Diet-induced modification of the acid-base balance in rats

Toxicological implications

Ben Lina

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Verandering van de zuur-base balans bij de rat onder invloed van de voeding
Toxicologische implicaties

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
maandag 15 november 2004 des middags te 12.45 uur

door

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Geboren op 4 januari 1954, te Akersloot

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ISBN 90-393-3843-4
Printed by Febodruk BV, Enschede, The Netherlands

The studies described in this thesis were performed at TNO Nutrition and Food Research (Zeist, the Netherlands) and were financially supported by the Ministry of Health, Welfare and Sport (The Hague, the Netherlands).
Publication of this thesis was financially supported by TNO Nutrition and Food Research.

Contents

Chapter 1

General Introduction

Diet and the acid-base balance

It has been known for a long time that the composition of the diet can strongly affect the acid-base balance of the body. Diet-induced changes in the acidity of the urine have long been of interest to physiologists because they demonstrate the kidney's role in the maintenance of homeostasis. Many diet manuals including 'acid forming,' 'base forming' and 'neutral' foods have been published (Langendorf, 1963; Dwyer et al., 1985) and it is well-established that vegetarian diets are alkalogenic in comparison to the acidifying typical western diets containing high levels of animal protein (Ball and Maugham, 1997). Also standard laboratory chow diets vary considerably in composition, and this variability is reflected in the urinary pH (Cohen, 1995). For instance, in rats AIN-76A diet and other diets containing casein as protein source produce an acidic urine (Cohen, 1995), whereas Altromin 1321 diet produces an alkaline urine (Clayson et al., 1995).

Although diet pH and diet acidogenicity are often confused, it is clear that the pH is not a suitable indicator of diet acidogenicity or alkalinogenicity. The balance of fixed (non-metabolizable) inorganic anions (Cl, P, S) and fixed cations (Na, K, Mg, Ca) indirectly refers to the metabolic fate of metabolizable anions and cations, and can be used as an indication of the acid or base forming properties of a diet (Patience and Wolynetz, 1990; Remer, 2000). When, for instance an organic salt of Na^+, like citrate, is ingested, the organic anion will be metabolized to CO_2; in the course of this transformation it is necessary that a proton be detached from the buffers of the extracellular fluid for each monovalent organic ion metabolized. It is as if base has been added to the extracellular fluid; the overall effect is that extracellular HCO_3^- concentration increases as well as the extracellular Na^+ concentration. For this reason sodium is termed a base-forming element. Conversely, when chloride is ingested as ammonium salt, the NH_4^+ group (against which the Cl^- is matched) must transfer a proton to the extracellular fluid buffers before it can be incorporated into urea; the net effect is as if HCl had been added to the extracellular fluid. Similarly any other metabolizable group attached to Cl^- (e.g. arginine chloride) must transfer a proton to yield a neutral product. Dietary chloride is therefore termed an acid forming element (Hills, 1973). According to the traditional dietary ash hypothesis, foods containing an excess of inorganic (or fixed) anions (Cl, P, S) over an excess of inorganic cations (Na, K, Mg, Ca) have acidifying properties (Dwyer et al., 1985). Conversely, an excess of inorganic cations over inorganic anions has alkalizing effects. In the animal feed industry, dietary

undetermined anion (dUA) is calculated as the balance of fixed anions and cations in the diet [dUA = (Na + K + 2Ca + 2Mg) - (Cl + 1.8P + $2SO_4$) mEq/kg] and is carefully controlled because imbalance can affect the growth rate of animals (Hulan et al., 1986; Patience and Wolynetz, 1990). The dUA, however, is only a rough estimate of the dietary acid load (Dwyer et al., 1985). One shortcoming is that intestinal absorption rates vary considerably among individual dietary components. For example, intestinal absorption of calcium is less than half of that of most inorganic anions. If $CaCl_2$ is ingested, an excess of chloride over calcium enters the blood. The portion of calcium not absorbed reacts with bicarbonate secreted by the pancreas and is excreted in the stool. Since sodium from the panceatic secretion is reabsorbed as NaCl (which is neither an acid nor a base) and not as $NaHCO_3$ (which is a base), a net loss of base equivalents occurs. Thus $CaCl_2$ (a 'neutral' salt, providing equimolar amounts of acid and base-forming elements) has an acidifying effect when given orally (Remer, 2000). Another shortcoming is that other substances are eaten that are not accounted for, such as organic acids from fruits which give an acid reaction *in vivo* because they are incompletely metabolized. In addition, the metabolism of proteins and other substances results in the generation of acids and bases. The liver, in particular, produces large amounts of hydrogen ions and alkali ions by oxidizing sulphur containing amino acids (methionine and cystine) and a number of organic anions (such as citrate and lactate), respectively. For the above reasons, the dUA gives only a rough indication of the acidity of foods. Recently, however, a physiologically based calculation model that corrects for intestinal absorption of minerals and sulphur containing protein and assumes a rate of urinary excretion of organic acids has been shown to accurately predict diet-induced generation of acidity or alkalinity (Remer and Manz, 1994; Remer, 2001).

After release into the circulation, hydrogen ions and alkali ions add to the diet-derived acid-base pool in the blood, which is buffered by the extracellular fluid buffers and by pulmonary mechanisms before the kidney excretes the ions to maintain acid-base balance (Guyton and Hall, 1996; Rose and Post, 2001). The extracellular and intracellular buffers are the body's primary defence against acid-base disturbances. The plasma buffers are protein, inorganic phosphates, and, most importantly, bicarbonate. The primary intracellular buffers are proteins, phosphates and, in the erythrocyte, haemoglobin. In addition, bone represents an important site of buffering in acute acidosis (Rose and Post, 2001). The bicarbonate system is the major blood buffer. The lungs maintain the blood pH within a narrow range by altering the rate at which CO_2 is

excreted in proportion to the actual alteration in the blood bicarbonate level (respiratory compensation) according to the Henderson-Haselbalch equation. This equation defines the pH of blood in terms of the interrelationship between the metabolic component (HCO_3^-) and the respiratory component (pCO_2):

$$pH = pK + \log \frac{\text{plasma bicarbonate}}{0.03 \times pCO_2}$$

where pK is the dissociation constant, and 0.03 is the solubility constant of carbonic acid.

Though the lungs can maintain or modify pH by changing the partial pressure of CO_2 (pCO_2) by adapting the ventilation rate, this process cannot cause any loss or gain in hydrogen ions. It is the kidney that controls acid-base conditions in the body by disposal of metabolically produced non-volatile acids (Remer 2000; Rose and Post, 2001).

Metabolic acidosis is characterized by acidemia that is due to a reduced plasma HCO_3^- concentration. As a consequence, renal net acid excretion is increased. Although able to excrete urine of varying pH, the kidney does so within certain fixed limits. The lowest urinary pH it can achieve is 4.4, thus the kidney is unable to excrete significant amounts of acid in the form of free hydrogen ions. Consequently, appropriate hydrogen ion acceptors must buffer most of the secreted hydrogen ions. The most important renal buffer systems are phosphate buffer (HPO_4^{2-} /$H_2PO_4^-$) which becomes concentrated in the tubular fluid, and ammonia buffer which is synthesized from glutamine in the tubular cell and secreted into the lumen. Combining excess hydrogen ions with these buffers results in the generation of bicarbonate that will help to replenish the bicarbonate lost from the extracellular fluid in acidosis. Metabolic alkalosis on the other hand is characterized by an alkaline extracellular pH that results from an elevation of the plasma HCO_3^- concentration. The response to a HCO_3^- load is to excrete the excess HCO_3^- in the urine, both by diminishing its rate of tubular reabsorption and by HCO_3^- excretion in the cortical collecting tubule.

Biological effects of 'poorly digestible carbohydrates'

More than half of the dry matter in the diet of man and many animal species consist of carbohydrates and about 60% of this carbohydrate fraction is composed of various starches. Most of the starches and of the other major dietary carbohydrates (sucrose, maltose) are easily digested in the small intestine by amylases and glycosidases and are subsequently absorbed. Native starches are contained in granules which are

(particularly in the case of raw potato starch) too large to allow the amylases to digest the granules completely during intestinal transit, but they are completely digestible after disintegration of the granules by heating. Many carbohydrates, however, are incompletely hydrolysed and/or incompletely absorbed in the small intestine, and are referred to as 'dietary fibre', or 'poorly digestible carbohydrates'. These substances belong to a chemically heterogeneous family of products, such as disaccharides (e.g. lactose, maltitol, isomalt), sugar alcohols (e.g. xylitol, sorbitol), resistant starch, various oligosaccharides, celluloses, and many other compounds (Hodkinson et al., 1982; De Groot, 1987; Til et al., 1986; Newberne, 1988; Brommage et al., 1993; Bär et al., 1995). Upon their passage through the intestinal tract, the unabsorbed parts of these carbohydrates reach the large intestine where they can be fermented by intestinal micro flora. The fermentation results in the production of gases, lactate and, most importantly, short chain fatty acids (SCFA), and is accompanied by a decrease in the pH of the luminal content. The major SCFAs that are produced by colonic fermentation are acetate, propionate and butyrate. Butyrate is a metabolic substrate for the colonocytes, and the liver completely removes absorbed butyrate and propionate, but 20-30% of portal venous concentrations of acetate may be found in peripheral blood. The acetate in peripheral blood is used as energy source by the muscles, and eventually metabolized into neutral products (Demigné et al., 1986; Fleming and Arce, 1986; Roberfroid, 1993). However, acetic acid, a weak acid with a pK of 4.8, is dissociated at physiological pH. It has been postulated that the continuous supply of SCFA from fermentation may act as an acid load on the body (De Groot, 1987).

Physiological, biochemical and toxicological changes observed in rats or mice after chronic feeding of high dietary levels of lactose or other low digestible carbohydrates are diarrhoea, caecal enlargement, transient aciduria, hypercalciuria, renal pelvic calcification and hyperplasia of the renal pelvic epithelium. Moreover, in long-term studies, an increased incidence of neoplastic changes has been observed in the adrenal medulla of rats fed diets containing high levels of sorbitol, xylitol, mannitol, lactitol or lactose (De Groot 1987; Roe, 1989). Also an increased incidence of Leydig cell tumours in the testes has been reported in rats fed lactose or lactitol (Sinkeldam et al., 1985; Roe, 1989).

De Groot (1987) postulated that the increased production of SCFA is of fundamental significance for the development of the adverse effects found in the kidneys, adrenals and testes. De Groot hypothesized that the increased urinary calcium levels, the calcification and hyperplasia of the epithelium of the renal pelvis and the neoplasia of

adrenals and testes were related to the permanently increased acid levels in the blood resulting from microbial fermentation of poorly digestible carbohydrates in the large intestine (De Groot, 1987).

To test this hypothesis a long-term feeding study was carried out in rats dealing with the effects of a dietary load of acid or base on the changes induced by lactose. This study is described in Chapter 2.

Toxicity and carcinogenicity of a dietary load of acid or base

The interpretation of the results obtained in the study described in Chapter 2 was seriously hampered by the lack of information on the short- and long-term effects of ingesting an acidogenic or an alkalogenic diet in rats. Therefore, short- and long-term toxicity studies, and a carcinogenicity study were performed in rats fed diets supplemented with $NaHCO_3$ as the alkalizing, NH_4Cl as the acidifying and KCl as the neutral salt. These studies are reported in Chapter 3.

Role of urinary pH and sodium and potassium ion concentrations in urinary bladder carcinogenesis in rats

Dietary administration of a wide variety of alkalizing salts such as the sodium salts of ascorbate, *o*-phenylphenate, phytic acid, glutamate, aspartate, citrate, erythorbate or bicarbonate has been shown to enhance urinary bladder carcinogenesis in rats, whereas the corresponding parent compounds (free acids) were less active or inactive (Fukushima et al., 1983[a]; Fukushima et al., 1984; Fukushima et al., 1986; De Groot et al., 1988; Shibata et al., 1989; Cohen et al., 1995; Kitamura et al., 1996). The clear difference in hyperplasia-inducing potency between the free acid and the sodium salt suggested that sodium ions play an important role in the promoting properties of these alkalizing salts. Enhancing effects of an alkalizing supplement (HCO_3^-) on the promotion of urinary bladder carcinogenesis by the free acid *o*-phenylphenol (Fuji et al., 1987) or ascorbic acid (Fukushima et al., 1988), and inhibiting effect of an acidifying supplement (NH_4Cl) on the promotion by the sodium salts of these acids have also been reported (Shibata et al., 1989). Apparently, the simultaneous occurrence of a high urinary pH and high urinary sodium concentration is crucial for enhancing urinary bladder carcinogenesis in rats.

In our Institute, it was found that feeding the flavour enhancer monosodium glutamate to rats had a marked alkalizing effect on the urine, and was associated with hyperplasia of the epithelium of the urinary bladder (De Groot et al., 1988). Variation of the urinary

pH, by the feeding of either purified or natural ingredient diet, modulated this urothelial response. Moreover, hyperplasia of the urothelium could also be induced by feeding the alkalizing salt $KHCO_3$, while the urothelial response to an alkalizing substance was prevented by reducing the urinary pH through simultaneous feeding of the acidifying salt NH_4Cl. These findings indicated that both sodium and potassium salts with alkalizing properties are capable of inducing hyperplasia of the urothelium in rats, and that the alkalizing properties rather than the cations are responsible for the hyperplasia observed, since NH_4Cl prevented the urothelial response. However, to further complicate the interplay between ions and acidity, Shibata et al. (1986) reported that NaCl, which elevates the urinary sodium ion concentration without affecting the pH, promoted urinary bladder carcinogenesis in rats, while Fukushima et al. (1987) found only a weak promoting activity of K_2CO_3 despite its clear induction of an alkaline urine. Also, it has been reported that an increase in urinary pH without a concomitant increase in sodium concentration, induced by the carbonic anhydrase inhibitor acetazolamide did not promote urinary bladder carcinogenesis (Fukushima et al., 1983[b]). Against this complex body of partially conflicting data, the role of low- and high urinary sodium or potassium ion concentrations in urinary bladder carcinogenesis was examined under both neutral and alkaline urinary conditions, by feeding rats equimolar amounts of NaCl, $NaHCO_3$, KCl or $KHCO_3$ following initiation with N-butyl-N-(4-hydroxybutyl) nitrosamine. This study is described in Chapter 4.

The study described in Chapter 4 showed that both potassium and sodium ions are strong mitogenic bladder tumour promotors under conditions of elevated urinary pH, and that both ions may exert weak promoting activity when the urine is neutral. Since it is widely accepted that mitogenic tumour promotors (inducing sustained hyperplasia) by themselves may induce tumours without prior initiation, the effects of dietary administration of alkalizing and neutral potassium salts ($KHCO_3$ and KCl, respectively) on the urinary bladder were examined in rats not treated with a bladder tumour initiator. These studies are described in Chapter 5.

Effect of urinary pH on tumour progression

Since long, systemic alkalosis has been associated with a variety of malignant neoplasms, and has been implicated as an enhancing factor in tumorigenesis. In contrast, a favourable influence of systemic acidosis on tumour control and regression has long been implicated (reviewed by Harguindey, 1982; Harguindey and Gragoe 1992; Harguindey et al., 1995). Several reports have indicated that metabolic acidosis

inhibits tumour growth of experimentally induced tumours. Verne and Roth (1963) reported inhibition of the carcinogenicity of subcutaneous implantations of estradiol benzoate in rabbits by acidification of drinking water. In another study it was found that lowering the systemic pH by NH_4Cl inhibited the growth of solid tumours and leukaemias in mice (Anghileri, 1975). Hydrochloric acid added to laboratory food has resulted in an increased rate of regression of subcutaneously implanted sarcoma 180 (Harguindey et al., 1979, 1995). In humans, some cases of complete spontaneous regression of breast, cervix and bladder tumours under circumstances of severe metabolic acidosis of different aetiologies have been reported (Harquindey et al., 1995). Spontaneous cell death has been observed within regions of solid tumours. Although the causes of spontaneous cell death within tumours are not known, the poorly developed vasculature may contribute to this process, by failing to provide adequate nutrients or to remove catabolytes (Vaupel et al., 1989). Due to the limited range of diffusion of oxygen within tissues, regions within solid tumours tend to become hypoxic. It is well known that the extracellular fluid in malignant tumours is acidic relative to that in normal tissue (Wike-Hooley et al., 1984; Jähde et al., 1990). Hypoxic regions of tumours are likely to be acidic because of dependence on glycolysis as a major source of metabolic energy, leading to accumulation of lactic acid, with consequent reduction of extracellular pH (Hochachka and Mommsen, 1983; Maidorn et al., 1993). It has been hypothesized that the combination of hypoxia and lower extracellular pH may be responsible for cell death (Rotin et al., 1986; Boyer et al., 1993). Also in tissue culture, low environmental pH has been shown to inhibit cell proliferation, survival and activity of tumour cells. Most mammalian cells will not proliferate in medium at a pH lower than about 6.6 (Wike-Hooley et al., 1984; Tannock and Rotin, 1989). Harquindey and Gragoe (1992) postulated that spontaneous regression of cancer may occur only in cases of severe systemic acidosis, a condition which is practically never observed because of the poor tolerance to uncorrected systemic acidosis. To investigate whether tumour progression can be inhibited by a chronically decreased environmental pH, we selected the urinary bladder as target organ because, in contrast to the pH of blood and interstitial fluid, the urinary pH can be significantly manipulated. We studied the effect of urinary pH on the progression of N-butyl-N-(4-hydroxybutyl) nitrosamine-initiated urinary bladder tumours in rats, using $NaHCO_3$ as the alkalizing and NH_4Cl as the acidifying dietary component (Chapter 6).

Chapter 1

Scope of the Thesis

The studies described in this thesis deal with toxicological implications of dietary modulation of the acid-base balance in rats. These studies can be distinguished in two groups. One group deals with the toxicity and carcinogenicity of acidogenic or alkalogenic diets (Chapters 2 and 3). The other group of studies examines the role of the urinary pH and urinary potassium and sodium ions in urinary bladder carcinogenesis (Chapters 4, 5 and 6).

In the first study (Chapter 2) we investigated whether the effects of poorly digestible carbohydrates (hypercalciuria, calcification and hyperplasia of the renal pelvic epithelium, and hyperplasia and tumours of the adrenals and testes) could be ascribed to acidosis resulting from the production of SCFA. For this purpose the effects of a dietary load of acid or base on lactose-induced changes were examined in a long-term feeding study.

Interpretation of the results of this long-term study, was hampered by the lack of systematic information on the effects of disturbances in the acid-base balance on parameters routinely used in toxicity studies. To fill in this gap in knowledge, short- and long-term toxicity studies and a carcinogenicity study were performed in rats fed acidogenic, alkalogenic or neutral diets. These studies are reported in Chapter 3.

As a sequel to earlier work conducted in our Institute (De Groot et al., 1988) in which sodium or potassium salts with alkalizing properties were found to induce hyperplasia of the epithelium of the urinary bladder, a 37-week initiation-promotion study was conducted, using N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) as tumour initiating agent (Chapter 4). In this study, the role of urinary sodium or potassium in promoting urinary bladder carcinogenesis under both neutral and alkaline urinary conditions was examined in rats which, after initiation with BBN, were fed diets containing equimolar amounts of NaCl, $NaHCO_3$, KCl or $KHCO_3$.

The study described in Chapter 4 showed that both potassium and sodium ions are strong mitogenic bladder tumour promotors under conditions of elevated urinary pH, and that both ions may exert weak tumour promoting activity when the urine is neutral. Subsequently, we examined whether these tumour promoters (inducing sustained hyperplasia) were capable of inducing urinary bladder tumours without prior initiation (Chapter 5). For this purpose rats were fed alkalizing or neutral potassium salts ($KHCO_3$ and KCl, respectively) for periods up to 30 months.

Systemic alkalosis has been postulated to enhance carcinogenesis, whereas it has been suggested that metabolic acidosis may inhibit tumour growth and may lead to tumour

regression. While the influence of systemic acidosis or alkalosis on the occurrence of spontaneous tumours was reported in Chapter 3, the study described in Chapter 6 addresses the effects of acidosis or alkalosis on the early and late progression of experimentally-induced tumours. The study focused on the urinary bladder because, in contrast to the systemic pH, the urinary pH can easily and strongly be manipulated. Urinary bladder tumours were initiated with BBN, and $NaHCO_3$ and NH_4Cl were used as alkalizing and acidifying salt, respectively. Finally, in Chapter 7, results obtained in the various studies are discussed and summarized, and conclusions are formulated.

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Chapter 2

Does the production of short chain fatty acids in the large intestine affect the acid-base balance in rats?

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Reprinted from Food and Chemical Toxicology 33, pp 1-14, 1995

Chapter 3

Toxicity and carcinogenicity of acidogenic or alkalogenic diets

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Reprinted from Food and Chemical Toxicology 42, pp 135-153, 2004

Chapter 4

Interplay of urinary sodium and potassium concentration with urinary pH in urinary bladder carcinogenesis in rats

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Reprinted from Carcinogenesis 10, pp 1733-1736, 1989

Chapter 5

Effects of long-term feeding of alkaline and neutral potassium salts on the urinary bladder of rats

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Reprinted from Carcinogenesis 15, pp 523-527, 1994

Chapter 6

Effect of urinary pH on urinary bladder tumour progression in rats

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Reprinted from Food and Chemical Toxicology 37, pp 1159-1166, 1999

Chapter 7

General Discussion

In this thesis, studies in rats are described that address the toxicological implications of dietary modulation of the acid-base balance. The studies are distinguished in two groups. The first group deals with the short- and long-term effects of disturbances of the acid-base balance (Chapters 2 and 3), the second group concerns the role of the urinary pH and urinary potassium and sodium ion concentrations in urinary bladder carcinogenesis (Chapters 4, 5 and 6).

Acidifying properties of lactose

In Chapter 2, it was hypothesised that short chain fatty acids (SCFA), the acid end products of carbohydrate fermentation, act as a systemic acid load, and that the acidity of SCFA plays a role in the induction of certain lactose effects. This hypothesis would be strongly supported if a dietary acid load would enhance, and a dietary base load would reduce these effects. For this purpose the effects of a high-lactose diet were compared with those of high-lactose diets supplemented with NH_4Cl or $KHCO_3$ in a chronic rat study. The study produced some evidence suggestive of SCFA acting as an acid load on the body. However, a number of findings discussed in Chapter 2 need reconsideration.

SCFA and systemic pH Lactose when given at high dietary levels to rats is incompletely digested and absorbed in the small intestine and is fermented by the bacterial flora in the large intestine, a feature which lactose shares with other low digestible, fermentable substances such as dietary fibre, modified starches and polyols (Hodkinson et al., 1982; Bär 1985; Newberne et al., 1988; Brommage et al., 1993). The fermentation results in the production of gases, lactate and, most importantly, SCFA. The major SCFA that are produced by fermentation in the large intestine are acetate, propionate and butyrate. Butyrate is almost completely used by the enterocytes as metabolic substrate, and hence acetate and propionate are the major SCFA that reach the circulation. The liver takes up these acids, and completely removes propionate and butyrate, but 20-30% of portal venous concentrations of acetate may be found in peripheral blood. Acetic acid is a weak acid with a pK of 4.8 and is dissociated at physiological pH. Although it is ultimately used as energy source by the muscles and metabolized into neutral products (Demigné et al., 1986; Fleming and Arce, 1986; Roberfroid, 1993), a constant supply of large quantities of acetate in peripheral blood could theoretically pose a threat to acid-base homeostasis. However, a number of

arguments plead against acetate acting as a systemic acid load. Firstly, it has been reported that the liver homeostatically regulates the systemic acetate concentration both in rat and man; the acetate uptake by the liver being higher in rats fed a high dietary fibre diet than in those fed a low dietary fibre diet (Hermann et al., 1985; Demigné et al., 1986; Roberfroid, 1993). Secondly, the production of endogenous acids increases with alkaline systemic pH and decreases with acidic pH. This modification of the rate of endogenous acid production can attenuate the effect of an acid challenge in physiologic and pathologic situations (Romeh and Tannen, 1986; Hood and Tannen, 1998). Finally, copious quantities of acid would alter the systemic pH. When such disturbance in systemic pH occurs, shifts in body buffers and ventilatory adjustment of the partial pressure of carbon dioxide promptly attenuate the change in pH, until it can be corrected by appropriate changes in renal acid excretion, but in most cases compensation is never completely effected (Beetham, 1982). In our study, however, the feeding of lactose did neither induce significant changes in blood pH, bicarbonate or base excess nor did it affect urinary pH or acid excretion. The transient decline in urinary pH after feeding lactose reported by de Groot (1986, 1987) is most probably due to the simultaneously occurring diarrhoea. Diarrhoea and soft stools result in a loss of electrolytes particularly sodium, potassium and bicarbonate (Lord and Newberne, 1990), which explains the temporary dip in urinary pH. In this respect it is striking that the effect disappeared as soon as the animals had adapted to lactose and diarrhoea was no longer observed. In summary, the above considerations argue against accumulation of SCFA at sufficient concentrations to depress systemic pH.

Mechanisms of urinary calcium excretion The observation that the lactose-induced increase in urinary calcium excretion was further elevated by dietary NH_4Cl whereas $KHCO_3$ diminished this effect was initially regarded as another indication that SCFA acted as an acid load on the body (Chapter 2). However, the effects of lactose, NH_4Cl and $KHCO_3$ on urinary calcium excretion result from separate mechanisms. Numerous studies have shown that lactose, and many other carbohydrates that are resistant to digestion in the small intestine but are fermented in the large intestine, increase the intestinal absorption of calcium and hence its urinary excretion (Hodkinson et al., 1982; Bär 1985; Newberne et al., 1988; Brommage et al., 1993; Younes et al., 1996). The mechanism by which polyols and non digestible but fermentable carbohydrates stimulate calcium absorption is not yet entirely elucidated, but several mechanisms have been proposed. Indigestible saccharides may directly affect epithelial tissue and

open tight junctions thereby promoting Ca absorption in both the small and large intestine (Mineo et al., 2004). In addition, many indirect mechanisms in which fermentable carbohydrates promote calcium absorption in the large intestine have been postulated (Brommage et al., 1993; Roberfroid and Slavin, 2000; Sakuma, 2001; Scholz-Ahrens and Schrezenmeir, 2002). One of these mechanisms is the stimulation of passive calcium transport by increasing its solubility via lowering the pH in the large intestine. Also SCFA may directly stimulate calcium uptake across the colon by formation of calcium salts of these acids; calcium propionate being more effective than calcium acetate probably due to its greater chain length and lipid solubility. Fermentable carbohydrates might indirectly induce cell growth and growth of the gut's absorptive area by increasing the microbial butyrate production, a substrate for cell growth and proliferation. Another way in which especially butyrate production might contribute to enhanced mineral absorption is by stimulating the active transcellular calcium transport via a higher expression of calcium binding protein and of 1,25 $[OH]_2$vit D receptor activity (Sakuma, 2001; Scholz-Ahrens and Schrezenmeir, 2002). Also, the osmotic effect resulting from fermentation and production of SCFA might promote calcium absorption, because the volume of fluid required to maintain isotonicity favours passive calcium transport by increasing distension and permeability of the intracellular junctions between enterocytes. Although the exact mechanisms have not yet been clarified, it is clear and generally accepted that the increased urinary calcium excretion in lactose-fed rats is due to enhanced calcium absorption.

The aetiology of the NH_4Cl-induced hypercalciuria is, however, different. Metabolic acidosis induces increased glomerular filtration and decreased tubular reabsorption of calcium (Lehman et al., 1967; Heusel et al., 1999). The further enhancement of lactose-induced urinary calcium excretion by dietary NH_4Cl as discussed in Chapter 2 and the hypercalciuria observed with dietary NH_4Cl in Chapter 3 are therefore ascribed to decreased renal reabsorption of calcium.

Finally, HCO_3^- depresses intestinal calcium absorption (Goulding et al.,1984) and elevates renal tubular calcium reabsorption (Peraino and Suki,1980), which explains the observation that $KHCO_3$ supplement diminished the urinary calcium concentration and excretion (Chapters 2 and 3). In conclusion, the different aetiology of increased urinary calcium excretion induced by lactose (increased gastrointestinal absorption) and NH_4Cl (decreased tubular reabsorption) does not support the hypothesis postulated in Chapter 2 that acidic end-products of carbohydrate fermentation act as a systemic acid load.

Renal mineralization Besides inappropriate Ca:P ratio and magnesium supply (Ritskes-Hoitinga et al., 1991), hypercalciuria appears to be a major etiological factor in the development of renal pelvic mineralization (Hodkinson et al., 1982; Bär, 1985; Roe, 1989; Lord and Newberne 1990). Surprisingly, both NH_4Cl and $KHCO_3$ ameliorated the lactose-induced mineralization of the renal pelvis, despite their opposite effects on urinary calcium output. The ingestion of urinary acidifiers such as NH_4Cl has been found to reduce nephrocalcinosis (Goulding and Malthus, 1970). The decreased renal mineralization in the NH_4Cl-supplemented lactose group in our study is probably due to the lower urinary pH in this group which may have increased the solubility and prevented the deposition of calcium salts, despite the higher urinary calcium concentration. Our observations are in line with those of Cohen et al. (1998) who showed in a recent 2-year rat bioassay that pelvic mineralization and hyperplasia induced by the feeding of sodiumascorbate was mitigated by co administration of NH_4Cl.

The amelioration by $KHCO_3$ of lactose-induced pelvic mineralization seems, however, surprising. Elevated urinary pH is considered a risk factor for the precipitation of calcium salts (Bär, 1985). In nephrocalcinogenic diets addition of $NaHCO_3$ has been shown to further increase nephrocalcinosis in the corticomedullary area (Forbes, 1966; Goulding and Malthus, 1970). However, in our study, $KHCO_3$ supplement diminished the lactose-induced elevation of urinary calcium concentration and excretion. Because hypercalciuria enhances the risk of mineral precipitation in the renal pelvis, it is tempting to speculate that the amelioration of pelvic mineralization in the $KHCO_3$ - supplemented lactose group was due to the lowered urinary calcium levels.

Comparison of lactose and NH_4Cl effects Finally, the different results obtained with lactose (Chapter 2), and with NH_4Cl (Chapter 3) do not support the hypothesis that lactose effects are the result of systemic acidification by SCFA. Firstly, the administration of NH_4Cl did not induce adrenomedullary hyperplasia and/or neoplasia . The increases in the incidence of these changes observed with lactose and with other compounds such as mannitol, xylitol, sorbitol, or lactitol (Bär, 1988; Lina et al., 1996; Lynch et al., 1996; Sinkeldam et al., 1992; Yoshida et al., 1995) can, therefore, not be ascribed to systemic acidification. There is evidence that increased calcium absorption due to the ingestion of low-digestible carbohydrates is a causal factor in the development of adrenomedullary proliferative disease in rats (Yoshida et al., 1995).

The first evidence came from a study showing that reduction of the dietary calcium level partly abolished a xylitol-induced increase in the incidence of medium-size and large pheochromocytomas (Bär, 1988). Further evidence for a role of calcium homeostasis in the aetiology of adrenomedullary proliferative disease of rats is provided by studies in which a decreased dietary calcium supply was associated with decreased adrenal catecholamine levels (Baksi & Hughes, 1984; Hagihara et al., 1990). Furthermore, it has been demonstrated that the ingestion of elevated doses of vitamin D stimulates the proliferation of adrenomedullary cells *in vivo* and results in the formation of hyperplastic nodules in the adrenals after treatment for 26-weeks (Tischler et al., 1996; Tischler et al., 1999).

A second difference between results obtained with lactose and with NH_4Cl is that the administration of NH_4Cl did not induce calcification and hyperplasia of the epithelium of the renal pelvis. Both lactose and NH_4Cl induced hypercalciuria, but as discussed above, the aetiology of this phenomenon was different (increased gastrointestinal absorption versus decreased tubular reabsorption). Both substances induced an increase in plasma alkaline phosphatase activity. The increased alkaline phosphatase levels induced by lactose and other poorly digestible substances such as isomalt, sorbitol, raw potato starch or carboxymethylcellulose, are probably of intestinal origin (Dupuis et al., 1977; Bär et al., 1995). For NH_4Cl the origin is not known, but alkaline phosphatase is found in many tissues, including bone, liver, intestine and kidney (Endres and Rude, 1999). The unspecificity of increased total plasma alkaline phosphatase activity does therefore not allow a conclusion with respect to similarities in working mechanism of lactose and NH_4Cl. Finally, NH_4Cl did not induce Leydig cell hyperplasia and neoplasia as noted in the study described in Chapter 2 and in a chronic feeding study in rats with 10% lactitol or 20% lactose (Sinkeldam et al., 1992).

Overall, the above considerations allow the conclusion that SCFA, the acid end products of carbohydrate fermentation in the intestines, do not act as an acid load on the body, and thus, that the effects of lactose are not due to a systemic acid load.

Lactose and bone status

As discussed above, lactose stimulates intestinal calcium absorption and urinary calcium excretion in rats when added to diets at concentrations of 10% or greater. Lactose-induced elevated calcium absorption also occurs in vitamin D deficient rats, leading to correction of most of the skeletal defects associated with vitamin D deficiency (Miller et al., 1988; Schaafsma et al., 1988). Increased calcium

concentration in the femur of rats has been reported after short term (2-5 wk) feeding of xylitol, sorbitol (Knuuttila et al., 1989) and oligosaccharides (Ohta et al., 1996). Ovariectomy-induced bone loss in rats (an accepted method to simulate human postmenopausal state) can be prevented by galactooligo-saccharides and oligofructose, the dose needed being inversely related to the dietary calcium level (Scholz-Ahrens and Schrezenmeir, 2002). The administration of 20% lactose in a diet adequate in mineral and vitamin levels to healthy rats in our chronic study did, however, not affect bone status (Chapter 2).

Acidification and osteoporosis

In Chapter 3, it has been postulated that the increased renal acid excretion and hypercalciuria in animals and man during experimental or pathological acidosis, reflects dissolution of alkaline bone salts to buffer excess acid. It has often been assumed that life-long ingestion of acidifying, high-protein diets might play a role in the causation of osteoporosis in post-menopausal women and the elderly. Because administration of bicarbonate has been shown to prevent the increase in urinary Ca and P excretion that accompanies the increase in acid production caused by a high-protein diet, it has been claimed that the administration of bicarbonate, or alkali from fruits and vegetables to healthy subjects, may improve the calcium balance and might have a beneficial effect on bone mineral mass (Lutz, 1984; Sebastian et al., 1994; Tucker et al., 2001). However, our life-span studies in rats did not reveal reduced or enhanced bone mineral content resulting from the administration of NH_4Cl or $KHCO_3$. The role of acid versus basic components of the diet in the long-term status of the human skeleton remains a topic of debate. In a recent longitudinal cohort study higher, rather than lower, protein intakes were associated with lower age-related bone loss (Tucker et al., 2001). Similar results were obtained in recently published studies showing either no differences in bone mineral density between vegetarians and omnivores, or significantly lower bone mass in groups consuming vegetable-based diets (reviewed by New, 2002). These findings contradict the hypothesis of acid-induced bone loss. Recently, Lemann et al. (2000) concluded that in patients with renal dysfunction, metabolic acidosis alone is not sufficient for the development of osteomalacia, and that additional pathophysiological mechanisms are required. The pathogenesis of osteoporosis is multi-factorial and is determined by a combination of genetic, endocrine, mechanical and nutritional factors (Heaney et al., 2000). The results obtained in our life-span studies in rats indicate that metabolic acidosis alone does not induce bone loss.

Urinary bladder proliferative changes; possible mechanisms

Sodium or potassium salts of a broad group of non-genotoxic anions, including ascorbate, glutamate, erythorbate, aspartate, citrate, succinate, bicarbonate and carbonate have been found to induce hyperplasia and tumours in rat urinary bladder epithelium (de Groot et al., 1988; Fukushima et al., 1986; Shibata et al., 1989a ; Otoshi et al., 1993; Lina et al., 1994; Cohen et al., 1995). All of these treatments resulted in alkalinization of the urine and elevation of the urinary sodium or potassium concentrations. Also, increased urinary pH, associated with increased sodium excretion, induced by the administration of carbonic anhydrase inhibitors, such as acetazolamide or 4-ethyl sulfonylnaphthalene-1-sulfonamide, is accompanied by urothelial proliferation (Clayson and Cooper, 1970; Durand-Gavagna et al., 1992; Clayson et al., 1995). For a number of these chemicals it has been shown that acidification of the urine abolishes the proliferative and tumorigenic activity (Clayson et al., 1995; Cohen, 1995). Sodium- and potassium chloride which do not increase the urinary pH, have been found to induce only weak proliferative changes (Shibata, 1986; Lina and Woutersen, 1989; Lina et al., 1994). The mechanism by which high urinary pH and sodium or potassium ion concentrations affect the urothelial response is not clear, but several hypotheses have been formulated (Cohen, 1995) and it may be that more than one of them pertain.

An increased urinary pH is a well known risk factor of urolithiasis, and mechanical irritation of the epithelium by urinary crystals or bladder stones may provide the stimulus for the hyperplastic response (Clayson et al., 1995). The formation of calcium phosphate containing precipitates seems to be essential in the carcinogencity of sodium saccharin and sodium ascorbate (Cohen et al., 1998). Studies with other sodium salts, however often failed to show the presence of uroliths. Also in our studies, urinary precipitates or calculi were not observed in the urinary bladder. In this respect it is significant that the urinary calcium and phosphate concentration were consistently lower in all groups fed potassium- or sodium salts than in controls (see Chapter 4.1). Moreover, probably because HCO_3^- decreases urinary calcium excretion (Goulding et al., 1984; Peraino and Suki, 1980), the lowest urinary calcium concentrations were found with sodium and/or potassium bicarbonate, which were most potent in promoting urinary bladder carcinogenesis. These lower calcium and phosphorus concentrations in the urine seem to rule out the occurrence of uroliths as a major risk factor.

There is some evidence that low calcium concentrations in the medium may increase

the proliferative rate and the morphological appearance of urothelial cells in both cell and organ culture (Reese and Friedman, 1978). The overall mean urinary calcium concentrations in the control-, 4% $KHCO_3^-$ and 3% KCl group in our chronic studies were approx. 2.0, 1.3 and 1.8 mMol/L for males and 3.6, 1.4 and 3.1 mMol/L for females, respectively. Historical control data from 40 studies in male and 22 studies in female rats showed overall means of 2.0 (lowest mean 1.2, highest mean 4.6) mMol/L for males and 4.3 (lowest mean 1.8, highest mean 8.3) mMol/L for females. Thus, the urinary calcium concentrations in the $KHCO_3^-$ group were lower than most of our historical control values that were not associated with urothelial alterations. It can not be excluded, therefore, that the lower urinary calcium concentration in our studies may have contributed to the proliferative effects in the $KHCO_3^-$group.

Polyuria, bladder distension and decreased urinary density (Anderson, 1988; Shioya et al., 1994) have been suggested as other possible causes of urinary bladder proliferative lesions. These factors may have played a role in our studies in which the urinary volume was increased and the density decreased. On the other hand, administration of diuretics such as furosemide, which produces a greater urinary volume than seen with sodium salts, is not associated with increased urothelial proliferation or tumorigenesis (Fukushima et al., 1983; Shibata et al., 1989b). Moreover, in our studies urinary volume and density were comparable in all groups with added salts, which would not explain the clear difference in urothelial response between bicarbonate-fed and chloride-fed rats.

A plausible hypothesis is that elevated urinary pH, and sodium or potassium ions directly affect the urothelium. Recent studies focus on the role of elevated intracellular pH (pH_i) in neoplastic transformation. Acidic cytoplasmic conditions are usually associated with a quiescent or dormant cellular state while an increase in pH_i often accompanies cellular activation (Frelin et al., 1988). An abnormally high pH_i is directly involved in entering cells into the S-phase cell cycle and also in maintaining them in a status of permanent proliferation (Harquindey, 2002). An abnormally high pH_i and micro-environmental extracellular alkalinization play an essential role in tumorigenic transformation, and malignant and transformed cells of many origins, from leukaemias to solid tumours, systematically show highly elevated pH_i (Harquindey, 2002). The Na^+/H^+ antiporter is the most recognized membrane-based ion transport mechanism in the control of intracellular acid-base homeostasis. Overstimulation of the Na^+/H^+ antiporter by a variety of carcinogenic stimuli is generally accepted to be an essential mechanism in the elevation of the intracellular pH (Harquindey, 2002). