 15 marks on the micrometer scale (22 to 30 µm)
+++ 16 to 20 marks on the micrometer scale (32 to 40 µm)
++++ $\geq$ 21 marks on the micrometer scale ($\geq$ 42 µm)

b) Observations

Some variations in configuration of the uterine lumen and the height of the epithelium were observed mainly between the control animals and animals treated with low and medium doses. However, in the absence of histological sections of vagina which would provide a more accurate diagnosis of the estrous cycle stage we cannot comment on these variations in the morphology.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: REPRODUCTIVE

TESTICLES	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	0	10	0	10	0	10	0
NUMBER OF EXAMINED TESTICLES	10	0	10	0	10	0	10	0
LOCALIZED DEGENERATION	0	0	1	0	1	0	2	0

SEMINAL VESICLES	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	0	10	0	10	0	10	0
NUMBER OF EXAMINED VESICLES	10	0	10	0	10	0	10	0

PROSTATE	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	0	10	0	10	0	10	0
AUTOLYSIS	0	0	1	0	0	0	0	0
NUMBER OF EXAMINED SPECIMENS	10	0	10	0	10	0	10	0
LOC. GLANDULAR EPITH. ATROPHY	4	0	5	0	4	0	3	0
UNIFOCAL LYMPHOID INFILTRATION	2	0	1	0	2	0	0	0
MULTIFOCAL LYMPHOID INFILTRATION	2	0	7	0	5	0	2	0
POLYMORPHONUCLEAR CELLS, GLANDS LUMEN	0	0	0	0	0	0	1	0

OVARIES	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	0	10	0	10	0	10	0	10
NUMBER OF EXAMINED OVARIES	0	10	0	10	0	10	0	10
PARTIAL OVARIAN SECTION	0	1	0	1	0	3	0	1
GRAAFIAN FOLLICLES	0	10	0	10	0	10	0	10
CORPORA LUTEA	0	10	0	10	0	10	0	10
LUTEINIZING CYST	0	1	0	2	0	1	0	0
LUTEINIZING MULTICYSTIC FORMATION	0	0	0	0	0	0	0	1

UTERINE HORNS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	0	10	0	10	0	10	0	10
NUMBER OF EXAMINED HORNS	0	10	0	10	0	10	0	10
NARROW LUMEN	0	5	0	6	0	8	0	7
LUMEN WITH UNEVEN THICKNESS	0	3	0	3	0	1	0	2
DILATED LUMEN	0	2	0	1	0	1	0	2
HEIGHT OF THE EPITHELIUM (+)	0	1	0	3	0	0	0	3
HEIGHT OF THE EPITHELIUM (++)	0	6	0	5	0	9	0	7
HEIGHT OF THE EPITHELIUM (+++)	0	3	0	3	0	1	0	1
PROLIFERATIVE EPITHELIUM	0	6	0	6	0	10	0	9
NON-PROLIFERATIVE EPITHELIUM	0	5	0	6	0	1	0	1

CARDIOVASCULAR SYSTEM

HEART

No lesions that could be interpreted as an intolerance to the treatment were noted.

Small foci of inflammatory mononuclear cells or fibrosis, situated mostly in the ventricular myocardium in the subepicardial region and sometimes in the papillary muscle, were noted in many animals, both in control and treated animals, particularly in males. This is the common observation and due to their distribution in the different groups they don't have any toxicological significance.

RESPIRATORY SYSTEM

LUNGS

No lesions that could be interpreted as an intolerance to the treatment were noted.

All observed lesions, without any particular dominance in the treated animals, are common observations in the rat and cannot be interpreted as provoked by the treatment (focus of alveolar macrophages, perivascular edema, juxtabronchial or juxtavascular lymphoid clusters, perivascular inflammatory sheaths, hemorrhagic exudative pneumonia and septal hyperplasia).

The blood presence in alveoli (alveolar hemorrhage) without any inflammatory sign is related to bronchial aspiration of blood at the time of the animal's sacrifice.

No elastosis or proliferation of elastic fibers in alveolar walls was revealed by fuchsin paraldehyde staining.

SUMMARY OF OBSERVATIONS BY GROUP

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

CARDIOVASCULAR

HEART	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	10	10	10	10	10	10	10
NUMBER OF EXAMINED SPECIMENS	10	10	10	10	10	10	10	10
MONONUCLEAR CELLS INFILTR. IN MYOCARDIUM	4	0	2	2	4	0	2	0
FIBROUS INFILTRATION IN MYOCARDIUM	3	1	0	0	0	0	0	0

SUBSTANCE: HYPOTHIOCYANATE SPECIES: RAT

SYSTEM: RESPIRATORY

LUNGS	1 M	2 F	3 M	4 F	5 M	6 F	7 M	8 F
NUMBER OF SPECIMENS	10	9	10	10	10	10	10	10
ABSENT	0	1	0	0	0	0	0	0
NUMBER OF EXAMINED SPECIMENS	10	9	10	10	10	10	10	10
ALVEOLAR HEMORRHAGE	0	0	1	0	0	0	1	0
FOCUS OF ALVEOLAR MACROPHAGES	0	1	0	4	1	0	0	1
PERIVASCULAR EDEMA	0	1	0	0	0	0	0	0
JUXTABRONCHIAL LYMPHOID CLUSTERS	2	2	2	4	7	3	4	4
JUXTAVASCULAR LYMPHOID CLUSTERS	0	0	0	0	0	0	0	2
PERIVASCULAR INFL. SHEATHS	1	1	0	5	0	0	1	1
ELASTIC FIBERS: NORMAL ASPECT	10	9	10	10	10	10	10	10
HEMORRHAGIC EXUDATIVE PNEUMONIA	0	0	0	0	0	0	1	0
SEPTAL HYPERPLASIA	0	0	1	0	0	0	0	0

III - CONCLUSION

Histopathological examinations performed in the sacrificed rats after 12-week oral administration of three concentrations of the HYPOTHIOCYANATE ($SCNO^-$) Generating Mixture have enabled us to make the following findings.

1* **The histological examinations** did not reveal any lesions or any functional change attributable to treatment in following organs:

. Stomach, small intestine, colon, salivary glands and pancreas.
. Kidneys, bladder.
. Thymus, spleen, lymph nodes.
. Thyroids, adrenal glands.
. Testicles, seminal vesicles, prostate, ovaries, uterine horns.
. Heart, lungs.

Regarding the liver, the increased vacuolization of the hepatocytes cytoplasm was noted for all three groups of treated males.

2* **The histochemical examinations led to the following results:**

a) Detecting the presence of ferric iron with Perls' Prussian blue in the liver, kidneys and spleen did not show any increase that could be interpreted as a localized hemosiderosis.

b) Staining with fuchsin paraldehyde noted the cytoplasmic granulation of the B-cells in the islets of Langerhans in pancrea but showed no difference in its significance that could reflect a degranulation phenomenon or disappearance of these cells in the treated animals. In lungs the staining of elastic fibers using the same method did not reveal any elastosis reaction.

c) Analysis of lipids in frozen sections with the Sudan Red showed no change in significance of these locations in kidneys between the control and treated animals. In particular, no renal steatosis was noted. For the liver, the increase in the significance of lipid deposits in hepatocytes in the perilobular zones was seen in males, in particular in males treated with high doses.

In conclusion no intolerance reaction to the treatment itself was seen in animals treated with the three concentrations of the Hypothiocyanate Generating Mixture. The lipid excess in hepatocytes (in all cases very discrete and observed only in males) without associated necrotic cell lesions appeared to us to be related to the lipid metabolism phenomenon. The increase of lymphoid infiltration in the prostate in some treated animals as well as some variation in the configuration of uterine mucosa observed in groups treated with medium and low doses does not appear to us to be considered as signs of a response to the treatment in the absence of additional data.

IV. - APPENDICES

Our histopathological diagnoses are made using a computer program. Apart from the various observable lesions, information for each organ includes the following:

Number of specimens	
Absent	AB
Dead animal	M
Autolysis	A
Number of examined	specimens

The mention "Number of specimens" followed by the words "Number of examined specimens" without further reference to a lesion means that the examined specimen is normal.

When the number of specimens is followed by the letter R (eg. 1R), this means:

- For the pancreas: too few islets of Langerhans to enable their analysis.
- For the lungs: no bronchi in the specimen.
- For the node: the cutting angle prevents observing the paracortex and medulla.

In addition, the following letters are used to categorize some lesions or structures:

Localized inflammatory lesions

A: focal, discrete (minimal)	D: multifocal, discrete
B: focal, moderate	E: multifocal, moderate
C: focal, significant	F: multifocal, significant

Other aspects or structures (vacuolization, congestion, etc.)

