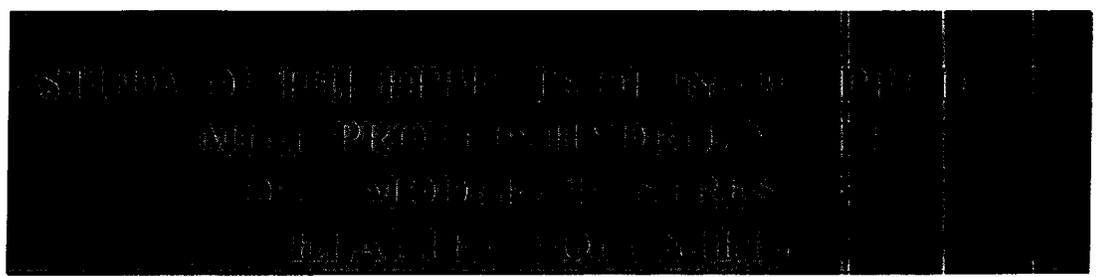


Confidential

FINAL REPORT



Biomedical Research Without Direct Individual Benefit

STUDY No. INGREDIA-04/0799/ING 911/SVS

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Study of the effects of ING 911 product, milk protein hydrolysate, on a moderate stress in healthy volunteer

Phase number: I

Name and addresses of the Sponsor: INGREDIA

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2 - INTRODUCTION

Over these last years, many work showed the biological properties of some products derived from milk proteins, in particular anti-hypertensive properties.

Maruyama *et al.* (1982, 1985 and 1987) have evidenced α S and β casein sequences which can inhibit, *in vitro*, the ACE (Angiotensin Converting Enzyme), privileged target against hypertension, by blocking its active site. Afterwards, peptides from human β casein (Kohmura *et al.*, 1989) and from β -lactoglobuline and α -lactalbumin enzymatic hydrolysis (Mullally *et al.*, 1997), were detected with the same inhibiting function.

In other studies, a blood pressure-lowering effect was observed but its mechanism was not studied. For instance, peptides from milk fermented by *L. helveticus* given to hypertensive rats, have lowered their blood pressure (Yamamoto *et al.*, 1994). It is the same with hypertensive rats which were given a single 5-ml/kg dose of milk fermented by *L. helveticus* and *S. cerevisiae* (Nakamura *et al.*, 1995); and anti-hypertensive peptides of this milk were found in the rat aorta (Masuda *et al.*, 1996).

In human, such fermented milk was given to hypertensive subjects and the same results were obtained: a significant lowering of systolic blood pressure compared to control group receiving an acidified milk as placebo.

A new peptide class extracted from milk shows protective properties against the stress in rat, mouse and pig.

The purpose of this study is to evaluate the milk protein hydrolysate anti-stress properties in human, through a physical test like the test of hand immersion in cold water (Weise *et al.*, 1993), and a psychological test like the Stroop test, a colour conflict test (Laude *et al.*, 1997), by measuring blood pressure, heart rate and plasma ACTH and cortisol content.

2.1 - Methods

2.1.1 - Subjects

Forty two subjects were recruited for the present double blind study (treatment/placebo in 2 parallel groups). They were healthy male caucasian volunteers, aged of 18 to 35 years with consent collection and satisfying various inclusion and non-inclusion criteria.

2.1.2 - Procedure

The subject swallows two 200-mg capsules of hydrolysate in the morning and two in the evening, on the day before the stress test. On the next morning, the study day, 90 to 120 minutes after having taken two new 200-mg capsules of hydrolysate, the volunteer is sitting down for the experiment time. The control subject is treated under the same conditions with two 200-mg capsules of powdered skim milk. After an explanation of what will be carried out and required, a digital sensor of a Finapres 2300 (Ohmeda, Maurepas) monitor is set up on the level of the second phalange of the left middle finger (for the right-handed persons). This hand will be kept at heart level. The right hand rests flat. The pressure signal analysis requires a high frequency digitalisation followed by a reprocessing intended for extracting the consecutive blood pressure (systolic and diastolic) and heart rate values. The first part of the recording starts with a five-minute control phase. Then the volunteer is asked to type with a finger of the dominant hand (right) on the coloured keys of the keyboard (blue, green, yellow or red) as series of four colour written with words of different colour are scrolling on the computer, while being stimulated by the experimenter.

Every error is recorded and indicated by a ringing. The test lasts for five minutes. At the end, a five-minute recovery phase is recorded.

Following the first test, the subject is resting for thirty minutes. The second test starts with a control pressure recording for five minutes. Then the left hand is immersed to the wrist in cold water at an average 2°C temperature. This nociceptive stimulation by cold lasts nearly five minutes during which the recording is continued (test) then the hand is withdrawn from cold water and the recording is continued for five minutes (recovery).

At the end of the second test, the digital sensor is withdrawn from the left hand. The subject can go home without having to stay in observation and without any particular recommendation.

2.1.3 - Studied variables

- Mean levels of systolic blood pressure, diastolic blood pressure and heart rate before and after tests (baseline variable);
- Plasma ACTH and cortisol content are assayed before and after test series (secondary variable).

2.1.4 - Expected effects

The expected effect such as lowering of blood pressure, heart rate and plasma ACTH and cortisol content variations would signify a protective activity against the stress.

3 - PROGRESS ACHIEVED

3.1 - Milk proteins and their functional peptides: recent discovery

Since thousand years, milk and dairy products represent a significant component of the food in nearly all the countries of the world. As these products were familiar, there was no question about their interest: it was implicit, not without reasons, but rather without evidence.

Thanks to exchanges between countries and the scientific progress which was able to control the production of this food regarding the biotechnological aspects, this food consumption increased very significantly. Moreover, thanks to their food and dietetic values, dairy products have an excellent image - very good nutrition at every age. They do bring to the body significant protein, carbohydrate, lipid, vitamin and trace element contents.

The α S1, α S2, β and κ caseins (resulting from the milk casein fraction) and the β -lactoglobuline, the α -lactalbumin, the lactoferrin and the lactotransferrin (resulting from the whey) are the eight main milk proteins whose specific properties on health have been evidenced, for two decades, by various researcher teams (Lorient *et al.*, 1991). Their presence or absence as their content is depending on the milk-producing species.

The concept according to which the essential function of these proteins was limited to the nitrogen supply necessary for the biosynthesis of tissue and circulating proteins and to that of nucleic acids was questioned (Hambraeus, 1985). Actually, the physiological and biochemical data of the nitrogen food allow us to think that dairy proteins contain in their structure peptide segments with a biological activity which can start some reactions of anticipatory regulation (Mendy, 1984). So they are called "functional peptides". This physiological role of dairy proteins is becoming every day more evident and β casein, the main protein, was called pro-hormone by some people (Migliore-Samour & Jollès, 1988).

During the milk protein digestion, the proteolytic enzymes from gastro-intestinal tract release peptides, which are then divided into amino acids by peptidases. Besides, the same result is obtained during milk fermentation by enzymes from lactic bacteria.

Distinct amino acid sequences, inactive within their original proteins but having particular properties following release by enzyme action, are called "functional peptides". They are also called "active peptides". These peptides are small and generally made up of about three to ten amino acids, except the caseinomacropptide (CMP) which is made up of 64 amino acids. This small size partly explains that they are well absorbed by the intestinal mucous membrane.

They have two physiological roles either remotely in the body or locally in the digestive tract. In the first case, these peptides must be absorbed at the intestine level then carried by blood circulation, in the second case, they must resist digestive enzymes during the time necessary to their action.

3.2 - Biological effects of functional peptides from milk

The current data on the identified physiological roles of functional peptides from milk allow differentiating five activity fields:

- calcium bio-transfer activity;
- opiate activity;
- immunomodulating activity;
- anti-hypertensive activity;
- anti-thrombotic activity

3.2.1 - Functional peptide intervention in the mineral nutrition

Milk and its derivatives are regarded as the main source of calcium supply to the human body (1,200 mg of Ca per litre of milk). In addition, the milk calcium intestinal absorption is higher than that of calcium present in vegetables (Renner, 1983). For long time, we have thought that milk was well assimilated only thanks to the lactose which would increase the intestine epithelial cell permeability to calcium salts, according to Wasserman (1964). In fact, complex soluble calcium salts would be formed by the lactose degradation and the subsequent transformation of resulting compounds by the wall enzymes and these salts would be absorbed mainly at the last segment of the small intestine level: the ileum (Armbrecht & Wasserman, 1976; Renner, 1983). The absence of β -galactosidase or the loss of induction capacity of this enzyme, blocking the first stage of this biotransformation, would explain the relations observed between lactose intolerance and osteoporosis (Kocian, 1986).

However, the results obtained thereafter showed that original casein peptide segments: caseinophosphopeptides (CPPs) would take a significant part in the milk calcium intestinal absorption.

The α S, β and κ caseins do contain phosphorylated serine sequences which confer to these peptides a very marked chelating value towards earth alkali (Ca^{++} and Mg^{++}) and trace elements. Thus, all the phosphorus and micellar calcium combine with CPPs allowing casein micelle stability and facilitating milk coagulation (Brulé & Lenoir, 1987).

In testings carried out on tied up intestinal loop of rats, Lee *et al.* (1979a and 1979b), using radiolabelled CaCl_2 , have noted that not only the calcium intraluminal solubility but also its absorption was increasing. Moreover, Gerber & Jost (1986) have observed that femur, tibia and metatarsus were well mineralised when they mixed CPPs with embryonic extracts of these bones. This property was lost if a CPP enzymatic dephosphorylation was performed.

According to Sato *et al.* (1986), the CPPs are involved in the biochemical mechanism of the calcium intestinal absorption by inhibiting the calcium salt precipitation in the small intestine and

by thus contributing to a passive-type absorption at the ileum level.

3.2.2 - Opiate activity of functional peptides

Some food origin peptides were called exorphins because their action was similar to that of peptides with opiate activity (enkephalins and endorphins) secreted by the brain and pituitary gland (Klee *et al.*, 1978). These exorphins were observed in pepsin hydrolysates of wheat gluten, α S casein, (Zioudrou *et al.*, 1979) and β casein (Brantl *et al.*, 1979). As their primary sequences have been elucidated, research on exorphin activities of dairy proteins has made rapid progress. Consequently, it was quickly possible to detect a morphinomimetic activity in the 60-66 sequence of bovine β casein and its derivatives (Henschen *et al.*, 1979); this sequence was called β -casomorphine 7.

Because the Tyr-aa-phe or Tyr-aa1-aa2-Phe (aa: amino acid) sequences must be necessarily present in peptides with opiate activity, from endogenous or exogenous origin, it was possible to show the existence of many exorphins in the sequence of milk proteins produced by several mammals (Chiba & Yoshikawa, 1986).

So, these peptides were found in caseins of human, bovine, ewe, buffless and she-camel milk (Richardson & Mercier, 1979; Petrilli *et al.*, 1984; Beg *et al.*, 1986); it should be noted that they are also present in the sequence of two main proteins of the whey: the α -lactalbumin and the β -lactoglobuline (Table 1).

Table 1
Opiate peptide derived from human and bovine milk proteins
(Chiba & Yoshikawa, 1986)

<u>Opiate peptides</u>	<u>Origin</u>
β -Casomorphine 7	Bovine β casein 60-66
β -Casomorphine 5	Bovine β casein 60-64
α -Exorphin casein	Bovine α casein 90-96
β -Casomorphine	Human β casein 51-58; 51-56; 51-55 and 51-54
β -Casomorphine	Human β casein 59-63
α -Lactorphin	α -lactalbumin η and β 50-53
β -Lactorphin	Bovine β -lactoglobuline 102-105

The analgesic activity of exorphins from β casein was widely studied using the "tail withdrawal" test in rats after pinching, the intra-cerebroventricular injection of the different β -casomorphines allowing to quantify their anti-pain effect. Consequently, it was shown that the 1-4 NH₂ β -casomorphine, also called morphiceptin, is ten times more active than morphine does (Chan *et al.*, 1982). Moreover, Sturner & Chang (1988) have connected the very high β -casomorphine and morphiceptin content of the predigested infantile milks to the significant reduction of the tears

together with the increase in the sleep of children receiving these milks. These results would show that the analgesic effect of the dairy exorphins is related to the daily behavioural observations made on the new born, after feed or after feeding-bottle absorption; in other words calm and sleep induction.

In addition, it was noted that depending on the nature of its terminal nitrogen amino-acid, opiate peptides interact with such or such receptors. Consequently, when the Arg residue takes up this position, these exorphins interact with the δ receptors located in the spinal marrow and in the limbic system (Chiba & Yoshikawa, 1986), seat of emotional feelings and acquired memorisation reflexes (reward and punishment).

On the contrary, the presence of a Tyr residue in this position allows the opiate peptides to interact with the μ receptors of the brain, especially present at the hypothalamus and thalamus level (Teschmacher, 1987; Paroli, 1988). However, these receptors are involved in the supraspinale analgesia, the prolactin and acetylcholine salting-out, the intestinal motricity regulation. This would partly explain the results described in the literature regarding the β casomorphine effect on gastro-intestinal motricity and on the electrolytic transport at this level.

These results show that milk exorphins can reduce in rats the velocity of the gastric emptying and the intestinal motility (Daniel *et al.*, 1993; Defilippi *et al.*, 1995). These peptides are also involved in the modulation of the postprandial insulinemia in the dog (Schusdziarra *et al.*, 1983a). A test meal providing saccharose added with β -casomorphine multiplied the plasma insulin content by a factor 6 or 7, compared with the contents observed following a meal only providing saccharose (Nieter & Schatz, 1981). Moreover, from *in vitro* testings on rabbit ileum, it was evidenced that natural β -casomorphines and their analogues increased the electrolytic absorption, like enkephalins, and had, consequently, a potential anti-diarrhoeal action (Hautefeuille *et al.*, 1986; Tomé *et al.*, 1987; Ben Mansour *et al.*, 1988; Mahé *et al.*, 1989).

The list of physiological effects of opioid peptides is not exhaustive; Chiba & Yoshikawa (1986) consider that these exorphins would have the following effects at the central level:

1 – analgesia; 2 – catalepsy; 3 – sedation and torpor; 4 – respiratory depression; 5 – hypotension; 6 – thermoregulation; 7 – regulation of the food intake; 8 – suppression of gastric secretion; 9 – increase in the GH, PRL and ADH contents; 10 – reduction in the LH, FSH, TSH and ACTH contents; 11 – regulation of the sexual behaviour.

The peripheral effects are related to the suppression of intestinal motricity and the potentiation of the MSH activity.

3.2.3 - Effect of functional dairy peptides on defence mechanisms of the body.

The role of functional peptides in this field is a very promising approach which was rather recently observed; it concerns both the antimicrobial effect (inhibition of pathogenic bacteria) and the immunomodulator effect.

Many factors present in milk, and especially in the colostrum, help the new-born baby, whose immune system is immature, to fight against viral and bacterial infections (Spik, 1988).

In addition to these defence factors from protein origin, the colostrum and human milk contain high quantities of leucocytes, of which 80 to 90 % are the macrophages and 10 to 20 % the T lymphocytes, which manufacture interferon, and B lymphocytes which cross the intestinal mucous membrane to secrete then immunoglobulins (Pitt *et al.*, 1974; Lestradet 1988).

Recently, dairy peptides with an antimicrobial effect were discovered. For instance, the lactoferricin (a lactoferrin sequence), the casocidin I (sequence 165-203 of α S2 casein and the isracidin (fragment of α S1 casein) inhibit *in vitro* the pathogenic strain growth (Tomita *et al.*, 1994; Zucht *et al.*, 1995).

In addition, several functional peptides, with immunomodulator effects, were isolated. Fractions 54-59 of the human β casein and 51-53 of the α -lactalbumin stimulate the phagocytosis of the red blood cells of sheep by the peritoneal macrophages of mouse (Parker *et al.*, 1984). Fragments of α -lactalbumin and bovine κ casein stimulate *in vitro* the human lymphocytic proliferation (Kayser & Meisel, 1996).

3.2.4 - Anti-hypertensive activity of functional peptides

A state of shock, an haemorrhage or any other reason cause blood pressure decrease resulting in renin secretion by the kidneys. This renin will act on a plasmatic protein manufactured by the liver and will release an inactive decapeptide: the angiotensin I. Under the ACE (Angiotensin Converting Enzyme) action this peptide is then hydrolysed and gives rise to a vasoconstrictor octapeptide: the angiotensin II. The key role of the ACE, its localisation in plasma and in many tissues like lungs and intestinal cells (Stevens *et al.*, 1988) justify that it was selected as privileged target of the therapeutic agents likely to fight against hypertension. The first identified active molecules were peptides present in a Brazilian snake venom (Ferreira *et al.*, 1970). These peptides inhibit the ACE action by blocking its active site. They contain a particular amino acid sequence including one proline in C-terminal position.

Due to the high proline residue content of caseins, Maruyama *et al.*, (1982 and 1985) began to search for such inhibitors in tryptic hydrolysates of bovine casein. For instance, they showed α S and β casein sequences capable of inhibiting the ACE *in vitro* (Maruyama *et al.*, 1985 and 1987) by blocking its active site. Afterwards, peptides from human β casein sequence (Kohmura *et al.*, 1989) and from β -lactoglobuline and α -lactalbumin enzymatic hydrolysate (Mullally *et al.*, 1997) were detected with the same inhibiting function.

In other studies, a blood pressure-lowering effect was observed but its mechanism was not studied. For instance, peptides from milk fermented by *L. helveticus* ingested by hypertensive rats, lowered their blood pressure (Yamamoto *et coll.*, 1994). The same was observed with hypertensive rats which received a single 5-ml/kg dose of milk fermented by *L. helveticus* and *S. cerevisiae* (Nakamura *et al.*, 1995); in addition, anti-hypertensive peptides of this milk were

found in the rat aorta (Masuda *et al.*, 1996).

In human, 95 ml of such fermented milk were given daily to 36 hypertensive subjects for 8 weeks and the same results were obtained: a significant lowering of systolic blood pressure compared to control group receiving an acidified milk as placebo. In this study, both groups of subjects were receiving in addition a medical treatment (Hata *et al.*, 1996).

3.2.5 - Anti-thrombotic activity of functional peptides

The intravascular coagulations represent one of the most frequent causes of the population mortality in the industrialised countries (Zucker, 1980).

The mechanisms involved in the formation of venous or arterial thrombi are nearly identical to those involved in the haemostasis.

During an haemorrhage, the thromboxane A₂ highly stimulates the platelet aggregation to the collagen fibres contained in the subendothelium. The resulting plasma prothrombin is then transformed into thrombin leading to ADP secretion thanks to the granules present at the platelet surface, which not only stimulates their aggregation but induced also the manifestation of specific fibrinogen receptors at membrane level (Zucker, 1980). The platelets then fix fibrinogen as well as other proteins, such as the fibronectin and the von Willebrand's factor.

This protein binding, called adhesine, takes place at the level of glycoprotein receptors – GPIIb-GPIIIa – which are found on the activated platelet surface (Plow, 1985).

The fibrinogen peptide sequences involved in the interaction with the GPIIb-GPIIIa receptors were identified. This is the tetrapeptide RGD/X (fragment 572-575) of the chain a and the sequence 400-411 of the chain g of fibrinogen (Kloczewiak *et al.*, 1984; Shebuski *et al.*, 1989). *In vitro* and *in vivo* tests carried out showed that synthetic analogues of these sequences could inhibit platelet aggregation and thus thrombus formation (Cadroy and Al 1989; Shebuski *et al.*, 1989).

The similarity of blood and milk coagulation phenomena led Jollès *et al.* (1986) then Drouet *et al.* (1990) to search for the existence of inhibiting sequences within milk proteins.

Two sequences similar to those contained in the fibrinogen molecule were identified. This is on the one hand an analogue of the fibrinogen tetrapeptide RDD/X located in the domain 39-42 of the human lactotransferrin. The tests carried out after chemical synthesis of this fragment, showed that this sequence has an *in vitro* anti-platelet activity and an *in vivo* anti-thrombotic activity (rat and guinea-pig). In addition, a similar domain to the fragment 400-411 of the fibrinogen g was identified in domain 106-116 of bovine k casein. This sequence can inhibit *in*

vitro the fibrinogen fixation and the platelet aggregation.

In the same research field, two anti-thrombotic peptides, the human and bovine caseinoglycopeptide, were found in the plasma of human newborn babies, fed either with the mother's milk or with infantile milk based on cow's milk (Chabance *et al.*, 1995). Their content likely to be active on a physiological level (10 to 20 µmol/ml) suggests that these peptides are released from milk proteins during digestion.

A more recent work from the same team (1998) showed that, for the first time, potentially active peptides with anti-thrombotic sequences were found in the stomach, then in the blood of adults having ingested 500 ml of milk or yoghurt. This observation does confirm that in adult rather long peptides can cross the intestinal barrier. Moreover, this peptide content was higher after yoghurt ingestion than after milk ingestion, thus confirming the assumption according to which fermentation plays a significant role in the formation of functional milk peptides.

3.2.6 - Functional peptide protective activity against stress

Recent preclinical studies, carried out at the Research Centre ETAP-Ethologie Appliquée in Nancy (1998 and 1999), at the "Laboratoire de Neurobiologie de l'Apprentissage" of the University of Rouen (Prs J CASTON and R. LALONDE, 1999) and at the "Institut Technique du Porc" in Le Rheu (1999) have showed a significant anxiolytic-like activity of the milk protein hydrolysate (ING 911) in the laboratory rodents (conditioned defensive burying test in rat and elevated plus maze test in mouse) and in the pig. Besides, no side effects like sedation (Irwin test in rat), memory disorders (social memory test in rat) and addiction to the product ING 911 (conditioned preferential site test in rat) were observed by the ETAP team.

4 - MAIN OBJECTIVE

The main objective of this study was to evaluate, through a psychological test (Stroop test of colour conflict) and a physical test (test of the hand immersion in cold water) the protective properties against stress of a milk protein hydrolysate by measuring blood pressure and heart rate. The secondary objective was to evaluate the stress-responding variations of plasma ACTH and cortisol contents.

The research was without direct individual benefit.

5 - METHODOLOGY

5.1 - Experimental programme

- This was a double blind trial versus placebo.
- Two parallel groups comprising 21 male subjects.
- Conditions of subject reception: volunteers were recruited by the CIC for clinical and serological examinations. On the study day, the subject was accommodated at 7.30 AM in a room of the CIC for a four-hour period.
- Products given: the ING 911 product is manufactured by INGREDIA Company in St Pol-sur-Ternoise (62). It is presented as 200-mg capsules of powdered hydrolysate, known as food substrate without residues or toxicity risks (See appendix 3). This is a naturally *in vivo* synthesised product; no adverse reaction is expected (See appendix 2).
- The placebo is presented as 200-mg capsules of powdered skim milk.
- Administration: three doses before measurements, two 200-mg capsules in the morning and two capsules in the evening on the day before and a 3rd dose of 2 capsules on the study morning under control at the CIC.
- products are coded so that treated and placebo group are balanced by 7: 1-14, 15-28 and 29-42 (7 of the one and 7 of the other, every time). The products must be used preferably within 12 months from the manufacture date and stored in a fresh and dry room, at a temperature lower than 25°C and a relative humidity lower than 70%.

5.2 - Stress model rationale

The cold test (cold pressor test) was described first in 1932. It ranks, together with the forearm isometric contraction test (handgrip) or the dynamic stress tests (bicycle or treadmill), among physical provocative tests as opposed to mental tests like mental arithmetic, mirror drawing test, Stroop test of colour conflict (Tulen *et al.*, 1994; Grillot *et al.*, 1995), which are also triggering hypertension, but by originally central mechanisms. The immersion of the hand in cold water would product a cutaneous heat-sensory stimulation equivalent to a pain, which would cause a sympathetic nervous activation, as with any other stressing agent. The response to a mental constraint involves psychological mechanisms which are different depending on the subjects. From this point of view a psychotropic agent could act differently depending on the constraint (physical or mental) applied. This is a first justification to the choice of both tests (Stroop and cold test).

The second reason is that responses to stressing agents differ: one subject can react to a constraint more than to another whereas another subject reacts conversely to the precedent. Parati *et al.* (1988) failed to connect to the 24-hour pressure variability the responses to stressing agents (cold, handgrip, mental arithmetic, mirror). It is obviously not possible to determine a variability profile, basically complex and multiple-factor dependent, from a response to a single stressing agent.

However, this response involves autonomous nervous processes interesting to be studied. A particular effect, limited to a test, could be detected by diversifying the tests.

Thirdly, their good reproducibility inclines us to choose these tests preferably to the many others. The Stroop test reproducibility was controlled (Tulen *et al.*, 1994; Mounier-Vehier *et al.*, 1995; Laude *et al.*, 1997; Elghozi *et al.*, 1997a, 1997b) and we have experienced the cold test reproducibility, already known from another source (Weise *et al.*, 1993; Girard *et al.*, 1993). The Tulen's work interest is to show the benzodiazepine-like effects (lorazepam) on cardiovascular variables at rest and during a Stroop test.

The vagotonic effect of the tested benzodiazepine is thus evidenced. Concerning the cold we use a work showing that a benzodiazepine, the midazolam, does not affect the cold perception when the hand is immersed in cold water (Zacny *et al.*, 1995). We don't already know the information about the benzodiazepine-like effect on the cold pressure response.

5.3 - Measurements

On the day before the test, the subject takes two 200-mg capsules of hydrolysate in the morning and two in the evening. On the study day, 90 to 120 minutes after having taken two new 200-mg capsules of hydrolysate, the volunteer is sitting down for the experiment time. After an explanation of what will be carried out and required, a digital sensor of a Finapres 2300 (Ohmeda, Maurepas) monitor is set up on the level of the second phalange of the left middle finger (for the right-handed persons). This hand will be kept at heart level. The right hand rests flat. The pressure signal analysis requires a high frequency digitalisation followed by a reprocessing intended for extracting the consecutive blood pressure (systolic and diastolic) and heart rate values. The first part of the recording starts with a five-minute test phase. Then the volunteer is asked to type with a finger of the dominant hand (right) on the coloured keys of the keyboard (blue, green, yellow or red) as series of four colour written with words of different colours are scrolling on the computer, while being stimulated by the experimenter.

Every error is recorded and indicated by a ringing. The test lasts for five minutes. At the end, a five-minute recovery phase is recorded.

Following the first test, the subject is resting for thirty minutes. The second test starts with a control pressure recording for five minutes. Then the left hand is immersed to the wrist in cold water at an average 2°C temperature. This nociceptive stimulation by cold lasts nearly five minutes during which the recording is continued (test) then the hand is withdrawn from cold water and the recording is continued for five minutes (recovery).

At the end of the second test, the digital sensor is withdrawn from the left hand and a 15-ml blood sample is taken in order to assay cortisol (RIA method cortisolemie) and peptide (peptide content by ELISA method).

The subject can go home without having to stay in observation and without any particular recommendation.

Variation of average levels of systolic and diastolic blood pressure and heart rate by spectral analysis is the main criterion.

6 - STATISTICAL ANALYSIS

6.1 – Population rationale

The number of necessary subjects to detect a 20 % effect of the average frequency oscillation of the systolic blood pressure, owing to the reproducibility of this measurement (Girard *et al.*, 1994) with a power = 0.8 and a 5 % first-order risk is of 42 subjects for a parallel group study, i.e. 21 subjects per group.

7 - SUBJECT SELECTION

7.1 - Ethical rationale

This project was subjected to the CCPPRB's approval of the "Necker-Enfants Malades" hospital. The clinical study complies with the declaration of Helsinki (revised version Hong-Kong, 1989), the European Community note of July 11, 1990 concerning the recommendations of "Good clinical practices for clinical trials within the European Community", as well as the 27 December 1990 decree which implements the amended law of December 20, 1988 relating to the protection of persons involved in biomedical research (Huriet Law). The subjects' informed consent is a prerequisite, mandatory for their participation in clinical studies. Before signature of the informed consent the volunteer was given an information paper (See appendix 1).

Voluntary subjects recruited by the "CIC Groupe Necker-Enfants Malades" will be informed by Dr N. BERESSI and Pr J.-L. BRESSON which will collect their informed consents.

7.2 - Main inclusion criteria

- caucasian male volunteers aged 18 to 35 years old after consent collection;
- normal clinical examination;
- normal electrocardiogram
- heart rate at rest between 60 and 80 bpm;
- HBP: SBP <140 mmHg and DBP <80 mmHg;
- body mass index < 25;
- body weight between 65 and 75 kg.

7.3 – Non-inclusion criteria

- dairy product allergy;
- Raynaud's syndrome;
- coronary artery disease;
- current therapy (psychotropic substances, anti-epileptic drugs, β -blocking, analgesics);
- significant consumption of fermented dairy products;
- night-work;
- alcohol consumption;
- nicotineism;
- positive HIV, HCV or HBV serology.

8 - RESULTS

* Note: Some subjects were withdrawn from statistical study because of technical failure of the recording equipment or vagal malaise expressed by the subject.

In Stroop test, four subjects were eliminated for the following reasons:

- N° 10: voluntary stop of the subject;
- N° 21: too slow breathing generating management problems;
- N° 31: irregular breathing and value instability;
- N° 37: too irregular recording.

In cold pressor test, eight subjects were eliminated for the following reasons:

- N° 9: malaise;
- N° 17: value instability (unusable);
- N° 20: malaise;
- N° 23: malaise;
- N° 32: malaise;
- N° 33: voluntary stop of the subject;
- N° 37: malaise;
- N° 40: value instability (unusable).

8.1 - Stroop test

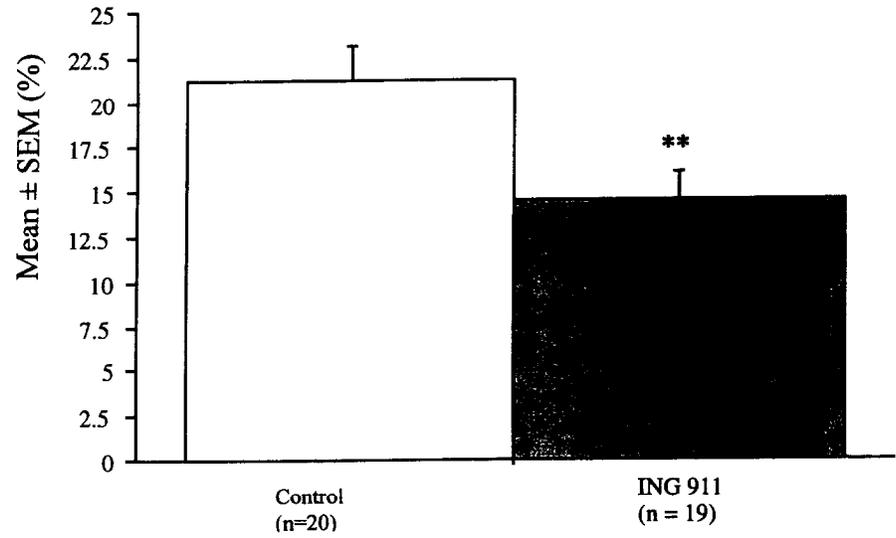
8.1.1 - Systolic blood pressure during Stroop test

Systolic blood pressures of control and ING 911 subjects increase significantly during Stroop test compared to rest values ($t = 13.03$; $p < 0.0001$ and $t = 10.11$; $p < 0.0001$, respectively). However, the deviation percentage of systolic blood pressure of control group subjects is significantly higher than that of the ING 911 group subjects (Tab. 2, fig. 1).

Table 2
Effect of ING 911 product on systolic blood pressure during Stroop test

Group	Systolic blood pressure at rest (mmHg)	Systolic blood pressure during stress (mmHg)	Deviation percentage
Control (n = 20)	135.02 ± 4.12	163.31 ± 4.81	+ 21.27 ± 1.78
ING 911 (n = 19)	138.40 ± 3.09	157.86 ± 2.68	+ 14.47 ± 1.59
Unpaired T test (bilat. prob.)			T = 2.84 p < 0.01

Figure 1
Effect of ING 911 product on the deviation percentage of systolic blood pressure (SBP) during
Stroop test
[SBP (stress) - SBP (rest)/ SBP (rest)] x 100



Unpaired T test (bilat. prob.) ** p < 0.01

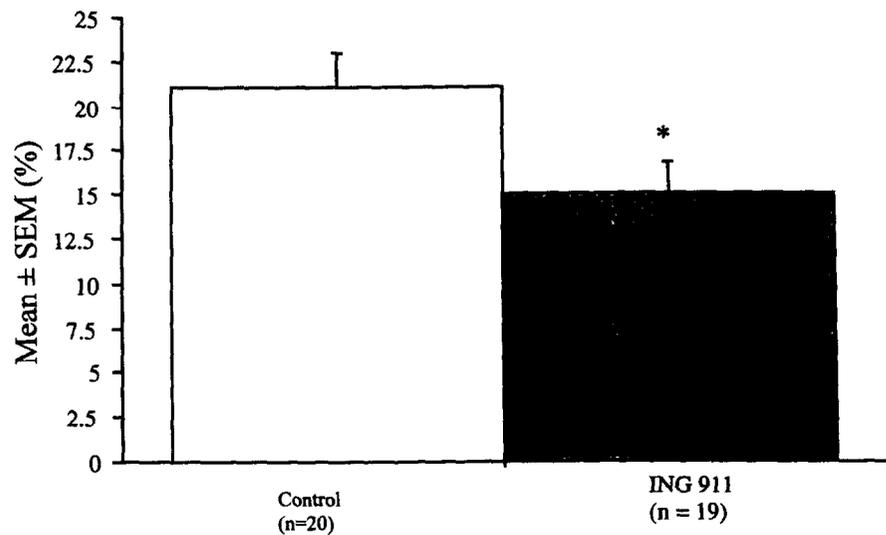
8.1.2 - Diastolic blood pressure during Stroop test

Diastolic blood pressures of control and ING 911 subjects increase significantly during Stroop test compared to rest values ($t = 11.69$; $p < 0.0001$ and $t = 8.56$; $p < 0.0001$, respectively). However, the deviation percentage of diastolic blood pressure of reference group subjects is significantly higher than that of the ING 911 group subjects (Tab. 3, fig. 2).

Table 3
Effect of ING 911 product on diastolic blood pressure during Stroop test

Group	Diastolic blood pressure at rest (mmHg)	Diastolic blood pressure during stress (mmHg)	Deviation percentage
Control (n = 20)	69.23 ± 1.89	83.79 ± 2.44	+ 21.14 ± 1.79
ING 911 (n = 19)	71.68 ± 2.05	82.31 ± 2.44	+ 15.04 ± 1.79
Unpaired T test (bilat. prob.)			t = 2.42 p < 0.05

Figure 2
Effect of ING 911 product on the evolution of diastolic blood pressure (DBP) during Stroop test
[DBP (stress) - DBP (rest)/ DBP (rest)] x 100



Unpaired T test (bilat. prob.) * $p < 0.05$

8.1.3 - Heart rate during Stroop test

Heart rates of control and ING 911 subjects increase significantly during Stroop test compared to rest values ($t = 4.48$; $p < 0.001$ and $t = 5.48$; $p < 0.001$, respectively). No significant difference is noted in the deviation percentage of heart rate of both group subjects (Tab. 4).

Table 4
Effect of ING 911 product on heart rate during Stroop test

Group	Heart rate at rest (bpm)	Heart rate during stress (bpm)	Deviation percentage
Control (n = 20)	65.89 ± 1.56	74.98 ± 2.81	+ 13.70 ± 2.80
ING 911 (n = 19)	66.38 ± 1.75	75.70 ± 2.49	+ 14.20 ± 2.60
Unpaired T test (bilat. prob.)			t = 0.14 N.S.

8.2 - Test of the hand in cold water (cold pressor test)

8.2.1 - Systolic blood pressure during the test of the hand in cold water

Systolic blood pressures of control and ING 911 subjects increase significantly during the test of the hand in cold water compared to rest values ($t = 8.93$; $p < 0.0001$ and $t = 8.35$; $p < 0.0001$, respectively). No significant difference is noted in the deviation percentage of systolic blood pressure of both group subjects (Tab. 5).

Table 5
Effect of ING 911 product on systolic blood pressure during the test of the hand in cold water

Group	Systolic blood pressure at rest (mmHg)	Systolic blood pressure during stress (mmHg)	Deviation percentage
Control (n = 16)	135.57 ± 4.46	171.89 ± 5.39	+ 27.60 ± 3.30
ING 911 (n = 16)	131.45 ± 4.61	160.45 ± 4.01	+ 23.20 ± 3.20
Unpaired T test (bilat. prob.)			t = 0.96 N.S.

8.2.2 - Diastolic blood pressure during the test of the hand in cold water

Diastolic blood pressures of control and ING 911 subjects increase significantly during the test of the hand in cold water compared to rest values ($t = 9.62$; $p < 0.0001$ and $t = 8.13$; $p < 0.0001$, respectively). However, the deviation percentage of diastolic blood pressure of control subjects tends to increase compared to that of ING 911 subjects (Tab. 6).

Table 6
Effect of ING 911 product on diastolic blood pressure during the test of the hand in cold water

Group	Diastolic blood pressure at rest (mmHg)	Diastolic blood pressure during stress (mmHg)	Deviation percentage
Control (n = 16)	70.11 ± 1.90	91.78 ± 2.90	+ 31.40 ± 3.30
ING 911 (n = 16)	70.14 ± 2.78	86.22 ± 3.45	+ 23.40 ± 3.20
Unpaired T test (bilat. prob.)			t = 1.71 p < 0.10

8.2.3 - Heart rate during the test of the hand in cold water

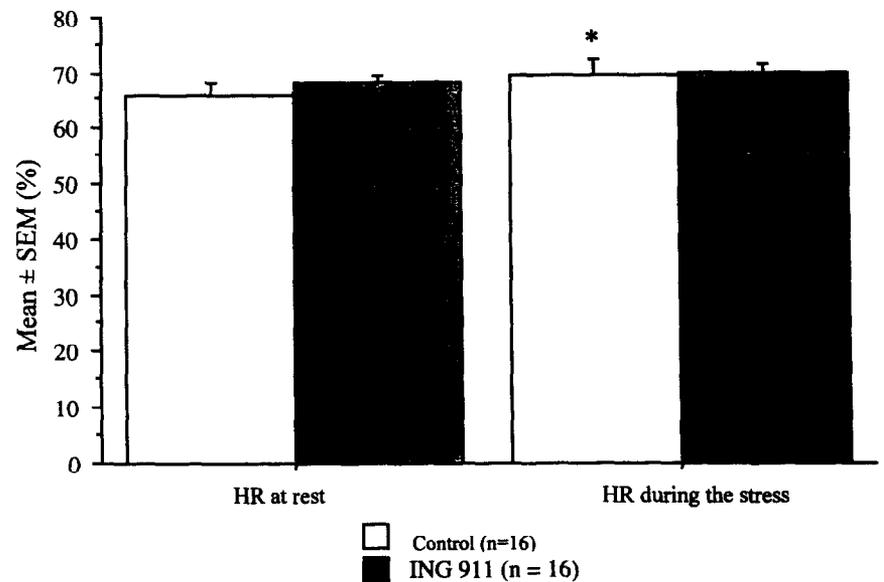
Whereas heart rate of ING 911 group subjects remains statistically stable during the test of the hand in cold water ($t = 1.13$; N.S.), that of control subjects increases significantly ($t = 2.75$; $p < 0.02$). No significant difference was noted in the deviation percentage of heart rate of both group subjects (Tab. 7; Fig. 3).

Table 7
Effect of ING 911 product on heart rate during the test of the hand in cold water

Group	Heart rate at rest (bpm)	Heart rate during stress (bpm)	Deviation percentage
Control (n = 16)	66.00 ± 2.35	69.39 ± 2.86*	+ 5.04 ± 1.74
ING 911 (n = 16)	68.03 ± 1.70	69.92 ± 1.87	+ 3.17 ± 2.57
Unpaired T test (bilat. prob.)			t = 0.60 N.S.

Paired T test: * $t = 2.75$; $p < 0.05$

Figure 3
Effect of ING 911 product on the evolution of heart rate (HR) during the test of the hand in cold water



Paired T test: * $p < 0.05$

8.3 - Blood pressure and heart rate evolution between the rest phase before Stroop test and the stress phase during the test of the hand in cold water

8.3.1 - Evolution of systolic blood pressure

Systolic blood pressures of control and ING 911 subjects increase significantly between the rest phase before Stroop test and the stress phase during the test of the hand in cold water ($t = 7.70$; $p < 0.0001$ and $t = 6.35$; $p < 0.0001$, respectively). However, the deviation percentage of systolic blood pressure of control group subjects is significantly higher than that of the ING 911 group subjects (Tab. 8, fig. 4).

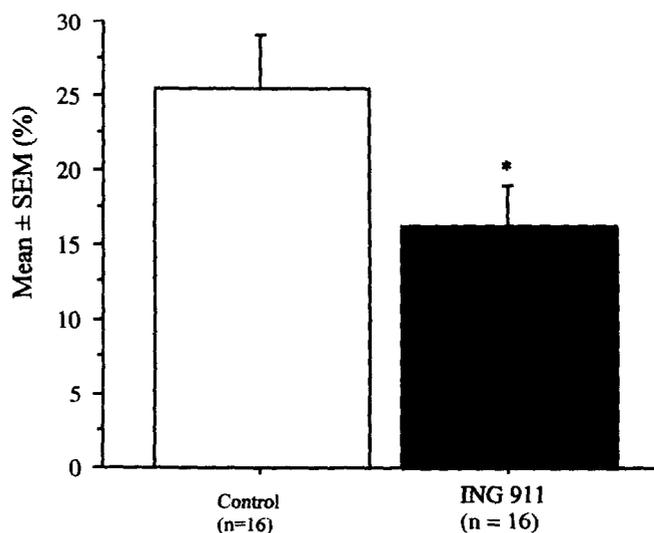
Table 8

Effect of ING 911 product on the evolution of systolic blood pressure between the rest phase before Stroop test and the stress phase during the test of the hand in cold water

Group	Systolic blood pressure at rest before Stroop test (mmHg)	Systolic blood pressure during stress of the hand in cold water (mmHg)	Deviation percentage
Control (n = 16)	137.44 ± 4.55	171.89 ± 5.39	+ 25.94 ± 3.40
ING 911 (n = 16)	138.32 ± 3.28	160.45 ± 4.01	+ 16.39 ± 2.70
Unpaired T test (bilat. Prob.)			t = 2.20 p < 0.05

Figure 4

Effect of ING 911 product on the deviation percentage of systolic blood pressure (SBP) between the rest phase before Stroop test and the stress phase during the test of the hand in cold water
 $[(SBP (Cold stress) - SBP (rest Stroop)) / SBP (rest Stroop)] \times 100$



Unpaired T test (bilat. prob.): * $p < 0.05$

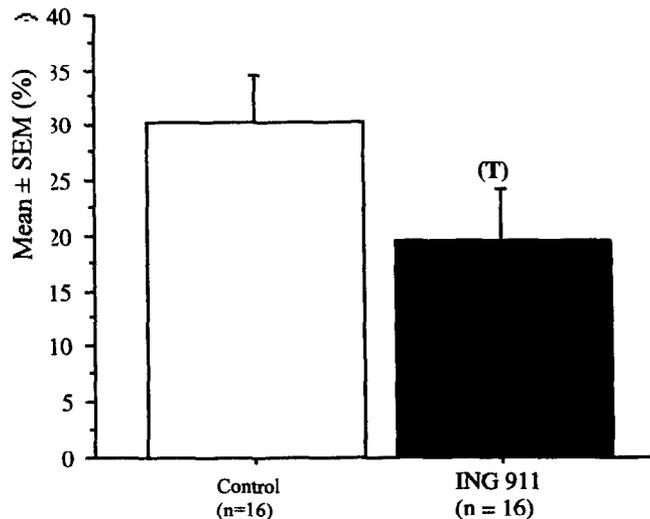
8.3.2 - Evolution of diastolic blood pressure

Diastolic blood pressures of control and ING 911 subjects increase significantly between the rest phase before Stroop test and the stress phase during the test of the hand in cold water ($t = 7.98$; $p < 0.0001$ and $t = 4.92$; $p < 0.0002$, respectively). However, the deviation percentage of diastolic blood pressure of control subjects tends to increase compared to that of ING 911 subjects (Tab. 9; Fig. 5).

Table 9
Effect of ING 911 product on the evolution of diastolic blood pressure between the rest phase before Stroop test and the stress phase during the test of the hand in cold water

Group	Diastolic blood pressure at rest before Stroop test (mmHg)	Diastolic blood pressure during stress of the hand in cold water (mmHg)	Deviation percentage
Control (n = 16)	70.76 ± 2.08	91.78 ± 2.90	+ 30.63 ± 4.11
ING 911 (n = 16)	72.45 ± 2.38	86.22 ± 3.45	+ 19.66 ± 4.46
Unpaired T test (bilat. prob.)			t = 1.81 p < 0.10

Figure 5
Effect of ING 911 product on the deviation percentage of diastolic blood pressure (DBP) between the rest phase before Stroop test and the stress phase during the test of the hand in cold water
[DBP (Cold stress) - DBP (rest Stroop)] / DBP (rest Stroop) x 100



Unpaired T test (bilat. prob.): (T) p < 0.10

8.3.3 - Evolution of heart rate

Whereas heart rate of ING 911 group subjects remains statistically stable between the rest phase before Stroop test and the stress phase during the test of the hand in cold water ($t = 0.92$; N.S.), that of control subjects increases significantly ($t = 2.36$; $p < 0.04$). No significant difference is noted in the deviation percentage of heart rate of both group subjects (Tab. 10).

Table 10
Effect of ING 911 product on the deviation percentage of heart rate (HR) between the rest phase before Stroop test and the stress phase during the test of the hand in cold water

Group	Heart rate at rest before the tests (bpm)	Heart rate during stress of the hand in cold water (bpm)	Deviation percentage
Control (n = 16)	65.60 ± 1.91	69.39 ± 2.86*	+ 5.59 ± 2.33
ING 911 (n = 16)	68.24 ± 1.61	69.92 ± 1.87	+ 2.94 ± 2.97
Unpaired T test (bilat. prob.)			t = 0.70 N.S.

Paired T Test: * T = 2.36; p < 0.05

8.4 - Cortisol and plasma ACTH content

8.4.1 - Plasma cortisol content

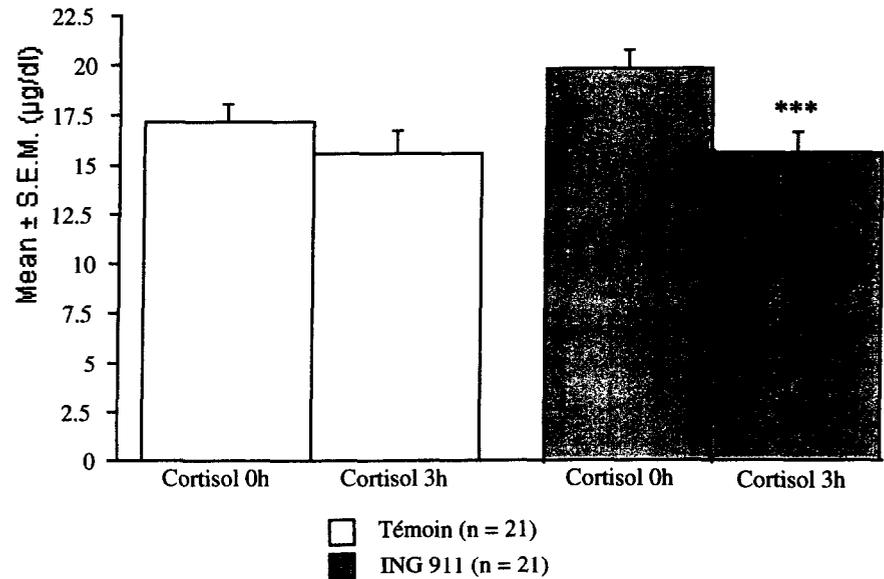
Whereas plasma cortisol content of control subjects remains statistically stable between the beginning and the end of the stress tests ($t = 1.35$; N.S.), that of ING 911 group subjects is significantly reduced ($t = 3.28$; $p < 0.005$). No significant difference was noted in the deviation percentage of plasma cortisol content of both group subjects (Tab. 11; Fig. 6).

Table 11
Effect of ING 911 product on the evolution of plasma cortisol content between the beginning and the end of stress tests

Group	Plasma cortisol content at rest before stress tests (µg/dl)	Plasma cortisol content after stress tests (µg/dl)	Deviation percentage
Control (n = 21)	17.17 ± 0.90	15.60 ± 1.08	- 5.88 ± 7.39
ING 911 (n = 21)	19.70 ± 0.92	15.50 ± 0.98***	- 17.89 ± 6.66
Unpaired T test (bilat. prob.)			t = 1.21 N.S.

Paired T test: *** T = 3.28; p < 0.005

Figure 6
Effect of ING 911 product on the evolution of plasma cortisol content between the beginning and the end of stress tests



*** p < 0.005 (Paired T test)

8.4.1 - Plasma ACTH content

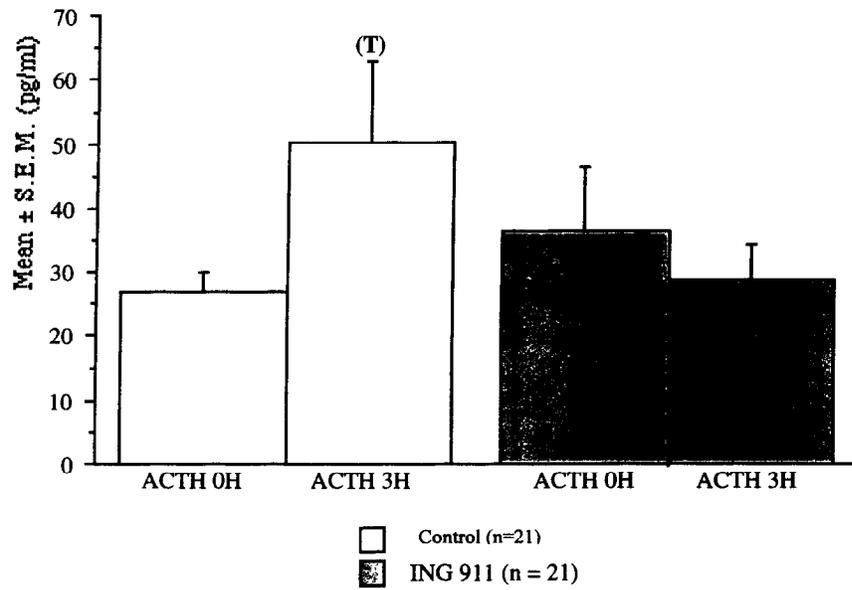
Whereas plasma ACTH content of control subjects tends to increase between the beginning and the end of stress tests (t = 1.79; p < 0.09), that of ING 911 group subjects is reduced, but not significantly (t = 0.67; N.S.). No significant difference was noted in the deviation percentage of plasma ACTH content of both group subjects (Tab. 12; Fig. 7).

Table 12
Effect of ING 911 product on the evolution of plasma ACTH content between the beginning and the end of stress tests

Group	Plasma ACTH content at rest before stress tests (pg/ml)	Plasma ACTH content after stress tests (pg/ml)	Deviation percentage
Control (n = 21)	26.86 ± 3.27	50.16 ± 12.84 (T)	+ 106.38 ± 56.30
ING 911 (n = 21)	35.59 ± 9.85	28.11 ± 5.75	- 24.86 ± 28.58
Unpaired T test (bilat. prob.)			t = 1.29 N.S.

Paired T test: (T) t = 1.79 ; p < 0.09.

Figure 7
Effect of ING 911 product on the evolution of plasma ACTH content
between the beginning and the end of stress tests



(T) Tendency; $p < 0.09$ (Paired T test).

9 - CONCLUSION

Blood Pressure and heart rate during Stroop test

Systolic and diastolic blood pressures of control and ING 911 subjects increase significantly during Stroop test compared to the rest values. However, deviation percentages of systolic and diastolic blood pressures of control subjects are significantly higher than those of ING 911 group subjects.

Heart rates of control and ING 911 subjects increase significantly during Stroop test compared to the rest values and no significant difference was noted in the deviation percentages of heart rate of both group subjects.

Blood pressure and heart rate during the test of the hand in cold water

Systolic and diastolic blood pressures of control and ING 911 subjects increase significantly during the test of the hand in cold water compared to the rest values before the test. If no significant difference is noted in the deviation percentage of systolic blood pressure of both group subjects, the deviation percentage of diastolic blood pressure of control subjects tends to increase compared to that of ING 911 subjects.

Whereas heart rate of ING 911 group subjects remains statistically stable during the test of the hand in cold water, that of control subjects increases significantly. No significant difference is noted in the deviation percentage of heart rate of both group subjects.

Evolution of blood pressure and heart rate between the rest phase before Stroop test and the stress phase during the test of the hand in cold water

Systolic and diastolic blood pressures of control and ING 911 subjects increase significantly between the rest phase before Stroop test and the stress phase during the test of the hand in cold water. However, the deviation percentage of systolic blood pressure of control subjects is significantly higher than that of ING 911 group subjects and their deviation percentage of diastolic blood pressure tends to increase compared to that of ING 911 subjects.

Whereas heart rate of ING 911 group subjects remains statistically stable between the rest phase before Stroop test and the stress phase during the test of the hand in cold water, that of control subjects increases significantly.

Plasma cortisol and ACTH content

Whereas plasma cortisol content of control subjects remains statistically stable between the beginning and the end of stress tests, that of ING 911 group subjects is significantly reduced. Plasma ACTH content of control subjects tends to increase between the beginning and the end of stress tests and that of ING 911 group subjects is reduced, but not significantly. No significant difference was noted in the deviation percentages of cortisol and plasma ACTH contents of both group subjects.

In Stroop test, situation of moderate stress, based on the value of systolic and diastolic blood pressures, the ING 911 product reduces significantly the stress of the treated subjects.

In the stress test of the hand in cold water, situation of stress associated with a strong pain, the ING 911 product does not show a significant activity on systolic and diastolic blood pressures. On the other hand it shows a heart rate-positive effect. Compared to blood pressure and heart rate values recorded at rest before Stroop test, the ING 911 product shows a protection from the stress during the test of the hand in cold water in the treated subjects.

Similarly, the ING 911 product significantly reduces plasma cortisol content between the beginning and the end of stress tests within the treated subjects.

11 - APPENDICES

APPENDIX No. 1

CONSENT FORM

AND

INFORMATION INTENDED TO VOLUNTEERS

**Study of milk protein effects on a light stress
in healthy volunteer**

Research without direct individual benefit

Investigator: Pr J.-L. BRESSON, Clinical Investigation Centre of Groupe Necker-Enfants Malades (CIC-NEM), Paris.

Sponsor: INGREDIA

Doctor....., investigator physician, gave me clear information on the sequence and objective of the research entitled: "Study of milk protein hydrolysate effects on a light stress in healthy volunteer", in which I was proposed to freely participate. He/she told me that the study lasts about thirty hours including 4 hours spent in the CIC of the "Groupe Hospitalier Necker-Enfants Malades".

He/she told me that I will have to take two capsules of product in the morning (8 am) and two in the evening (8 pm), on the day before the study. I will have eaten or drunk nothing before going to the Clinical Investigation Centre at 7.30 am on the study day where I will take again two capsules of product. I will rest in a room where I will remain for 90 to 120 minutes before the beginning of measurements.

Three hours after having taken the last capsule, a digital sensor will be set up on the level of the second phalange of the left middle finger to measure blood pressure and heart rate. Then I will be put through two tests: the test of colour conflict and the test of the hand in cold water.

At the end of the test session, the digital sensor will be withdrawn from the left hand and a 15-ml blood sample will be taken in order to assay cortisolometry and active peptide.

Following these measurements, I shall be given a lunch and I will leave the CIC without having to stay in observation and without any particular recommendation.

I was enabled to ask questions which seemed useful to me and I received clear answers. I was informed of legal provisions to protect people, in particular of the amended law of December 20, 1988 and the decree of application of September 27, 1990.

I freely and willingly accept to take part in the above described research. I am fully aware that I can withdraw my consent to participate in this research at any moment, whatever my reasons and without supporting any responsibility.

The relationships with my investigator physician will not be impaired by my research

withdrawal.

My consent does not discharge the investigator and the sponsor from their moral and legal liabilities and I preserve all my rights guaranteed by the law.

On September 13, 1999, the Paris-Necker CCPPRB gave a favourable opinion for the development of this research.

Made in....., on.....

Name, First name

Signature

(all the pages must be initialled)

Investigator's signature

CIC Groupe Hospitalier Necker-Enfants Malades, 149, rue de Sèvres 75743 Paris Cedex 15

Pavillon Maria Richard, Secteur Jaune, Porte 30

Pr Jean-Louis Bresson, Coordinator

Dr Nathalie BERESSI, Representative Doctor

INFORMATION INTENDED TO THE VOLUNTEERS

The purpose of the study in which you will take part is to evaluate the possible modifications of the physiological response to the stress associated with dairy protein consumption (hydrolysate). Indeed, actually it is known that small particular protein fragments (peptides) have biological actions and are capable of modulating certain biological phenomena. In particular, the product studied here showed a significant effect on the anxiety during tests carried out in the animal.

During the present study, these effects will be tested in human. During this study, the product will be given according to the following way: two 200-mg capsules of hydrolysate in the morning and two others in the evening, on the day before the test and two capsules on the next morning, 90 to 120 minutes before the stress test. Control subjects will be treated under the same conditions with capsules of powdered skim milk (inactive product). The products will be attributed randomly.

We require you to fully collaborate on this project and be present at the dates and hours fixed by the medical personnel of the Clinical Investigation Centre of the "Groupe Hospitalier Necker-Enfants Malades". You will not have to take any treatment in the 15 days before the first product administration and throughout the study. It is important that you indicate to the medical personnel if you are obliged to take some medicine.

To be allowed to take part in this study without direct individual benefit, you must be registered to a social security (your card will be required) and you should not be in period of exclusion from another therapeutic trial.

You commit yourselves taking no part in another therapeutic trial throughout this study. You will be allowed to take part in another therapeutic trial 2 weeks after the end of this one.

Your participation in this study is voluntary. If you wish to stop your participation in this study, you can do it at any moment by simply informing directly the doctor responsible for the study or via your attending practitioner. Your allowance for this study is 1,250 FF on the whole. To be entitled, you should not have reached the annual maximum limit of 25,000 FF including this study, which is fixed by the law for therapeutic test participation. In the event of study interruption for medical reason related to the protocol, all the allowance will be paid.

This study results will be transferred to INGREDIA. They could be published in a medical review and/or presented to the Administrative Authorities. In any case you will not be nominally identified.

If your health was impaired following your participation in the study, INGREDIA is committed compensating you within the framework of insurance, contracted for this purpose, insofar as it can be established that this injury is the direct consequence of the study product administration or of the performance conditions of the study.

The computer file used to carry out this research has received an authorization of the CNIL pursuant to articles 40-1 and following of the law "Informatique et Libertés". Your medical data, as well as the data about your life practices, which are necessary owing to the research purpose,

are the subject of a data-processing treatment and will only be transmitted to the sponsor or if necessary to the relevant medical authorities under conditions guaranteeing confidentiality. You can exert your access and correction rights near Pr J.-L. BRESSON.

The protocol of this trial (test n°99-07-11) was subjected to the CCPPRB's opinion (Consultative Committee of Protection of People involved in Biomedical Research) of the Groupe Hospitalier Necker-Enfants Malades, Paris. This committee gave a favourable opinion on September 13, 1999 for carrying out the study described in the protocol, which it considered to be in conformity with the provisions envisaged by law 88-1138 of 20/12/1988 relating to the protection of people involved in biomedical research.

APPENDIX No. 2

PROCESS TO OBTAIN HYDROLYSATE

The process to obtain hydrolysate comprises the following 5 phases:

- 1) The basic solution is enriched in Alpha S. casein;
- 2) The enriched solution is then hydrolysed using a pancreatic trypsin, the PTN 3.05 (Pancreatic Trypsin Novo, Novo Nordisk);
- 3) Enzyme inactivation;
- 4) Vacuum concentration of the solution;
- 5) Drying out in spray tower.

APPENDIX No. 3

VETERINARY CONTROL CERTIFICATE

PRODUCT: MILK PROTEIN HYDROLYSATE ING 911 (BATCH ING 99044)

We certify that goods intended for human consumption were manufactured by INGREDIA at St Pol-sur-Ternoise with the agreed number 62.767.30. This site is managed by Mr Jacques LANDA. Self-checkings, regularly controlled by our services, are carried out on the site of its laboratories by this Company.

The results presented hereafter were obtained within the framework of these self-checkings by the Quality Assurance department under the responsibility of Mr Jacques LANDA.

Veterinary Services of Arras (62)
Ministry of Agriculture

Doctor Catherine Rozo
Veterinary Inspector

ANALYSIS CERTIFICATE

INGREDIA Laboratories Saint Pol-sur-Ternoise (62)

Product name: ING 911

Product presentation: powder

Physicochemical analyses:

- Humidity: 5.3 %
- Fat content: < 0.1 %
- Nitrogen content: 74.9 %
- Mineral content: 13.1 %
- Lactose (by difference): 6.6 %

Bacteriological analyses:

- Total germs: 100/g.
- Coliform bacteria: Abs/g.
- E. Coli: Abs/g.
- Spores of clostridium SR: Abs/g (24 hours at 46°C).
- Staphylococcus aureus: Abs/g.
- Salmonellas: Abs/25 g
- Yeasts – Moulds: Abs/g.

APPENDIX No. 4

Table A

Variables and individual results at inclusion

Products	Subject No.	Age	Weight	Size	Body mass index	Systolic blood pressure	Diastolic blood pressure	Heart rate
P	1	20.0	77.2	1.86	22.4	132	68	72
T	2	30.0	75.0	1.79	23.4	115	67	60
T	3	33.0	84.0	1.89	23.5	124	67	56
T	4	30.0	70.0	1.77	22.5	127	69	82
P	5	23.0	62.0	1.74	20.5	127	54	60
P	6	21.0	64.0	1.74	21.1	126	68	60
T	7	28.0	74.0	1.73	24.7	110	68	68
P	8	21.0	64.0	1.74	21.1	130	70	80
P	9	25.0	67.0	1.82	20.2	139	82	68
P	10	37	82	1.73	27.4	135	92	68
T	11	22.0	84.0	1.81	25.6	120	60	64
T	12	27.0	73.0	1.74	24.1	130	75	68
P	13	20.0	65.0	1.80	20.1	124	72	60
T	14	25.0	62.0	1.75	20.2	110	60	72
P	15	28.0	67.5	1.73	22.6	120	60	60
T	16	29.0	80.0	1.81	24.4	130	70	64
P	17	23.0	70.0	1.73	23.4	125	69	68
T	18	21.0	67.0	1.76	21.6	139	74	60
P	19	26.0	69.0	1.82	20.8	137	74	68
P	20	25.0	65.0	1.73	21.7	121	75	64
T	21	32.0	72.0	1.70	24.9	110	70	80
P	22	35.0	67.0	1.80	20.7	130	70	60
T	23	28.0	64.0	1.77	20.4	117	61	75
P	24	21.0	74.7	1.79	23.3	118	65	66
P	25	30.0	78.0	1.77	24.9	130	70	60
T	26	34.0	87.0	1.89	24.4	135	78	62
T	27	22.0	69.0	1.81	21.1	123	64	72
T	28	26.0	70.0	1.79	21.8	132	63	60
P	29	21.0	70.0	1.76	22.6	138	57	80
T	30	28.0	68.0	1.76	22.0	135	75	60
T	31	19.0	60.0	1.82	18.1	127	76	68
P	32	30.0	74.0	1.82	22.3	130	70	76
T	33	30.0	62.0	1.65	22.8	110	60	76
P	34	22.0	70.5	1.73	23.6	130	70	76
T	35	21.0	64.0	1.72	21.6	140	70	72
P	36	21.0	73.0	1.85	21.3	120	60	67
P	37	21.0	74.7	1.84	22.1	135	75	76
P	38	21.0	63.5	1.73	21.2	130	80	72
T	39	23.0	72.0	1.81	22.0	135	68	72
T	40	23.0	76.0	1.77	24.3	114	62	60
P	41	25.0	69.0	1.75	22.5	130	75	62
T	42	26.0	75.0	1.84	22.2	132	85	67
T	43	22.0	72.5	1.79	22.6	105	68	60

P: Placebo (skim milk);

T: Treatment (ING 911 hydrolysate).

Table B
Individual results during Stroop test

Products	Subject No	Observation	Systolic blood pressure (Rest)	Systolic blood pressure (Stroop)	Diastolic blood pressure (Rest)	Diastolic blood pressure (Stroop)	Heart rate (Rest)	Heart rate (Stroop)
P	1		176.15	218.40	76.70	95.80	73.00	110.60
T	2		151.99	183.60	79.30	96.63	62.30	77.00
T	3		139.10	158.60	79.70	85.13	61.70	70.80
T	4		138.39	153.89	68.60	78.23	71.70	91.50
P	5		115.10	145.00	65.80	74.20	66.80	74.20
P	6		132.80	151.70	77.37	86.80	60.24	64.30
T	7		121.20	157.80	64.70	81.50	74.90	92.50
P	8		131.10	155.10	75.78	80.90	75.40	100.10
P	9		113.13	149.16	62.99	81.06	65.40	74.62
P	10	Withdrawal						
T	11		127.20	152.50	68.40	80.30	64.00	72.30
T	12		165.00	184.73	88.23	104.50	53.50	66.40
P	13		120.89	154.30	77.80	90.30	64.20	68.00
T	14		126.78	152.70	61.02	77.70	71.90	81.40
P	15		124.30	134.30	53.90	58.40	60.50	63.80
T	16		141.40	157.90	71.90	79.02	71.70	80.10
P	17		151.60	182.30	68.70	86.00	70.00	78.20
T	18		154.80	158.90	66.30	70.50	72.10	78.50
P	19		151.20	175.20	81.90	92.58	75.60	81.62
P	20		112.50	145.10	53.96	70.30	62.60	69.70
T	21	Stroop/UN						
P	22		129.34	157.10	68.60	84.20	69.70	80.70
T	23		160.10	178.50	68.90	81.30	61.60	65.97
P	24		141.32	169.80	68.20	85.60	50.60	60.30
P	25		167.60	189.80	81.10	103.10	63.40	72.30
T	26		146.90	171.56	71.30	88.10	75.50	81.60
T	27		129.35	156.70	61.90	70.31	62.10	67.40
T	28		141.00	156.55	74.50	78.70	78.40	91.10
P	29		139.70	154.30	69.90	97.70	66.20	77.70
T	30		121.10	147.40	61.50	71.70	69.80	71.60
T	31	Stroop/UN						
P	32		124.10	177.50	66.80	82.90	70.20	74.20
T	33		129.30	155.80	69.20	88.80	58.50	64.10
P	34		120.80	148.00	66.60	79.20	78.00	75.30
T	35		153.20	153.90	75.90	75.80	70.10	71.60
P	36		123.40	142.40	57.20	68.20	63.70	73.50
P	37	Stroop/UN						
P	38		119.60	143.20	64.90	76.40	65.60	63.40
T	39		136.60	151.00	88.40	98.40	67.30	80.60
T	40		127.00	143.10	64.50	72.80	49.40	57.70
P	41		154.00	189.60	66.00	85.50	53.50	61.50
T	42		148.50	171.30	89.00	105.70	62.90	83.00
T	43		122.60	136.30	67.90	75.30	64.20	58.60

P: Placebo (skim milk);

T: Treatment (ING 911 hydrolysate).

Withdrawal: subject voluntary stop;

Stroop/UN: Stroop /unusable Finapres recording.

Table C**Individual results during the test of the hand in cold water (CPT)**

Products	Subject No	Observation	Systolic blood pressure (Rest)	Systolic blood pressure (CPT)	Diastolic blood pressure (Rest)	Diastolic blood pressure (CPT)	Heart rate (Rest)	Heart rate (CPT)
P	1		172.60	234.90	73.47	114.10	75.60	86.90
T	2		154.26	199.00	84.15	113.85	60.00	66.10
T	3		125.99	164.60	78.43	95.51	59.20	60.00
T	4		151.60	176.10	77.50	94.10	74.10	75.90
P	5		132.00	168.30	68.90	88.90	65.50	60.60
P	6		116.80	169.90	77.50	98.10	60.40	59.20
T	7		95.80	138.90	53.90	75.10	74.20	82.20
P	8		135.60	160.59	73.00	91.90	78.60	92.20
P	9	CPT/M	119.29	130.50	67.58	76.04	66.14	65.24
P	10	Withdrawal						
T	11		126.40	167.70	66.00	84.80	65.10	67.80
T	12		169.88	179.50	88.97	97.30	60.10	72.60
P	13		117.90	160.40	71.30	102.90	61.00	65.70
T	14		117.60	146.50	51.30	69.10	72.90	76.20
P	15		128.70	171.70	60.80	86.50	57.30	61.40
T	16		142.74	163.40	75.20	90.98	73.40	71.89
P	17	CPT/UN						
T	18		152.30	165.20	66.60	76.30	66.20	70.00
P	19		134.60	176.30	71.90	88.30	83.20	84.30
P	20	CPT/M	114.86	153.70	57.29	78.62	57.03	73.39
T	21	Stroop/UN						
P	22		128.80	170.20	74.90	97.20	69.40	73.49
T	23	CPT/M	155.41	168.23	81.60	88.68	61.69	73.15
P	24		154.00	189.00	68.60	93.00	54.30	56.00
P	25		148.30	165.90	78.50	87.60	62.70	62.20
T	26		140.90	180.90	79.10	101.40	70.50	70.90
T	27		123.90	180.70	63.40	98.40	62.10	58.20
T	28		125.20	142.60	55.30	56.50	78.60	70.50
P	29		145.40	157.49	68.10	74.75	66.30	73.90
T	30		132.00	147.40	62.30	66.20	67.50	80.30
T	31	Stroop/UN						
P	32	CPT/M						
T	33	Withdrawal						
P	34		114.90	174.00	64.70	92.30	83.90	83.00
T	35		148.70	174.20	76.00	93.40	71.20	62.30
P	36		125.40	154.20	62.20	84.70	59.80	65.10
P	37	Stroop/UN CPT/M						
P	38		106.60	142.90	52.50	76.00	60.30	58.30
T	39		119.50	134.30	80.70	92.60	75.70	75.30
T	40	CPT/UN						
P	41		152.60	155.30	70.60	78.30	58.50	62.10
T	42		115.30	162.00	81.10	104.70	61.80	57.20
T	43		115.90	143.00	66.50	82.00	55.90	67.10

P: Placebo (skim milk);

T: Treatment (ING 911 hydrolysate).

Withdrawal: subject voluntary stop;

Stroop/UN: Stroop /unusable Finapres recording during the Stroop test.

CPT/UN: unusable Finapres recording during the test of the hand in cold water;

CPT/M: Malaise during the test of the hand in cold water (not taken into account in the statistical analysis).

Table D
Individual results: Plasma cortisol and ACTH

Products	SUBJECT No.	Observation	Cortisol/T0	Cortisol/ T+3 hours	ACTH/T0	ACTH/ T+3 hours
P	1		20.10	16.00	32.00	26.80
T	2		13.40	17.10	20.50	18.00
T	3		21.20	16.00	29.00	25.00
T	4		18.60	15.20	24.00	36.00
P	5		15.30	11.50	15.50	13.50
P	6		15.80	17.90	38.00	50.00
T	7		22.90	9.10	35.00	19.00
P	8		23.60	14.80	51.00	103.00
P	9		10.20	14.10	17.00	163.00
P	10	Withdrawal				
T	11		21.00	17.70	20.00	20.00
T	12		20.70	12.10	20.00	10.00
P	13		20.00	18.50	39.00	27.00
T	14		19.00	20.40	36.00	65.00
P	15		19.00	14.00	23.70	20.40
T	16		12.40	8.20	22.00	20.00
P	17		18.00	11.00	27.00	23.60
T	18		25.00	13.00	34.30	13.70
P	19		24.00	22.00	22.60	46.90
P	20		16.00	29.00	25.10	113.90
T	21		12.00	19.00	12.10	22.70
P	22		11.00	17.00	16.10	21.70
T	23		16.00	18.00	19.60	121.30
P	24		23.00	19.00	17.90	26.00
P	25		13.00	7.70	14.90	13.00
T	26		20.00	17.00	8.80	12.30
T	27		19.00	18.00	34.30	57.90
T	28		29.00	14.00	21.40	8.40
P	29		21.00	19.00	14.30	12.00
T	30		26.29	23.59	221.00	35.24
T	31		19.18	11.92	20.97	12.43
P	32		18.91	19.57	26.59	242.14
T	33		18.37	13.00	3.16	8.54
P	34		14.04	14.82	20.65	45.87
T	35		22.29	19.83	33.74	23.28
P	36		13.22	12.82	25.82	29.34
P	37	PSNR/M				
P	38		14.68	11.35	20.38	12.74
T	39		17.33	11.15	14.38	9.21
T	40		19.20	7.80	79.26	9.62
P	41		21.25	13.84	17.70	14.21
T	42		20.65	21.79	35.14	24.63
T	43		13.57	18.76	23.20	36.00

P: Placebo (skim milk);

T: Treatment (ING 911 hydrolysate).

Withdrawal: subject voluntary stop;

PSNR/M: blood sampling not carried out because of malaise experienced during the test

10 - BIBLIOGRAPHY

- Armbrecht H.J & Wasserman R.H. (1976): Enhancement of Ca⁺⁺ uptake by lactose in the rat small intestine. *J. Nutr.* 106, 1265-1271.
- Beg O.V. Von Bahr-Lindstrom H., Zaidi S.H., & Jornvall H. (1986): Characterization of a camel milk protein rich in proline identifies a new β -casein fragment. *Regul. Peptides* 15, 55-62.
- Ben Mansour A., Tomé D., Rautureau M., Bisalli A. & Desjeux J.-F (1988): Luminal anti-secretory effects of a casomorphin analogue on rabbit ileum treated with cholera toxin. *Pediatr. Res.* 24, 751-755.
- Brantl V., Teschemacher H., Henschen A., & Lottspeich F. (1979): Novel opioid peptides derived from casein. *HoppeSeyler's Z. Physiol. Chem.* 360, 1211-1216.
- Brulé G., & Lenoir J., (1987) : La coagulation du lait. In : *Le Fromage* (Eck A., ed.). Technique et Documentation, Lavoisier, Paris, pp. 1-20.
- Cadroy Y., Houghten R.A. & Hanson S.R (1989): RGDV peptide selectively inhibits platelet-dependent thrombus formation in vivo. Studies using a baboon model. *J. Clin. Invest.* 84, 939-944.
- Chabance B., Jollès P., Izquierdo C., Mazoyer E., Francoual C., Drouet L. & Fiat A.M (1995): Characterization of an antithrombotic peptide from κ -casein in newborn plasma after milk ingestion. *Brit. J. Nut.* 73, 582-590.
- Chabance B., Marteau P., Rambaud J.C, Migliore-Samour D., Boynard M., Perrotin P., Guillet R., Jollès P. & Fiat A.M (1998): Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* 80, 155-165.
- Chang K.J., Cuatrecasas P., Wei E.T. & Chang J.K. (1982): Analgesic activity of intracerebroventricular administration of morphiceptin and β -casomorphins : correlation with the morphine (μ) receptor binding affinity. *Life Sci.* 30, 1547-1551.
- Chiba H. & Yoshikawa M. (1986): Biologically functional peptides from food proteins : new opioid peptides from milk proteins. In : *Protein Tailoring for Food and Medical Uses* (Feeney R.E. & Wytaker J.R., eds). Marcel Dekker, New York, pp. 123-153.
- Coller B.S., Scudder L.E, Berger H.J. & Lulicci J.D. (1988): Inhibition of human platelet function *in vivo* with a monoclonal antibody: with observations on the newly dead as experimental subject. *Ann. Int. Med.* 109, 635-638.
- Daniel H., Vohwinkel H. & Rehner G. (1993): Effects of casein and β -casomorphin on gastrointestinal motility in rats. *J. Nutr.* 120, 252-257.
- Defilippi C., Gomez E., Charlin V. & Silva C. (1995): Inhibition of small intestinal motility by casein : a role of β -casomorphins ? *Nutr.* 11, 751-554.
- Drouet L., Bal dit Sollier C., Cisse M., Pignaud G., Mazoyer E., Fiat A.M., Jollès P. & Caen J.P., (1990): The antithrombotic effect of KRDS, a lactotransferrin peptide, compared with RGDS. *Nouv. Rev. Fr. Hematol.* 32, 59-62.
- Elghozi J.L., Laude D., Girard A. & Mounier-Vehier C. (1997a): Heart rate and blood pressure

spectra during mental stress. In: M. Di Rienzo *et al.* (Eds) *Frontiers of Blood Pressure and Heart Rate Analysis*, IOS Press, Amsterdam, pp.143-153.

Elghozi J.L., Laude D., Girard A., Consoli S. & Mounier-Vehier C. (1997b): Anger-coping types and cardiovascular reactivity to a mental stress. *Scripta Medica* 70, 157-161.

Ferreira S.H., Bartelt D.C., & Greene L.J. (1970): Isolation of bradykinin-potentiating peptides from *Bothrops jaraca* venom. *Biochemistry* 9, 2583-2593.

Geller N. & Pocock S.J. (1987): Interim analyses in randomized clinical trials: ramifications and guidelines for practitioners. *Biometrics* 43, 213-223.

Gerber H.W. & Jost R. (1986): Casein phosphopeptides: their effect on calcification of *in vitro* cultured embryonic rat bone. *Calcif. Tissue Int.* 38, 350-357.

Girard A., Weise F., Laude D. & Elghozi J.L. (1993) : Variabilité tensionnelle au cours de la réponse pressive au froid. *Arch. Mal. Coeur* 86, 1159-1162.

Girard A., Laude D. & Elghozi J.-L. (1994) : Reproductibilité des indices de variabilité tensionnelle à court terme. *Arch. Mal. Coeur* 87, 1079-1082, 1994.

Grillot M., Fauvel J.P., Cottet-Emard J.M., Laville M., Peyrin L., Pozet N. & Zech P. (1995): Spectral analysis of stress-induced change in blood pressure and heart rate in normotensive subjects. *J Cardiovasc Pharmacol* 25: 448-452.

Hambraeus L. (1985): Importance of milk proteins in human nutrition: physiological aspects. In : *Milk Proteins '84*. Pudoc, Wageningen, pp. 63-79.

Hata Y., Yamamoto M., Ohni M., Nakajima K., Nakamura Y. & Takano T. (1996): A placebo controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* 64, 767-771.

Hautefeuille M., Brantl V., Dumontier A.M. & Desjeux J.F. (1986): *In vitro* effects of β -casomorphins on ion transport in rabbit ileum. *Am. J. Physiol.: Gastrointest. Liver Physiol.* 250, G92-G97.

Henschen A., Lottspeich F., Brantl V. & Teschemacher H. (1979): Novel opioid peptides derived from casein. II. Structure of active components from bovine casein peptone. *Hoppe-Seyler's Z. Physiol. Chem.* 360, 1217-1224.

Jollès P., Levy-Toledano S., Fiat A.M., Soria C., Gillessen D., Thomaidis A., Dunn F.W. & Caen J.P. (1986): Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from k-casein on platelet function. *Eur. J. Biochem.* 158, 379-382.

Kayser H. & Meisel H. (1996): Stimulation of human peripheral blood lymphocytes by bioactive peptides derived from bovine milk proteins. *FEBS Letters* 383, 18-20.

Klee W.A., Zioudrou C. & Streaty R.A. (1978): Exorphin peptides with opioid activity isolated from wheat gluten and their possible role in the etiology of schizophrenia. In: *Endorphins in Mental Health Research* (Usdin E., Bunney W.E. & Kline N.S., eds). MacMillan, New York, pp. 209-218.

Kloczewiak M., Timmons S., Lukas T.J. & Hawiger J. (1984): platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to

the carboxy-terminal segment of the g-chain. *Biochemistry* 23, 1767-1774.

Kocian J. (1986): Lactose intolerance. Its complications. *Cs. Gastroenterol. Vyz.* 40, 252-257.

Kohmura M., Nio N., Kubo K., Minoshima Y., Munekata E. & Suzuki H. (1989): Inhibition of angiotensin-converting enzyme by synthetic peptides of human β -casein. *Agric. Biol. Chem.* 53, 2107-2114.

Laude D., Girard A., Consoli S., Mounier-Vehier C. & Elghozi J.L. (1997): Anger expression and cardiovascular reactivity to mental stress: a spectral analysis approach. *Clin. Exp. Hypertension* 19, 901-911.

Lee Y.S., Noguchi T. & Naito H. (1979 a): An enhanced intestinal absorption of calcium in the rat directly attributed to dietary casein. *Agric. Biol. Chem.* 43, 2009-2011.

Lee Y.S., Noguchi T. & Naito H. (1979 b): Abstracts of papers. Annu. Meeting of food and Nutr. Soc. Japan, Tokyo, May, 41.

Lestradet H. (1988) : Laits et immunité. *Cah. Nutr. Diet.* 23, 297-300.

Lorient D., Closs B. & Courthaudon J.L. (1991) : Connaissances nouvelles sur les propriétés fonctionnelles des protéines du lait et des dérivés. *Le lait*, 71, 141-171.

Mahé S., Tomé D., Dumontier A.M. & Desjeux J-F (1989): Absorption of intact β -casomorphins in rabbit ileum *in vitro*. *Reprod. Nutr. Develop.* 29, 725-732.

Maruyama S. & Suzuki H. (1982): A peptide inhibitor of Angiotensin-I Converting Enzyme in the tryptic hydrolysate of casein. *Agric. Biol. Chem.* 46, 1393-1394.

Maruyama S., Nagakami K., Tomizuka N. & Suzuki H.K. (1985): Angiotensin I converting inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentating activity on the uterus and the ileum of rats. *Agric. Biol. Chem.* 49, 1405-1409.

Maruyama S., Mitachi H., Tanaka H., Tomizuka N. & Suzuki H.K. (1987): Studies of active site and hypertensive activity of Angiotensin-I Converting Enzyme inhibitors derived from casein. *Agric. Biol. Chem.* 51, 1581-1586.

Masuda O., Nakamura Y. & Takano T. (1996): Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J. Nutr.* 126, 3063-3068.

Mendy F. (1984): Fragmentation des protéines laitières. Interview recueillie par J. Rajnchapel Messaï. *Biofutur* 24, 60-61.

Migliore-Samour D. & Jollès P. (1988): Casein, a prohormone with an immunomodulating role for the newborn ? *Experientia* 44, 188-193.

Mounier-Vehier C., Girard A., Consoli S., Laude D., Vacheron A. and Elghozi J.L. (1995): Cardiovascular reactivity to a new mental stress test: the maze test. *Clin. Auton. Res.* 5, 145-150.

Mullally M., Meisel H. & Fitzgerald R. (1997): Angiotensin-I-Converting enzyme inhibitory activities of gastric and pancreatic proteinase digests of whey proteins. *Int. Dairy J.* 7, 299-303.

Nakamura Y., Yamamoto N., Sakai K. & Takano T. (1995): Antihypertensive effect of sour milk

and peptides isolated from it that are inhibitors to Angiotensin I Converting Enzyme. *J. Dairy Sci.* 78, 1253-1257.

Nieter M. & Schatz H. (1981): Insolutropic action of endorphins and β -casomorphin, an opioid-like fragment of milk casein. *Acta Endocrinol.* 85, 249-252.

Parati G., Pomidossi G., Casadei R., Ravogli A., Gropelli A., Cesana B. & Mancina G. (1988): Comparison of the cardiovascular effects of different laboratory stressors and their relationship with blood pressure variability. *J. Hypertension* 6: 481-488.

Parker F., Migliore-Samour D., Floc'h F., Zerial A., Wemer G.H., Jollès J., Casaretto M., Zahn H. & Jollès P. (1984): Immunostimulating hexapeptide from human casein : aminoacid sequence, synthesis and biological properties. *Eur. J. Biochem.* 45, 677-682.

Paroli E. (1988): Opioid peptides from food (the exorphins). *Wld Rev. Nutr. Diet.* 55, 58-97.

Petrilli P., Picone D., Caporale C., Addeo F., Auricchio S. & Marino G. (1984): Does casomorphin have a functional role ? *FEBS Lett.* 169, 53-56.

Pitt J., Barlow D., Hend W.C. & Snatrilli T.V. (1974): Macrophages and the protective action of breast milk on necrotic enterocolitis. *Pediatr. Res.* 8, 384.

Plow E.F. (1985) : Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. *Blood.* 66 (3), 724-727.

Renner E. (1983): Milk and Dairy Products in Human Nutrition. *Volkswirtschaftlicher Verlag, München.*

Richardson B.C. & Mercier J.C. (1979): The primary structure of the ovine β -caseins. *Eur. J. Biochem.* 99, 285-297.

Sato R., Noguchi T. & Naito H. (1986): Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *J. Nutr. Sci. Vitaminol.* 32, 67-76.

Schusdziarra V., Holland A., Schick R., De la Fuente A., Lier M., Maier V., Brantl V. & Pfeiffer E.F. (1983a): Modulation of post-prandial insulin release by ingested opiate-like substances in dogs. *Diabetologia* 24, 113-116.

Schusdziarra V., Specht J., Schick R., De la Fuente A., Holland A., Brantl V. & Pfeiffer E.F. (1983b): Effect of β -casomorphins on somatostatin release in dogs. *Endocrinology* 112, 1948-1951.

Shebuski R.J., Berry D.E., Bernnett D.B., Romoff T., Storer B.L., Ali F. & Samanen J. (1989): Demonstration of Ac-Arg-Gly-Asp-Ser-NH₂ as an antiregulatory agent in the dog by intracoronary administration. *Throm. Haemostasis* 61, 183-188.

Spick G. (1988) : Rôle de la lactotransferrine dans la nutrition martiale du nourrisson. *Cah. Nut. Diet.* 23, 121-125.

Stevens B.R., Fernandez A., Kneer C., Cerda J.J., Phillips M.I. & Moodmard E.R. (1988): Human intestinal brush border Angiotensin Converting Enzyme activity and its inhibition by antihypertensive Ramipril. *Gastroenterology* 94, 942-947.

Sturner R.A. & Chang K.J. (1988): Opioid peptide content in infant formulas.

Pediatr. Res. 23, 4.

Teschemacher H. (1987): Casein derived opioid peptides : physiological significance ? *Adv. Biosci.* 65, 41-48.

Tomé D., Dumontier A.M., Hautefeuille M. & Desjeux J.F. (1987): Opiate activity and trans-epithelial passage of intact β -casomorphins in rabbit ileum. *Am. J. Physiol. Gastrointest. Liver Physiol.* 253, G737-G744.

Tomita M., Takase M., Bellamy W. & Shimamura S. (1994): A review : the active peptides of lactoferrin. *Acta Paed. Jap.* 36, 585-591.

Tulen J.H.M., Mulder G., Peplinkhuizen L., Man in't Veld A.J., van Steenis H.G. & Moleman P. (1994): Effects of lorazepam on cardiac vagal tone during rest and mental stress: assessment by means of spectral analysis. *Psychopharmacol* 114: 81-89.

Wasserman R.H. (1964): Lactose stimulated intestinal absorption of calcium: a theory. *Nature* 201, 997-999.

Weise F., Laude D., Girard A., Zitoun P., Siché J.P. & Elghozi J.L. (1993): Effects of the cold pressor test on short-term fluctuations of finger arterial blood pressure and heart rate in normal subjects. *Clin. Auton. Res.* 3, 303-310.

Yamamoto N., Akino A. & Takano T. (1994): Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Biosci. Biotech. Biochem.* 58, 776-778.

Zacny J.P., Coalson D., Young C., Klawns J., Rupani G., Thapar P., Choi M. & Apfelbaum J.L. (1995): A dose-response study of the effects of intravenous midazolam on cold pressor-induced pain. *Anesth. Analg.* 80: 521-525.

Zioudrou C., Streaty R.A. & Klee W.A (1979): Opioid peptides derived from food proteins: the exorphins. *J. Biol. Chem.* 254, 2446-2449.

Zucht H.D., Raida M., Dermann K., Magert H.J. & Forssmann W.G. (1995): Casocidin-I: a casein α S₂-derived peptide exhibits antibacterial activity. *FEBS Letters* 372, 185-188.

Zucker M. (1980) : Plaquettes sanguines et coagulation. *Pour Sci.* 34, 37-47.