K: discrete L: moderate M: significant

Group 1: control – males

HISTOPATHOLOGICAL DIAGNOSES

SUBSTANCE: HYPOTHIOCYANATE GROUP 1: CONTROL

SPECIES: RAT SEX: M

SYSTEM: DIGESTIVE

LIVER	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	D	D	D	D	D	D	-	D	D	-	8
SINUSOIDS ECTASIA	-	-	-	-	K	-	-	-	-	-	1
ECTASIA OF LYMPHATIC CAPILLARIES	-	-	-	-	K	-	-	-	-	-	1
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	-	-	K	-	-	-	-	K	-	K	3
LIPID VACUOLIZATION	-	-	-	-	-	-	-	-	-	K	1

LIVER IRON	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	-	9
HEPATOCYTES: (+)	-	-	-	-	-	-	-	-	-	+	1

LIVER - LIPIDS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	+	+	-	+	-	+	+	-	-	-	5
PERILOBULAR ZONE: (+)	-	-	+	-	-	-	-	-	-	+	2
PERILOBULAR ZONE: (++)	-	-	-	-	-	+	-	-	+	-	2
PERILOBULAR ZONE: (+++)	-	-	-	-	-	-	-	+	-	-	1

STOMACH	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	-	+	-	+	-	+	+	+	7
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	-	-	-	K	-	+	-	-	-	-	1

SMALL INTESTINE	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10

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NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10

COLON	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	-	1	1	1	1	1	1	1	1	9
ABSENT	-	AB	-	-	-	-	-	-	-	-	1
NUMBER OF EXAMINED SPECIMENS	1	-	1	1	1	1	1	1	1	1	9
SUBMUCOSAL LYMPHOID FOCI	D	-	-	-	D	-	D	-	-	-	3
GLAND. ECTASIA & MUCUS RETENTION	-	-	-	-	K	-	-	-	-	-	1

SALIVARY GLANDS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	2	2	10
MUCOUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
ACINAR ATROPHY	-	-	-	2D	1A	-	1A	-	-	-	3

PANCREAS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: URINARY

KIDNEYS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
TUBULONEPHROSIS	-	-	-	-	-	-	-	-	1A	1A	2
MEDULLARY TUBULAR CYST	-	-	1	-	-	-	-	-	-	-	1
SINGLE TUBULAR CYLINDER	-	-	-	-	-	-	-	-	1	1	2
MULTIPLE TUBULAR CYLINDERS	-	-	-	-	-	1 K	2 K	-	-	-	2
UNIFOCAL MIXED NEPHRITIS	-	-	-	-	-	-	-	-	-	1 K	1

KIDNEY - IRON	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	-	-	-	+	+	+	-	+	+	6
PROXIMAL TUBULES: NORMAL (+)	-	+	+	+	-	-	-	+	-	-	4

BLADDER	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SYSTEM: HEMATOLYMPHOID

THYMUS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
CILIATED CYST	-	-	-	-	+	-	-	-	-	-	1
EPIDERMOID CYST	-	-	-	-	-	+	-	+	+	-	3

SPLEEN	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (+)	+	+	-	+	+	+	-	-	+	-	6
RED PULP: (++)	-	-	-	-	-	-	+	+	-	-	2
RED PULP: (+++)	-	-	+	-	-	-	-	-	-	+	2

NODES	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: ENDOCRINE

THYROIDS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	2	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	2	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	2	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	2	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	2	10
EPIDERMOID CYST	-	1	-	-	-	-	-	1	1	-	3
PARATHYROID PRESENT	1	-	1	1	1	1	2	2	2	-	8

ADRENAL GLANDS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	2	2	2	2	1	2	2	2	2	10
CORTICAL CYST	-	-	-	-	-	-	-	1	-	-	1

SYSTEM: REPRODUCTIVE

TESTICLES	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED TESTICLES	2	2	2	2	2	2	2	2	2	2	10

SEMINAL VESICLES	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	1	2	2	2	10
NUMBER OF EXAMINED VESICLES	2	2	2	2	2	2	1	2	2	2	10

PROSTATE	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
LOC. GLANDULAR EPITH. ATROPHY	-	-	-	-	+	+	+	-	+	-	4
UNIFOCAL LYMPHOID INFILTRATION	-	-	-	-	-	-	-	K	K	-	2
MULTIFOCAL LYMPHOID INFILTRATION	K	-	-	-	-	K	-	-	-	-	2

SYSTEM: CARDIOVASCULAR

TESTICLES	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MONONUCLEAR CELLS INFILTR. IN MYOCARDIUM	-	-	-	A	-	E	D	-	-	D	4
FIBROUS INFILTRATION IN MYOCARDIUM	A	-	-	-	-	D	-	-	A	-	3

SYSTEM: RESPIRATORY

LUNGS	15788	15789	15790	15791	15792	15793	15794	15795	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
JUXTABRONCHIAL LYMPHOID CLUSTERS	-	A	D	-	-	-	-	-	-	-	2
PERIVASCULAR INFL. SHEATHS	-	-	-	-	-	A	-	-	-	-	1
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10

Group 2: control – females

SUBSTANCE: HYPOTHIOCYANATE GROUP 2: CONTROL

SPECIES: RAT SEX: F

SYSTEM: DIGESTIVE

LIVER	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	-	D	D	-	-	D	D	D	D	-	6
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	-	-	-	-	-	-	K	-	K	-	2
LIPID VACUOLIZATION	-	K	K	-	-	K	-	-	-	-	3

LIVER IRON	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES: (+)	+	+	+	+	+	+	+	+	-	+	9
KUPFFER CELLS: (+)	+	-	-	+	+	+	+	+	+	+	8

LIVER - LIPIDS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
PERILOBULAR ZONE: (+)	-	-	-	-	+	-	+	+	-	+	4
PERILOBULAR ZONE: (++)	-	+	+	+	-	+	-	-	-	-	4
PERILOBULAR ZONE: (+++)	+	-	-	-	-	-	-	-	+	-	2

STOMACH	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	-	K	-	-	-	-	K	-	-	-	2
GLANDULAR SUBMUCOSA EDEMA	+	-	-	-	+	+	-	-	-	+	4

SMALL INTESTINE	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
SUBMUCOSAL LYMPHOID FOCI	-	-	-	-	-	-	-	-	-	10	1
LUMINAL DISTENSION	-	-	-	-	-	-	-	1	-	-	1

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COLON	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SALIVARY GLANDS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	2	2	10
MUCOUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
ACINAR ATROPHY	-	-	-	-	1A	1A	-	-	-	-	2

PANCREAS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: URINARY

KIDNEYS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
FOCI OF BASOPHILIC TUBULES	1A	-	-	-	1A	-	-	1A	-	-	3
SINGLE TUBULAR CYLINDER	-	-	-	1	-	-	-	-	-	-	1
MULTIPLE TUBULAR CYLINDERS	-	-	-	-	-	-	-	-	1 K	-	1
MINERALIZATION	-	-	-	-	-	-	-	-	1 K	-	1

KIDNEY - IRON	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
PROXIMAL TUBULES: NORMAL (+)	-	-	+	+	-	-	-	+	+	+	5
PROXIMAL TUBULES: NORMAL (++)	+	+	-	-	+	+	+	-	-	-	5

BLADDER	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

HISTOPATHOLOGICAL DIAGNOSES

SYSTEM: HEMATOLYMPHOID

THYMUS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
EPIDERMOID CYST	+	+	+	+	+	+	+	+	-	-	8

SPLEEN	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (++)	+	+	-	-	-	+	+	+	-	-	5
RED PULP: (+++)	-	-	+	-	+	-	-	-	+	+	4
RED PULP: (++++)	-	-	-	+	-	-	-	-	-	-	1

NODES	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

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HISTOPATHOLOGICAL DIAGNOSES

SYSTEM: ENDOCRINE

THYROIDS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	1	2	1	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	1	2	1	2	2	2	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	1	2	1	2	2	2	10
NORMAL FOLLICLES	2	2	2	2	1	2	1	2	2	2	10
SECRETORY ASPECT	2	2	2	2	1	2	1	2	2	2	10
CHROMOPHILE COLLOID	2	2	2	2	1	2	1	2	2	2	10
EPIDERMOID CYST	-	-	-	-	-	-	-	2	1	-	2
UNIFOCAL LYMPHOID FOCUS	1 K	-	-	-	-	-	-	-	-	-	2
PARATHYROID PRESENT	1	1	-	1	-	-	-	-	1	-	4

ADRENAL GLANDS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	-	2	2	-	2	2	2	2	2	8

SYSTEM: REPRODUCTIVE

OVARIES	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED OVARIES	2	2	2	2	2	2	2	1	2	2	10
PARTIAL OVARIAN SECTION	-	-	-	-	-	-	-	1	-	-	1
GRAAFIAN FOLLICLES	2	2	2	2	2	2	2	1	2	2	10
CORPORA LUTEA	2	2	2	2	2	2	2	1	2	2	10
LUTEINIZING CYST	-	-	-	1	-	-	-	-	-	-	1

UTERINE HORNS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED HORNS	2	2	2	2	2	2	2	2	2	2	10
NARROW LUMEN	-	-	2	-	2	2	2	-	-	2	5
LUMEN WITH UNEVEN THICKNESS	2	2	-	2	-	-	-	-	-	-	3
DILATED LUMEN	-	-	-	-	-	-	-	2	2	-	2
HEIGHT OF THE EPITHELIUM (+)	-	-	-	-	-	-	-	2	-	-	1
HEIGHT OF THE EPITHELIUM (++)	-	2	2	-	2	2	2	-	-	2	6
HEIGHT OF THE EPITHELIUM (+++)	2	-	-	2	-	-	-	-	2	-	3
PROLIFERATIVE EPITHELIUM	1	-	2	-	2	-	2	2	-	2	6
NON-PROLIFERATIVE EPITHELIUM	1	2	-	2	-	2	-	-	2	-	5

SYSTEM: CARDIOVASCULAR

HEART	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
FIBROUS INFILTRATION IN MYOCARDIUM	-	-	-	-	A	-	-	-	-	-	1

SYSTEM: RESPIRATORY

LUNGS	15798	15799	15800	15801	15802	15903	15804	15805	15806	15807	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	-	9
ABSENT	-	-	-	-	-	-	-	-	-	AB	1
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	-	9
FOCUS OF ALVEOLAR MACROPHAGES	-	-	-	-	-	-	-	-	A	-	1
PERIVASCULAR EDEMA	-	-	-	-	-	-	-	-	A	-	1
JUXTABRONCHIAL LYMPHOID CLUSTERS	-	-	-	B	-	A	-	-	-	-	2
PERIVASCULAR INFL. SHEATHS	-	-	-	-	-	-	-	-	A	-	1
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	-	9

Group 3: high dose of hypothiocyanate – males

HISTOPATHOLOGICAL DIAGNOSES

SUBSTANCE: HYPOTHIOCYANATE GROUP 3: HIGH DOSE

SPECIES: RAT SEX: M

SYSTEM: DIGESTIVE

LIVER	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	D	D	D	D	D	D	D	-	D	-	8
SINUSOIDS ECTASIA	-	-	-	-	K	-	-	K	K	-	3
PORTAL AREA EDEMA	-	-	-	-	-	-	-	-	K	-	1
SINUSOIDS CONGESTION	-	-	-	-	K	-	-	-	-	-	1
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	K	-	K	K	K	K	K	-	K	K	8
LIPID VACUOLIZATION	K	-	-	-	K	K	-	-	K	-	4

LIVER IRON	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

LIVER - LIPIDS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	-	+	-	-	-	-	-	-	-	-	1
PERILOBULAR ZONE: (+)	-	-	-	+	-	-	-	-	-	-	1
PERILOBULAR ZONE: (++)	-	-	-	-	+	+	+	+	+	-	5
PERILOBULAR ZONE: (+++)	+	-	+	-	-	-	-	-	-	+	3

STOMACH	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	-	+	+	+	+	+	+	-	+	+	8
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	-	-	-	-	-	-	K	-	-	K	2
GLANDULAR SUBMUCOSA EDEMA	-	-	-	-	-	+	-	-	-	-	1

SMALL INTESTINE	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	1	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	1	2	2	2	10

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HISTOPATHOLOGICAL DIAGNOSES

COLON	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	-	1	1	1	-	1	-	-	1	1	6
ABSENT	AB	-	-	-	AB	-	AB	AB	-	-	4
NUMBER OF EXAMINED SPECIMENS	-	1	1	1	-	1	-	-	1	1	6

SALIVARY GLANDS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	-	2	2	9
ABSENT	-	-	-	-	-	-	-	AB	-	-	1
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	-	2	2	9
MUCOUS ZONE PRESENT	2	2	2	2	2	2	2	-	2	2	9
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	-	2	2	9
ACINAR ATROPHY	-	1D	-	-	-	-	-	-	-	-	1

PANCREAS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
IL. B-CELLS GRANULATION:(++)	+	+	+	+	+	+	+	+	+	+	10

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HISTOPATHOLOGICAL DIAGNOSES

SYSTEM: URINARY

KIDNEYS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
TUBULONEPHROSIS	-	-	-	1A	-	-	2D	-	-	1A	3
FOCI OF BASOPHILIC TUBULES	-	1D	-	-	-	-	1A	-	-	-	2
SINGLE TUBULAR CYLINDER	-	-	-	-	-	-	-	-	1	-	1
MULTIPLE TUBULAR CYLINDERS	-	-	-	1 K	1 K	-	2 K	-	-	1 K	4
UNIFOCAL MIXED NEPHRITIS	-	-	-	-	-	-	-	-	-	1 K	1

KIDNEY - IRON	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	-	+	9
PROXIMAL TUBULES: NORMAL (+)	-	-	-	-	-	-	-	-	+	-	1

BLADDER	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
INFILTR. OF MONONUCL. CELLS IN THE LAMINA PROPRIA	-	-	-	-	-	-	-	A	-	-	1

HISTOPATHOLOGICAL DIAGNOSES

SYSTEM: HEMATOLYMPHOID

THYMUS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
CILIATED CYST	-	+	-	-	-	-	-	-	-	-	1
EPIDERMOID CYST	+	-	-	-	-	-	+	-	-	+	3

SPLEEN	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (+)	-	-	-	+	-	-	-	-	+	-	2
RED PULP: (++)	-	+	-	-	-	+	+	+	-	+	5
RED PULP: (+++)	+	-	+	-	+	-	-	-	-	-	3

NODES	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

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HISTOPATHOLOGICAL DIAGNOSES

SYSTEM: ENDOCRINE

THYROIDS	15808	15809	15810	15811	15812	15813	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	1	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	1	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	1	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	1	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	1	10
EPIDERMOID CYST	1	-	-	2	-	-	-	-	1	-	3
UNIFOCAL LYMPHOID FOCUS	-	-	1 K	-	-	-	-	-	-	-	1
PARATHYROID PRESENT	2	1	1	2	1	1	1	2	1	-	9
SPECIMEN FOR MUSCLE	-	-	-	-	-	-	-	-	-	1	1

ADRENAL GLANDS	15808	15809	15810	15811	15812	15813	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	1	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	1	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	1	2	2	2	2	2	2	2	2	10

SYSTEM: REPRODUCTIVE

THYROIDS	15808	15809	15810	15811	15812	15813	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED TESTICLES	2	2	2	2	2	2	2	2	2	2	10
LOCALIZED DEGENERATION	-	-	-	-	-	-	1	-	-	-	1

SEMINAL VESICLES	15808	15809	15810	15811	15812	15813	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	2	2	1	2	2	1	2	2	2	1	10
NUMBER OF EXAMINED VESICLES	2	2	1	2	2	1	2	2	2	1	10

PROSTATE	15808	15809	15810	15811	15812	15813	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
AUTOLYSIS	-	-	-	-	-	A	-	-	-	-	1
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
LOC. GLANDULAR EPITH. ATROPHY	+	-	+	+	+	-	-	-	+	-	5
UNIFOCAL LYMPHOID INFILTRATION	-	-	-	K	-	-	-	-	-	-	1
MULTIFOCAL LYMPHOID INFILTRATION	-	-	K	-	K	L	L	L	K	K	7

SYSTEM: CARDIOVASCULAR

HEART	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MONONUCLEAR CELLS INFILTR. IN MYOCARDIUM	-	-	-	-	D	-	-	-	E	-	2

SYSTEM: RESPIRATORY

LUNGS	15808	15809	15810	15811	15812	15913	15814	15815	15816	15817	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
ALVEOLAR HEMORRHAGE	-	-	-	-	-	-	-	+	-	-	1
JUXTABRONCHIAL LYMPHOID CLUSTERS	-	-	-	A	-	-	-	-	-	A	2
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10
SEPTAL HYPERPLASIA	-	-	-	-	-	K	-	-	-	-	1

Group 4: high dose of hypothiocyanate – females

HISTOPATHOLOGICAL DIAGNOSES

SUBSTANCE: HYPOTHIOCYANATE GROUP 4: HIGH DOSE

SPECIES: RAT SEX: F

SYSTEM: DIGESTIVE

LIVER	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	D	-	D	A	-	-	D	D	A	D	7
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	K	-	-	K	-	-	-	-	-	-	2
LIPID VACUOLIZATION	-	-	-	-	-	-	-	-	-	K	1

LIVER IRON	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	-	-	-	+	-	-	-	-	-	+	2
HEPATOCYTES: (+)	+	+	+	-	-	+	+	+	+	-	7
KUPFFER CELLS: (+)	+	+	+	-	+	+	-	-	-	-	5

LIVER - LIPIDS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
PERILOBULAR ZONE: (+)	-	-	-	-	+	-	-	+	-	-	2
PERILOBULAR ZONE: (++)	-	+	+	+	-	+	+	-	+	-	6
PERILOBULAR ZONE: (+++)	+	-	-	-	-	-	-	-	-	+	2

STOMACH	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	-	1	1	1	1	9
ABSENT	-	-	-	-	-	AB	-	-	-	-	1
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	-	1	1	1	1	9
SQUAMOUS MUCOSA PRESENT	+	+	+	+	-	-	+	+	+	+	8
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	-	+	+	+	+	9
GLANDULAR ECTASIA	K	-	K	-	-	-	K	K	-	-	4

SMALL INTESTINE	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	1	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	1	2	2	2	2	2	2	2	2	10
SUBMUCOSAL LYMPHOID FOCI	10	-	-	-	-	-	-	-	-	-	1

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COLON	15818	15819	15820	15821	15822	15923	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MUCOSAL LYMPHOID INFILTRATION	-	-	-	-	A	-	-	-	-	-	1
SUBMUCOSAL LYMPHOID FOCI	-	-	-	-	-	-	D	-	-	-	1

SALIVARY GLANDS	15818	15819	15820	15821	15822	15923	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	2	2	10
MUCOUS ZONE PRESENT	2	1	2	1	1	2	2	2	2	2	10
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
UNIFOCAL LYMPHOID INFILTRATION	-	-	-	-	-	-	-	-	-	1A	1
ACINAR ATROPHY	-	1A	-	-	1A	-	-	-	-	-	2

PANCREAS	15818	15819	15820	15821	15822	15923	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	-	1	1	-	1	8
ABSENT	-	-	-	-	-	AB	-	-	AB	-	2
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	-	1	1	-	1	8
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	-	+	+	-	+	8

SYSTEM: URINARY

KIDNEYS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
MEDULLARY TUBULAR CYST	-	-	-	1	-	-	-	-	-	-	1
MULTIPLE TUBULAR CYLINDERS	-	-	-	-	1 K	-	-	-	-	-	1
INTERSTITIAL FIBROSIS	-	-	-	-	-	-	-	-	-	1A	-
MINERALIZATION	-	-	-	-	-	-	-	-	1 K	1 K	2

KIDNEY - IRON	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
PROXIMAL TUBULES: NORMAL (+)	+	-	+	+	-	-	-	+	+	+	6
PROXIMAL TUBULES: NORMAL (++)	-	+	-	-	+	+	+	-	-	-	4

BLADDER	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SYSTEM: HEMATOLYMPHOID

THYMUS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
EPIDERMOID CYST	+	+	+	+	-	+	+	+	-	+	8

SPLEEN	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	-	-	-	-	-	-	-	-	+	-	1
RED PULP: (++)	-	-	+	-	-	-	+	-	-	-	2
RED PULP: (+++)	+	+	-	-	-	+	-	-	-	+	4
RED PULP: (++++)	-	-	-	+	+	-	-	+	-	-	3

NODES	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	-	1	1	1	9
ABSENT	-	-	-	-	-	-	AB	-	-	-	1
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	-	1	1	1	9

NODE - IRON	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	-	1	1	1	9
ABSENT	-	-	-	-	-	-	AB	-	-	-	1
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	-	1	1	1	9
NONE	+	+	+	+	+	+	-	+	+	+	9

SYSTEM: ENDOCRINE

THYMUS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	1	2	2	-	2	2	1	9
ABSENT	-	-	-	-	-	-	AB	-	-	-	1
NUMBER OF EXAMINED THYROIDS	2	2	2	1	2	2	-	2	2	1	9
CUBO-COLUMNAR EPITHELIUM	2	2	2	1	2	2	-	2	2	1	9
NORMAL FOLLICLES	2	2	2	1	2	2	-	2	2	1	9
SECRETORY ASPECT	2	2	2	1	2	2	-	2	2	1	9
CHROMOPHILE COLLOID	2	2	2	1	2	2	-	2	2	1	9
EPIDERMOID CYST	1	-	-	-	-	1	-	-	-	-	2
UNIFOCAL LYMPHOID FOCUS	-	-	1 K	-	-	-	-	-	-	-	1
PARATHYROID PRESENT	2	1	1	1	2	2	-	1	1	1	9

ADRENAL GLANDS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	-	2	2	2	9
ABSENT	-	-	-	-	-	-	AB	-	-	-	1
NUMBER OF EXAMINED ADRENAL	2	2	2	2	2	2	-	2	2	2	9
MEDULLA PRESENT	2	2	2	2	2	2	-	2	2	1	9
CORTICAL SINUSOIDS ECTASIA	-	-	-	-	1 K	-	-	-	-	-	1

SYSTEM: REPRODUCTIVE

OVARIES	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED OVARIES	1	2	2	2	2	2	2	2	2	2	10
PARTIAL OVARIAN SECTION	1	-	-	-	-	-	-	-	-	-	1
GRAAFIAN FOLLICLES	1	2	2	2	2	2	2	2	2	2	10
CORPORA LUTEA	1	2	2	2	2	2	2	2	2	2	10
LUTEINIZING CYST	1	-	-	-	-	-	-	-	-	1	2

UTERINE HORNS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED HORNS	2	2	2	2	2	2	2	2	2	2	10
NARROW LUMEN	-	-	-	2	2	2	-	2	2	2	6
LUMEN WITH UNEVEN THICKNESS	-	2	2	-	-	-	2	-	-	-	3
DILATED LUMEN	2	-	-	-	-	-	-	-	-	-	1
HEIGHT OF THE EPITHELIUM (+)	-	-	-	-	-	1	-	2	-	2	3
HEIGHT OF THE EPITHELIUM (++)	2	-	-	2	2	1	-	-	2	-	5
HEIGHT OF THE EPITHELIUM (+++)	-	2	2	-	-	-	2	-	-	-	3
PROLIFERATIVE EPITHELIUM	-	-	-	2	2	2	1	1	2	-	6
NON-PROLIFERATIVE EPITHELIUM	2	2	2	-	-	-	1	1	-	2	6

SYSTEM: CARDIOVASCULAR

HEART	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MONONUCLEAR CELLS INFILTR. IN MYOCARDIUM	-	-	-	A	-	-	-	-	-	B	2

SYSTEM: RESPIRATORY

LUNGS	15818	15819	15820	15821	15822	15823	15824	15825	15826	15827	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
FOCUS OF ALVEOLAR MACROPHAGES	K	-	-	A	-	-	-	-	A	D	4
JUXTABRONCHIAL LYMPHOID CLUSTERS	-	-	-	-	A	-	B	-	E	D	4
PERIVASCULAR INFL. SHEATHS	D	-	-	D	A	E	-	-	E	-	5
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10

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Group 5: medium dose of hypothiocyanate – males

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SUBSTANCE: HYPOTHIOCYANATE GROUP 5: MEDIUM DOSE

SPECIES: RAT SEX: M

SYSTEM: DIGESTIVE

LIVER	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	D	D	D	D	D	D	D	D	D	D	10
SINUSOIDS ECTASIA	-	-	-	-	-	-	-	-	K	k	2
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	K	-	-	-	-	K	K	-	-	-	3
LIPID VACUOLIZATION	-	-	-	K	-	-	L	K	L	K	5

LIVER IRON	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	-	+	+	+	+	+	+	+	9
KUPFFER CELLS: (+)	-	-	+	-	-	-	-	-	-	-	1

LIVER - LIPIDS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	+	-	+	-	-	-	-	+	-	-	3
PERILOBULAR ZONE: (+)	-	-	-	+	-	-	-	-	+	-	2
PERILOBULAR ZONE: (++)	-	+	-	-	+	-	+	-	-	-	3
PERILOBULAR ZONE: (+++)	-	-	-	-	-	+	-	-	-	+	2

STOMACH	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	-	-	K	-	-	-	-	-	K	-	2
GLANDULAR SUBMUCOSA EDEMA	-	-	-	+	-	-	-	-	-	-	1

SMALL INTESTINE	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
SUBMUCOSAL LYMPHOID FOCI	-	-	-	-	-	-	1D	-	-	-	1

COLON	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	-	1	1	1	1	-	1	1	1	8
ABSENT	-	AB	-	-	-	-	AB	-	-	-	2
NUMBER OF EXAMINED SPECIMENS	1	-	1	1	1	1	-	1	1	1	8
SUBMUCOSAL LYMPHOID FOCI	-	-	-	-	-	-	-	D	-	D	2

SALIVARY GLANDS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	-	2	2	2	2	2	2	2	2	9
ABSENT	-	AB	-	-	-	-	-	-	-	-	1
NUMBER OF EXAMINED GLANDS	2	-	2	2	2	2	2	2	2	2	9
MUCOUS ZONE PRESENT	2	-	2	2	2	2	2	2	2	2	9
SEROUS ZONE PRESENT	2	-	2	2	2	2	2	2	2	2	9

PANCREAS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: URINARY

KIDNEYS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
FOCI OF BASOPHILIC TUBULES	1A	-	-	1A	-	-	-	-	-	-	2
SINGLE TUBULAR CYLINDER	-	-	1	-	-	-	-	-	-	-	1
UNIFOCAL MIXED NEPHRITIS	-	-	-	-	1 K	-	-	1 K	-	-	2

KIDNEY - IRON	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	-	+	+	-	+	-	-	+	6
PROXIMAL TUBULES: NORMAL (+)	-	-	+	-	-	+	-	+	+	-	4

BLADDER	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

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SYSTEM: HEMATOLYMPHOID

THYMUS	15828	15829	15830	15831	15832	15833	15834	15835	15796	15797	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
CILIATED CYST	-	-	-	+	-	+	-	-	-	+	3
EPIDERMOID CYST	+	+	+	-	+	-	+	+	+	-	7

SPLEEN	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (+)	+	-	-	-	+	-	-	-	-	-	2
RED PULP: (++)	-	-	-	+	-	+	+	+	+	+	6
RED PULP: (+++)	-	+	+	-	-	-	-	-	-	-	2

NODES	15828	15829	15830	15831	15832	15833	15834	15835	15836	15737	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: ENDOCRINE

THYROIDS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	2	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	2	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	2	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	2	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	2	10
EPIDERMOID CYST	-	-	-	-	-	1 K	-	-	1 K	-	2
PARATHYROID PRESENT	1	-	1	-	-	2	-	-	2	-	4

ADRENAL GLANDS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	2	2	2	2	2	2	2	2	2	10

SYSTEM: REPRODUCTIVE

TESTICLES	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED TESTICLES	2	2	2	2	2	2	2	2	2	2	10
LOCALIZED DEGENERATION	-	-	-	1	-	-	-	-	-	-	1

SEMINAL VESICLES	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	2	2	1	2	2	1	2	2	2	1	10
NUMBER OF EXAMINED VESICLES	2	2	1	2	2	1	2	2	2	1	10

PROSTATE	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
LOC. GLANDULAR EPITH. ATROPHY	+	-	-	-	+	-	+	+	-	-	4
UNIFOCAL LYMPHOID INFILTRATION	-	-	-	-	K	-	K	-	-	-	2
MULTIFOCAL LYMPHOID INFILTRATION	K	L	L	K	-	-	-	-	L	-	5

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SYSTEM: CARDIOVASCULAR

HEART	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MONONUCLEAR CELLS INF. IN MYOCARDIUM	A	-	-	-	D	D	-	D	-	-	4

SYSTEM: RESPIRATORY

LUNGS	15828	15829	15830	15831	15832	15833	15834	15835	15836	15837	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
FOCUS OF ALVEOLAR MACROPHAGES	-	-	-	-	-	-	A	-	-	-	1
JUXTABRONCHIAL LYMPHOID CLUSTERS	A	D	D	-	D	A	D	D	-	-	7
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10

Group 6: medium dose of hypothiocyanate – females

SUBSTANCE: HYPOTHIOCYANATE GROUP 6: MEDIUM DOSE

SPECIES: RAT SEX: F

SYSTEM: DIGESTIVE

LIVER	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	-	D	-	D	D	D	D	-	D	D	7
SINUSOIDS ECTASIA	-	-	-	-	K	K	-	-	-	-	2
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	K	-	-	-	-	-	-	-	-	-	1
LIPID VACUOLIZATION	-	-	-	-	-	-	-	K	-	-	1

LIVER IRON	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	-	-	-	-	-	+	-	-	-	-	1
HEPATOCYTES: (+)	+	+	+	+	+	-	+	+	+	+	9
KUPFFER CELLS: (+)	+	-	-	-	-	-	+	-	-	+	3

LIVER - LIPIDS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	-	-	-	-	-	+	-	+	-	-	2
PERILOBULAR ZONE: (+)	-	+	-	-	+	-	+	-	+	-	4
PERILOBULAR ZONE: (+++)	+	-	+	+	-	-	-	-	-	+	4

STOMACH	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	+	+	+	-	+	+	-	+	8
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	K	-	K	-	K	-	-	-	-	K	4

SMALL INTESTINE	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	1	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	1	2	2	2	2	2	2	2	10
SUBMUCOSAL LYMPHOID FOCI	-	-	-	-	-	1A	-	-	-	-	1

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COLON	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SUBMUCOSAL LYMPHOID FOCI	-	D	D	-	-	-	-	-	-	-	2
LUMINAL DISTENSION	+	-	-	-	-	-	-	-	-	-	1

SALIVARY GLANDS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	2	2	10
MUCOUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
ACINAR ATROPHY	1D	1A	-	-	-	-	-	-	-	-	2

PANCREAS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: URINARY

SYSTEM: URINARY

KIDNEYS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
GLOMERULONEPHROSIS	1A	-	-	-	-	-	-	-	-	-	1
FOCI OF BASOPHILIC TUBULES	1A	1A	-	-	-	-	-	-	-	-	2
SINGLE TUBULAR CYLINDER	-	-	1	-	1	-	-	-	-	-	2
MULTIFOCAL MIXED NEPHRITIS	-	-	1 K	-	-	-	-	-	-	-	1

KIDNEY - IRON	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
NONE	-	-	-	-	-	-	-	-	-	+	1
PROXIMAL TUBULES: NORMAL (+)	-	-	+	-	+	+	-	-	+	-	4
PROXIMAL TUBULES: NORMAL (++)	+	+	-	+	-	-	+	+	-	-	5

BLADDER	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
INFILTR. OF MONONUCL. CELLS IN THE LAMINA PROPRIA	-	-	-	-	A	-	-	-	-	-	1

SYSTEM: HEMATOLYMPHOID

THYMUS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
EPIDERMOID CYST	+	+	-	+	+	+	+	+	+	+	9

SPLEEN	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (++)	-	+	-	+	-	+	-	+	+	-	5
RED PULP: (+++)	-	-	+	-	-	-	+	-	-	+	3
RED PULP: (++++)	+	-	-	-	+	-	-	-	-	-	2

NODES	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

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SYSTEM: ENDOCRINE

THYROIDS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	2	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	2	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	2	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	2	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	2	10
EPIDERMOID CYST	1	1	-	-	-	-	-	-	-	-	2
FOLLICULAR CYST	-	-	-	1	-	-	-	-	-	-	1
UNIFOCAL LYMPHOID FOCUS	-	-	-	-	-	-	-	-	-	1 K	1
PARATHYROID PRESENT	1	-	-	-	-	2	2	1	1	2	6

ADRENAL GLANDS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	1	1	2	2	-	2	2	1	2	2	9

SYSTEM: REPRODUCTIVE

OVARIES	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED OVARIES	2	2	2	2	1	1	2	2	1	2	10
PARTIAL OVARIAN SECTION	-	-	-	-	1	1	-	-	1	-	3
GRAAFIAN FOLLICLES	2	2	2	2	1	1	2	2	1	2	10
CORPORA LUTEA	2	2	2	2	1	1	2	2	1	2	10
LUTEINIZING CYST	-	-	1	-	-	-	-	-	-	-	1

UTERINE HORNS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
NARROW LUMEN	2	-	2	2	2	2	-	2	2	2	8
LUMEN WITH UNEVEN THICKNESS	-	-	-	-	-	-	2	-	-	-	1
DILATED LUMEN	-	2	-	-	-	-	-	-	-	-	1
HEIGHT OF THE EPITHELIUM (++)	2	2	2	2	2	2	-	2	2	2	9
HEIGHT OF THE EPITHELIUM (+++)	-	-	-	-	-	-	2	-	-	-	1
PROLIFERATIVE EPITHELIUM	2	2	2	2	2	2	1	2	2	2	10
NON-PROLIFERATIVE EPITHELIUM	-	-	-	-	-	-	1	-	-	-	1

SYSTEM: CARDIOVASCULAR

HEART	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SYSTEM: RESPIRATORY

LUNGS	15838	15839	15840	15841	15842	15843	15844	15845	15846	15847	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
JUXTABRONCHIAL LYMPHOID CLUSTERS	-	-	-	-	A	A	-	A	-	-	3
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10

Group 7: low dose of hypothiocyanate – males

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SUBSTANCE: HYPOTHIOCYANATE GROUP 7: LOW DOSE

SPECIES: RAT SEX: M

SYSTEM: DIGESTIVE

LIVER	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	-	+	+	+	9
HETEROGENEOUS PARENCHYMA	-	-	-	-	-	-	+	-	-	-	1
CLEAR CENTRILOBULAR PARENCHYMA	-	-	-	-	-	-	+	-	-	-	1
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	D	D	-	D	D	A	-	-	D	D	7
SINUSOIDS ECTASIA	-	-	-	-	-	K	K	-	-	-	2
LOCALIZED HEMORRHAGIC NECROSIS	-	+	-	-	-	-	-	-	-	-	1
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	K	-	K	-	-	-	-	-	-	-	2
LIPID VACUOLIZATION	-	K	K	K	-	K	-	K	-	K	6
LOCALIZED FIBROSIS	-	-	-	-	-	-	-	-	K	-	1

LIVER IRON	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

LIVER - LIPIDS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	-	-	-	+	-	-	-	-	-	-	1
PERILOBULAR ZONE: (+)	-	+	+	-	-	-	-	+	-	+	4
PERILOBULAR ZONE: (++)	+	-	-	-	+	+	+	-	-	-	4
PERILOBULAR ZONE: (+++)	-	-	-	-	-	-	-	-	+	-	1

STOMACH	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	8
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
INTRAEPIDERMAL VESICLES	A	-	-	-	-	-	-	-	-	-	1
GLANDULAR ECTASIA	-	-	-	-	-	-	-	-	K	-	1

SMALL INTESTINE	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10

COLON	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	-	1	1	1	-	1	-	1	7
ABSENT	-	-	AB	-	-	-	AB	-	AB	-	3
NUMBER OF EXAMINED SPECIMENS	1	1	-	1	1	1	-	1	-	1	7

SALIVARY GLANDS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	-	2	9
ABSENT	-	-	-	-	-	-	-	-	AB	-	1
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	-	2	9
MUCOUS ZONE PRESENT	2	2	2	2	2	2	2	2	-	2	9
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	-	2	9
ACINAR ATROPHY	-	-	-	-	-	-	-	-	-	1A	1

PANCREAS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
B-CELLS IL. GRANULATION: (+++)	+	+	+	+	+	+	+	+	+	+	10

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SYSTEM: URINARY

KIDNEYS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
GLOMERULONEPHROSIS	-	-	-	1A	-	-	-	-	-	-	1
TUBULONEPHROSIS	-	-	-	2D	-	-	-	-	-	1D	2
FOCI OF BASOPHILIC TUBULES	-	-	-	-	1A	-	-	-	-	-	1
MULTIPLE TUBULAR CYLINDERS	-	2 K	-	2 K	-	-	-	1 K	-	2 K	4
UNIFOCAL MIXED NEPHRITIS	-	-	-	-	-	-	-	1 K	-	-	1

KIDNEY - IRON	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
NONE	-	-	+	+	+	+	+	-	-	+	6
PROXIMAL TUBULES: NORMAL (+)	+	+	-	-	-	-	-	+	+	-	4

BLADDER	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
EPITHELIAL VACUOLIZATION	-	-	-	-	-	-	-	-	K	-	1

SYSTEM: HEMATOLYMPHOID

THYMUS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	-	9
ABSENT	-	-	-	-	-	-	-	-	-	AB	1
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	-	9
CILIATED CYST	-	+	-	-	-	-	-	-	-	-	1
EPIDERMOID CYST	-	+	-	-	+	-	-	-	-	-	2

SPLEEN	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (+)	+	-	-	-	-	-	-	-	+	-	2
RED PULP: (++)	-	-	+	+	+	+	-	+	-	-	5
RED PULP: (+++)	-	+	-	-	-	-	+	-	-	+	3

NODES	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

SYSTEM: ENDOCRINE

THYROIDS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	1	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	1	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	1	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	1	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	1	10
EPIDERMOID CYST	-	-	-	-	1	-	-	-	-	1	2
PARATHYROID PRESENT	1	2	1	-	2	1	2	-	2	-	7
SPECIMEN FOR ANALYZING FAT	-	-	-	-	-	-	-	-	-	1	1

ADRENAL GLANDS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	2	2	2	2	1	2	2	2	2	10

SYSTEM: REPRODUCTIVE

TESTICLES	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED TESTICLES	2	2	2	2	2	2	2	2	2	2	10
LOCALIZED DEGENERATION	-	-	-	-	1	-	1	-	-	-	2

SEMINAL VESICLES	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	2	2	2	1	1	1	2	1	1	1	10
NUMBER OF EXAMINED VESICLES	2	2	2	1	1	1	2	1	1	1	10

PROSTATE	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
LOC. GLANDULAR EPITH. ATROPHY	-	-	+	+	-	-	+	-	-	-	3
MULTIFOCAL LYMPHOID INFILTRATION	K	-	-	K	-	-	-	-	-	-	2
POLYMORPHONUCLEAR CELLS, GLANDS LUMEN	-	-	-	-	-	-	A	-	-	-	1

SYSTEM: CARDIOVASCULAR

HEART	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
MONONUCLEAR CELLS INFILTR. IN MYOCARDIUM	A	-	-	-	A	-	-	-	-	-	2

SYSTEM: RESPIRATORY

LUNGS	15848	15849	15850	15851	15852	15853	15854	15855	15856	15857	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
ALVEOLAR HEMORRHAGE	-	-	-	-	-	-	-	+	-	-	1
JUXTABRONCHIAL LYMPHOID CLUSTERS	A	A	-	-	-	-	-	-	A	A	4
PERIVASCULAR INFL. SHEATHS	-	-	-	-	-	-	-	A	-	-	1
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10
HEMORRHAGIC EXUDATIVE PNEUMONIA	-	-	K	-	-	-	-	-	-	-	1

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Group 8: low dose of hypothiocyanate – females

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SUBSTANCE: HYPOTHIOCYANATE GROUP 8: LOW DOSE

SPECIES: RAT SEX: F

SYSTEM: DIGESTIVE

LIVER	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HOMOGENEOUS PARENCHYMA	+	+	+	+	+	+	+	+	+	+	10
HETEROGENEOUS HEPATOCYTES CYTOPLASM	+	+	+	+	+	+	+	+	+	+	10
MONONUCLEAR CELL FOCI	-	-	D	D	D	D	D	D	-	D	7
MULTIFOCAL HYPER- AND MICROVACUOLIZATION	-	-	K	-	-	-	K	-	-	-	2
LIPID VACUOLIZATION	-	-	-	-	-	K	K	-	-	-	2

LIVER IRON	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES: (+)	+	+	+	+	+	+	+	+	+	+	10
KUPFFER CELLS: (+)	+	+	+	-	-	+	-	-	+	+	6

LIVER - LIPIDS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
HEPATOCYTES NONE	-	-	-	-	-	-	-	-	+	-	1
PERILOBULAR ZONE: (+)	+	-	-	+	-	-	+	-	-	+	4
PERILOBULAR ZONE: (++)	-	+	-	-	+	+	-	+	-	-	4
PERILOBULAR ZONE: (+++)	-	-	+	-	-	-	-	-	-	-	1

STOMACH	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SQUAMOUS MUCOSA PRESENT	+	+	-	+	+	+	+	-	-	+	7
GLANDULAR MUCOSA PRESENT	+	+	+	+	+	+	+	+	+	+	10
GLANDULAR ECTASIA	K	-	-	-	K	-	-	-	K	-	3
GLANDULAR SUBMUCOSA EDEMA	-	+	-	-	-	-	-	+	-	-	2

SMALL INTESTINE	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	1	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	1	2	2	2	2	2	2	10

COLON	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
SUBMUCOSAL LYMPHOID FOCI	D	-	-	-	-	-	-	-	-	-	1

SALIVARY GLANDS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED GLANDS	2	2	2	2	2	2	2	2	2	2	10
MUCOUS ZONE PRESENT	2	2	2	2	2	1	2	2	2	2	10
SEROUS ZONE PRESENT	2	2	2	2	2	2	2	2	2	2	10
ACINAR ATROPHY	-	-	-	1A	-	-	-	-	-	-	1

PANCREAS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	-	1	1	9
ABSENT	-	-	-	-	-	-	-	AB	-	-	1
NUMBER OF EXAMINED SPECIMENS	1R	1	1	1	1	1	1	-	1	1	9
EX. UNIFOCAL ATROPHY	-	-	-	-	-	-	K	-	-	-	1
IL. B-CELLS GRANULATION: (+++)	-	-	+	+	+	+	+	-	+	+	7
IL. B-CELLS GRANULATION: (++)	-	+	-	-	-	-	-	-	-	-	1

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SYSTEM: URINARY

KIDNEYS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED KIDNEYS	2	2	2	2	2	2	2	2	2	2	10
EPITHELIAL HYPERTROPHY	-	-	1A	-	-	-	-	-	-	-	1
SINGLE TUBULAR CYLINDER	-	-	-	-	-	-	-	-	1	-	1
CORTICAL LYMPHOID FOCI	-	-	-	-	-	-	-	-	-	1A	1
MINERALIZATION	-	-	1 K	-	-	-	-	1 K	-	-	2

KIDNEY - IRON	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
IRON - NONE	2	2	2	2	2	2	2	2	2	2	10

KIDNEY LIPIDS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED KIDNEYS	1	1	1	1	1	1	1	1	1	1	10
PROXIMAL TUBULES: NORMAL (+)	+	+	-	+	+	+	+	+	+	+	9
PROXIMAL TUBULES: NORMAL (++)	-	-	+	-	-	-	-	-	-	-	1

BLADDER	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	-	1	1	1	1	1	1	1	1	1	9
ABSENT	AB	-	-	-	-	-	-	1	-	-	1
NUMBER OF EXAMINED SPECIMENS	-	1	1	1	1	1	1	1	1	1	9

SYSTEM: HEMATOLYMPHOID

THYMUS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
CILIATED CYST	-	+	-	-	-	-	-	-	-	-	1
EPIDERMOID CYST	-	+	+	+	+	+	-	+	-	+	7

SPLEEN	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SPLEEN IRON	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
RED PULP: (+)	-	+	-	-	-	-	-	-	-	-	1
RED PULP: (++)	+	-	-	-	+	-	+	+	-	-	4
RED PULP: (+++)	-	-	+	-	-	+	-	-	-	+	3
RED PULP: (++++)	-	-	-	+	-	-	-	-	+	-	2

NODES	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED NODES	1	1	1	1	1	1	1	1	1	1	10

NODE - IRON	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NONE	+	+	+	+	+	+	+	+	+	+	10

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SYSTEM: ENDOCRINE

THYROIDS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED THYROIDS	2	2	2	2	2	2	2	2	2	1	10
CUBO-COLUMNAR EPITHELIUM	2	2	2	2	2	2	2	2	2	1	10
NORMAL FOLLICLES	2	2	2	2	2	2	2	2	2	1	10
SECRETORY ASPECT	2	2	2	2	2	2	2	2	2	1	10
CHROMOPHILE COLLOID	2	2	2	2	2	2	2	2	2	1	10
EPIDERMOID CYST	-	2	-	1	-	-	-	1	1	1	5
PARATHYROID PRESENT	2	-	-	-	1	1	2	-	1	-	5

ADRENAL GLANDS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED ADRENAL GLANDS	2	2	2	2	2	2	2	2	2	2	10
MEDULLA PRESENT	2	2	2	2	2	1	2	2	-	2	9

SYSTEM: REPRODUCTIVE

OVARIES	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED OVARIES	2	2	2	1	2	2	2	2	2	2	10
PARTIAL OVARIAN SECTION	-	-	-	1	-	-	-	-	-	-	1
GRAAFIAN FOLLICLES	2	2	2	1	2	2	2	2	2	2	10
CORPORA LUTEA	2	2	2	1	2	2	2	2	2	2	10
LUTEINIZING CYST	-	-	1	-	-	-	-	-	-	-	1

UTERINE HORNS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	2	2	2	2	2	2	2	2	2	2	10
NUMBER OF EXAMINED HORNS	2	2	2	2	2	2	2	2	2	2	10
NARROW LUMEN	-	2	2	-	2	2	2	2	2	-	7
LUMEN WITH UNEVEN THICKNESS	2	-	-	1	-	-	-	-	-	-	2
DILATED LUMEN	-	-	-	1	-	-	-	-	-	2	2
HEIGHT OF THE EPITHELIUM (+)	-	-	-	-	-	-	2	2	1	-	3
HEIGHT OF THE EPITHELIUM (++)	2	2	2	-	-	2	2	-	1	2	7
HEIGHT OF THE EPITHELIUM (+++)	-	-	-	2	-	-	-	-	-	-	1
PROLIFERATIVE EPITHELIUM	2	2	2	-	2	2	2	2	2	2	9
NON-PROLIFERATIVE EPITHELIUM	-	-	-	2	-	-	-	-	-	-	1

SYSTEM: CARDIOVASCULAR

HEART	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10

SYSTEM: RESPIRATORY

LUNGS	15858	15859	15860	15861	15862	15863	15864	15865	15866	15867	Sum
NUMBER OF SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
NUMBER OF EXAMINED SPECIMENS	1	1	1	1	1	1	1	1	1	1	10
FOCUS OF ALVEOLAR MACROPHAGES	A	-	-	-	-	-	-	-	-	-	1
JUXTABRONCHIAL LYMPHOID CLUSTERS	D	-	D	-	A	-	-	-	-	A	4
JUXTAVASCULAR LYMPHOID CLUSTERS	-	-	-	D	D	-	-	-	-	-	2
PERIVASCULAR INFLAMMATORY SHEATHS	D	-	-	-	-	-	-	-	-	-	1
ELASTIC FIBERS: NORMAL ASPECT	+	+	+	+	+	+	+	+	+	+	10

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DISCUSSION AND CONCLUSION

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D. DISCUSSION AND CONCLUSION

3-month chronic toxicity study of the HYPOTHIOCYANATE Generating Mixture comprising sodium thiocyanate, lactoperoxidase, glucose oxidase and glucose with oral administration in male and female rats.

The HYPOTHIOCYANATE ion has bacteriostatic properties used in the food industry. Its instability requires an extemporaneous preparation.

Since the purpose of this study was to investigate the toxicity of the HYPOTHIOCYANATE ion,

the study of high doses was limited by two phenomena:

a/ There are optimal concentrations of the 4 components for producing the HYPOTHIOCYANATE ion. Higher concentrations of the reaction mixture components produce a smaller amount of the HYPOTHIOCYANATE ion.

b/ Increasing concentrations of the reaction mixture components causes an excessive release of hydrogen peroxide by the glucose oxidase/glucose combination. This excessive release has a dual effect: firstly, it destroys the lactoperoxidase that has an effect of decoupling the reaction producing the HYPOTHIOCYANATE and therefore increases the amount of hydrogen peroxide formed, and, secondly, it reduces the production of HYPOTHIOCYANATE as described above.

Therefore it is clear that in these conditions it was impossible to study a more concentrated mixture unless we wanted to study not the toxicity of the HYPOTHIOCYANATE ion but that of hydrogen peroxide, which is not the subject of this study.

However, if the administration of higher doses was not possible, the administration of the mixture at the time when the amount of the HYPOTHIOCYANATE ions is at maximum, gives a much higher level of exposure compared to normal conditions of use thereby providing a safety factor. Otherwise, administration in a volume of 10 ml/kg, which represents 700 ml for the human subject with an average weight of 70 kg, is much greater than the exposure levels considered under the normal conditions of use.

In this study the following aspects were observed:

- **In terms of clinical examinations**

  No mortality and no abnormal behavior was noted. The few changes in daily clinical examinations that were observed are either spontaneous or subsequent to sampling from retro-orbital sinus and are therefore not attributable to the treatment.

- **In terms of changes in animal body weight**

  A more significant weight increase in treated male animals, particularly the group treated with the medium dose, was noted as being attributable to the treatment. This effect was not observed in females animals.

- **In terms of feed consumption**

  An increase at different times of treatment was noted in all treated male animals, this increase was not observed in female animals.

  The caloric use factor was comparable between all treatment and control groups, therefore the feed consumption increase in male results

  in a corresponding increase in body weight.

- **In terms of drink consumption**

  No treatment-related effects were noted.

- **In terms of ophthalmic examinations**

  No treatment-related effects were noted.

- **In terms of hematological examinations**

  Only a statistically significant increase in the number of leukocytes was noted at 6 weeks in male animals. However, this increase was not found at twelve weeks and for the female animals the relation to the treatment was not even considered at these two time points. Other changes remained within physiological limits.

- **In terms of biochemical examinations**

  It should be noted the significant increase in triglyceride levels in treated male animals at six weeks with a significant dose-response relationship. Even if this increase was not found at twelve weeks or in female animals at different time points, this observation is undoubtedly linked to the increase of feed consumption and body weight observed in male animals.

- **In terms of urine tests**

  At two observation points, no change was observed.

- **In terms of anatomopathological examinations**

  No macroscopic lesions attributable to the treatment were noted. However, weighing the organs demonstrated an increase in liver weight in absolute and relative values in treated female animals with the existence of a dose-response relationship. This effect is therefore considered as attributable to the treatment.

- **In terms of histopathological examinations**

  The only histological change attributable to treatment appears as an increased vacuolization of the hepatocytes cytoplasm and discrete lipid excess with associated necrotic cell lesions in all treated male animals and more particularly in male animals treated with the higher dose.

In these circumstances, it appears that only changes were observed in treated male animals and these changes appear to be related. In fact, the significant increase in feed consumption in treated male animals without changing the caloric use factor appears rather premature. This results in a body weight increase proportional to the dose. At 6 weeks an increase in triglyceride levels is visible, while at the end of the treatment period the increased liver weight in proportion to the dose is noted in male animals. This is reflected histologically by increased vacuolization and discrete lipid excess in hepatocytes. These histological and biochemical changes may be due to a feed excess in male animals. All observed phenomena appear to be related to the treatment, however, they are benign effects that remain relatively limited in their amplitude. It must be noted that this type of effect was not seen in female animals.

In conclusion, a three-month oral administration in rats of the Hypothiocyanate Generating Mixture did not induce any significant toxic effects.

PART 7: LIST OF REFERENCES

Pursuant to 21 C.F.R. 170.255, the list of supporting data and information referenced in the GRAS notice is contained below.

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June 1, 2018

VIA ELECTRONIC MAIL

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Re: GRN 753: Response to FDA Request for Additional Information

Dear Dr. Wafula:

On behalf of our client Taradon Laboratory ("Taradon"), we submit this response to questions raised by the U.S. Food and Drug Administration ("FDA" or "Agency") in an email on May 17, 2018, regarding GRAS Notification 753 for the use of sodium thiocyanate as part of the lactoperoxidase system ("LPO") to extend the shelf life of a variety of dairy products, specifically fresh cheese including mozzarella and cottage cheese, frozen dairy desserts, fermented milk, flavored milk drinks, and yogurt. The intended use does not include the preservation of raw milk. We have included FDA's comment in bold italics, followed by the Taradon response.

1. ***Please provide the appropriate references for the statements provided on page 7:***
    a. ***"Concentrations have been reported to vary between 2.3 and 35 mg/l in milk from individual cows."***

The fluctuation in concentrations of milk that vary between 2.3 and 35 mg/l was reported in a study on seasonal fluctuations in thiocyanates in industrial milk over a period of 5 consecutive years. *See* Boulangé M, Han K, et Vert P, - C.R.Soc. Biol., 157, 1074-1076 (1963) ; Boulangé M, C.R.Soc.Biol. 153, 2019-2020 (1959). The article is provided in Appendix 1 (in English and French).

    b. ***"The SCN concentration is adult human gastric juice is high, 0.38 mM (22mg), and even higher than in salvia; up to 2.5 mM SCN (145 mg) has been found for the salvia of smokers."***

The concentration of SCN in adult human gastric juice (0.38 mM) and saliva of smokers (2.5. mM) was most recently reported in, Johannes Everse, Mathew B. Brisham, & Kathleen E. Everse,

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Peroxidase in Chemistry and Biology, Volume 1, (Nov. 1990) CRC Press, at p. 149. This source of this information also came from *Medical and Biological Significance of Thiocyanate* (Weuffen, 1982), which was provided in Annex 7 of the Notice.

2. ***Please provide a statement to confirm that sodium thiocyanate is manufactured under current good manufacturing practices using common food industry materials and processes in accordance with the applicable parts of 21 C.F.R. Part 110.***

Akzo Nobel Functional Chemicals GmbH is the supplier of the sodium thiocyanate. We have attached (Appendix 2 of this response) the product specifications of the sodium thiocyanate as well as the supplier's ISO 9001:2015 certificate demonstrating that the supplier as a sophisticated management system that would be compliant with cGMPs.

3. ***Taradon refers to dietary exposure from sodium thiocyanate in several parts of GRN 753 (see examples below) in different units of concentration (ppm, mg/L, etc.). Please report the estimated dietary intake (from background and from direct consumption of sodium thiocyanate) as mg/person/day, or mg/kg bodyweight/day, based on product of the concentration of sodium thiocyanate in food and the amount of those food/drink consumed.***

   Example 1: On Page 8, Taradon states that 'Cows grazing natural pastures with a complex flora of different grasses, weeds and clover were shown to give milk with the highest concentrations of SCN^- as between 0.26 mM (15 mg of SCN^- anion) to 0.35 mM (20 mg of SCN^- anion) … As to LPO system use in dairy products, the proposed maximum levels of thiocyanate, the estimated intake of SCN^- for the consumers of lactoperoxidase system-treated dairy products is estimated to be between 15 mg to 20 mg of SCN^- ions per liter of milk. Therefore, the intake of SCN^- anions for an average consumer of LPO system-treated dairy products would appear greater than the background from general milk consumption.' Please provide clarification.

As discussed on page 13 in Annex 9 of the Notice, the reaction and the kinetics of the lactoperoxidase system shows that the thiocyanate that is added for the preservation of dairy products will be transformed into hypothiocyanite (OSCN-) ions and that the concentration that is used in the lactoperoxidase system will not increase the level of thiocyanate already existing in the milk. As a result, ingestion of SCN^- anions for an average consumer of LPS-treated dairy products will not have a greater impact than the of overall milk consumption. Therefore, under the actual operating conditions proposed in the Notice, the total thiocyanate content, once the LPS is activated in the mixture, does not exceed the maximum natural concentration in a particular cow's milk.

In the study on the exposure assessment to thiocyanate, we did not take into account the transformation of thiocyanate into OSCN- ions. Although we are well aware that this will be the case, the maximum of thiocyanate (natural plus that proposed in the LPS) evaluated by the study carried out by Exponent (Annex 6 of the Notice) shows that the total use for the mean and 90th percentile users are well below those values established in the toxicology studies (Annex 7 of the Notice). As discussed on Page 19/Table 6 (and included below), notwithstanding the fact that the added thiocyanate will be transformed, the estimated dietary intake at the 90th percentile of users

is 7.24 mg/person/day, which includes both background and direct consumption of sodium thiocyanate in milk.

Table 6: Estimated Exposure

	Total U.S. Population					
			Per Capita (mg/day)		Per User (mg/day)	
EDI based on:	Unweighted N	% User	Mean	90th Percentile	Mean	90th Percentile
Proposed Use	7,576	49	0.59	2	1.2	3.33
Maximum Use (Natural + Proposed)	10,208	67	1.63	5.52	2.44	7.24

The levels from the proposed uses are below the naturally occurring levels found in milk, which range from 2.3 and 35 mg/l of thiocyanate in milk from individual cows.

Example 2: On Page 19/Table 6, Taradon reports that the maximum use (natural plus proposed) at the 90th percentile is 7.24 mg/day per user. It is not clear, therefore, what is meant by the statement "the intake of SCN^- anions for an average consumer of LPO system-treated dairy products would appear greater than the background from general milk consumption."

As stated above, the exposure assessment did not take into account the degradation of the SCN- ions, which will be consumed as part of the reaction of lactoperoxidase system. This creates the appearance that LPO-treated dairy products would be greater from the general milk consumption even though that is not the case.

Example 3: On Page 22, Taradon presents Table II to represent the amount of SCN^- in vegetables and concludes that the amount of thiocyanate encountered in the diet on a regular basis is not inconsequential, and can easily exceed the amount that will be used in the proposed system. However, Table II only gives the concentration (in ppm) and does not provide the potential dietary exposure to sodium thiocyanate from consumption of these vegetables. Please provide clarification.

Table II was added based on feedback from the Agency to demonstrate that thiocyanate is consumed as part of regular dietary patterns. The table is corroborative and was not intended to be part of the exposure assessment. The use of the lactoperoxidase system has been focused on the preservation of dairy products based on the intended use in the Notice. As discussed above, the addition of sodium thiocyanate to the lactoperoxidase system (See Annex 9 of the Notice) will have no impact on the overall consumption of thiocyanate to the consumer.

Example 4: On Page 33, the results from the acute toxicity studies included in the notice suggest that the LD50 values range from 8.5 g/kg/bw to greater than 16 g/kg/bw. The dietary exposure assessment included in Part 3, propose that these doses are orders of magnitude higher than the estimated daily intake of sodium thiocyanate, i.e. 3.33 mg/day at the 90th percentile. The cited study

was based on the LPO system with the actual doses of thiocyanate ranging from 68.9 mg to 14.3 g/L of sodium thiocyanate. Please clarify how the "total dose" of a 8.5 g-16 g/kg/bw can be related to estimated dietary exposure of sodium thiocyanate.

The total dose from the referenced studies were done with the complete lactoperoxidase system and not solely with thiocyanate. Therefore, when Taradon is referring to a total dose of 8.5 g/kg/bw and 16 g/kg/bw we are referring to the total mixture that includes glucose, lactoperoxidase, glucose oxidase, and thiocyanate. As indicated, the dose range specific to thiocyanate from these studies was from 68.9 mg (formula A) to 14.3 g/L. (formula B). See Page 30 and Annex 10 of the Notice. As noted in the responses above, the thiocyanate is degraded and will have no impact on the overall consumption to the consumer.

4. ***In establishing general recognition of safety of sodium thiocyanate for the indented uses in GRN 753, Taradon cites several international advisory bodies and regulatory agencies regarding thiocyanate and lactoperoxidase (LPO) system safety (examples below). However, the intended uses in those citations do not correspond to the proposed use in GRN 753.***

   Example 1: Taradon states that Codex guidelines provide specifications and practices for the addition of thiocyanate to lactoperxoidase already present in milk.

   Example 2: Taradon states that the FSANZ approved use of sodium thiocyanate for LPO system as an antibacterial agent for "meat and meat products," which are generally further processed or cooked.

   Example 3: Taradon states that the Swedish National Food Administration "... has decided to allow the use of LP-activation in milk where raw milk cannot be properly cooled" (pg. 24 of the notice).

   Please provide a statement indicating how the approved uses in other countries support the general recognition of the indented use of sodium thiocyanate in GRN 753.

As described in the Notice, the role of thiocyanate is to activate the lactoperoxidase (in the presence of percarbonate) system to produce an antimicrobial agent to protect milk products against post-contamination following pasteurization. The three examples referenced are corroborative and demonstrate the use of thiocyanate as a part of the diet. *See* Parveen, R., et al., J. Biotechnol. Biomater, 6(2), 1-6 (Appendix 3 of this response). The examples demonstrate that sodium thiocyanate has been evaluated by several international advisory bodies and regulatory agencies for its use in the lactoperoxidase system for the intention of extending the shelf-life of food products by producing antimicrobial agents that fight against contamination. While Taradon recognizes that the intended use of sodium thiocyanate in these applications may be slightly different, and that approval by foreign regulatory agencies does not establish a general recognition of safety in the United States, Taradon used these examples to demonstrate that sodium thiocyanate has a long history of use in the diet as part of the LPO system and its safety in these applications has long been evaluated by other regulatory agencies and qualified experts in the

field. This information was provided to give FDA a full context of its global uses and to demonstrate that it has been recognized as safe in other jurisdictions.

5. ***Taradon states (Pg. 24): "The lactoperoxidase system was approved by the National Expert Committee on Food Additives in the People's Republic of China as 'an acceptable preservative used for milk preservation.'" FDA notes that according to Yong et al. (2017), prior approval of adding 15.0 mg/L of sodium thiocyanate in raw milk in china was disallowed in 2007, with sodium thiocyanate being added to the list of inedible substances in 2008. Please provide an explanation on how this information does not impact your safety conclusion.***

Taradon has extensively reviewed this paper and we confirm that this information does not impact our safety conclusion. As noted in the abstract of the paper cited by FDA, "Thiocyanate in milk at this level [9.0 mg/kg] does not present a food safety concern." Notably, this level is higher than the estimated dietary intake of 7.4 mg/kg referenced in the Notice.

In the paper referenced by the Agency, the authors state that "When used as intended, the level of thiocyanate added to milk for treatment is **within the natural variation** in thiocyanate found in milks, and **the levels of thiocyanate in LPS treated milk do not present a food safety concern**" (emphasis added). This statement supports Taradon's conclusion that LPS-treated dairy products will have no impact on the overall consumption of thiocyanate.

It is our understanding that thiocyanate was not banned in China for reasons of safety, but because it was being misused to combat high microbial contamination in raw milk. This is supported by the paper, which states "misuse of the LPS preservation system by an excessive addition of sodium thiocyanate may give rise to certain health hazards." When sodium thiocyanate is misused through its excessive addition in milk with high levels of microbial contamination, the thiocyanate will not be converted to OSCN- ions and the level of thiocyanate in milk would be higher that what is naturally occurring. This paper is further distinguished from the intended use of thiocyanate identified in the Notice as part of the lactoperoxidase system because it is 1) not intended for the preservation of raw milk and 2) not characterized by an increase in the SCN- ions because the added thiocyanate is completely converted into OSCN- ions. Therefore, the change in regulatory status in China does not impact Taradon's safety conclusion.

6. ***Under section 6.3.2.1 "Acute Toxicity Studies of Sodium Thiocyanate," under section "c", Taradon states:***

   "While the Pasteur studies are not published and, therefore, merely collaborative [sic], we believe that they are of value because they are focusing on the Lactoperoxidase System." (pg. 29 of the notice)

   FDA notes that these studies would not be characterized as "acute toxicity studies of sodium thiocyanate" but rather as toxicity studies of the LPO system (or as stated in the enclosed Pasteur Institute document "Hypothiocyanate Generating Mixture). Given that Taradon states that the studies are unpublished and without appropriate controls, please provide a concise rationale or statement affirming that the GRAS conclusion by Taradon on the intended use of sodium thiocyanate was made without consideration of these studies.

Taradon did not use the corroborative studies referenced in section 6.3.2.1. as a basis for its GRAS conclusion of sodium thiocyanate. Taradon's GRAS conclusion is based on the fact that sodium thiocyanate has been studied in humans for over a century and is there is an extensive body of literature regarding its use, is present in many green vegetables that are consumed daily, and is almost completely consumed in the reaction that drives the antimicrobial protection of the LPO system. Taradon also based its conclusion on a two-year chronic toxicity/carcinogenicity study conducted by the National Cancer Institute demonstrating that sodium thiocyanate is not carcinogenic and there were no adverse effects observed in rats over a long exposure period. Published subacute toxicity was also evaluated in both *in vivo* and *in vitro* experiments and no adverse effects were found. These studies along with the other information provided in the Notice on the exposure of sodium thiocyanate formed the basis of Taradon's GRAS conclusion.

Taradon merely referenced the unpublished toxicity studies of the LPO system as an additional corroborative source to support the safe use of sodium thiocyanate under the conditions of its intended use. Per the preamble to the GRAS final rule, unpublished studies can be used to corroboratively support the intended nutritive effect of the substance under the conditions of its intended use. Taradon believes the evaluation of thiocyanate via the LPO system was important, although not necessary, as the sodium thiocyanate is intended for use as part of the system, and is consumed as part of the reaction.

7. Although a list of references was provided in Part 7, it is customary for a notifier to state that a thorough literature search was performed. Please provide the date through which literature searches for sodium thiocyanate safety information were performed, along with database(s) and search term(s) used.

Taradon conducted a literature search that was limited to January 1, 2016 to March 29, 2018, to reflect the time period since the FAO/WHO report was published. A search of "lactoperoxidase system" and "adverse" revealed no results. A search of "thiocyanate" AND "milk" AND "adverse" returned three papers, which related not to thiocyanate consumption, but rather concerns of perchlorate exposures in the environment. A search of "thiocyanate" in combination with "food safety" AND "milk" returned 5 results. A review of the resulting publication abstracts revealed one publication that had concerns over milk products for children in China (Yong L, Zhang L, et. al. 2017). This article does not impact Taradon's safety conclusion because thiocyanate is not intended to be used in raw milk. The search terms were chosen as they most closely relate to the intended use of thiocyanate as outlined in the Notice, and are not confounded by experimental uses of thiocyanate for therapeutic purposes. The abstract in the second paper by Eisenbrand as well as the abstract in the eight paper by Yong, indicate that the dietary intake of thiocyanate is of no toxicological concern.

Results

1. Leung AM, Braverman LE, HE X, Schuller KE, Roussilhes A, Jahreis KA, Pearce EN. Environmental perchlorate and thiocyanate exposures and infant serum thyroid function. Thyroid. 2012 Sep.; 22(9); 938-43.

2. Eisenbrand G, Gelbke HP. Assessing the potential impact on the thyroid axis of environmentally relevant food constituents/contaminants in humans. Arch Toxicol. 2016 Aug;90(8).

3. Blount BC, Valentín-Blasini L. Biomonitoring as a method for assessing exposure to perchlorate. Thyroid. 2007 Sept;(17)(9):837-47.

4. Peng CF, Pan N, Zhi-Juan Q, Wei XL, Shao G. Colorimetric detection of thiocyanate based on inhibiting the catalytic activity of cystine-capped core-shell. Talanta. 2017 Dec 1;175:114-120.

5. Tan Z, Lou TT, Huang ZX, Zong J, Xu KX, Li QF, Chen D. Single-Drop Raman Imaging Exposes the Trace Contaminants in Milk. J Agric Food Chem. 2017 Aug 2;65(30):6274-6281.

6. Rasti B, Erfanian A, Selamat J. Novel nanoliposomal encapsulated omega-3 fatty acids and their applications in food. Food Chem. 2017 Sep 1;230:690-696.

7. Yong L, Zhang L, Wang YB, Yang DJ, Liu ZP, Wang T, Huang JP, Zhou PP. Liquid milk exposure and risk assessment of thiocyanate in Chinese populations. Zhonghua Yu Fang Yi Xue Za Zhi. 2017 Apr 6;51(4):332-335.

8. Yong L, Wang Y, Yang D, Liu Z, Abernethy G, Li J. Investigation of concentration of thiocyanate ion in raw cow's milk from China, New Zealand and the Netherlands. Food Chem. 2017 Jan 15;215:61-6.

* * *

We hope that these responses adequately address the concerns of the Agency. However, if additional research or clarification is needed, please do not hesitate to contact me. I can be reached by email at gary.yingling@morganlewis.com or by phone at (202) 739-5610.

Sincerely,

(b) (6)

Gary L. Yingling

In your response to Question 6, you state:

> "Taradon merely referenced the unpublished toxicity studies of the LPO system as an additional corroborative source to support the safe use of sodium thiocyanate under the conditions of its intended use. Per the preamble to the GRAS final rule, unpublished studies can be used to corroboratively support the intended nutritive effect of the substance under the conditions of its intended use."

While we fully agree that unpublished information can be used as corroborative evidence for GRAS conclusion, experimental data, published or unpublished, must still be evaluated within the basic tenets of scientific method, one of which is the inclusion of appropriate controls [i.e. see (Johnson and Besselsen, 2002)]. Given that within the toxicity studies discussed under Section 6.3.2.1 section c you state "[n]o control group was included" for either the mouse or the rat study, please explain how this information contributes, if any, to your GRAS conclusion.

Johnson, P.D., and Besselsen, D.G. (2002). Practical aspects of experimental design in animal research. ILAR J *43*, 202-206.

Taradon Response: With regard to the acute toxicity study discussed in section 6.3.2.1, we reviewed the study and included it as corroborative information, to provide a comprehensive review of the available information. However, because of the limitations of its design, Taradon did not use this study in the development of the GRAS conclusion.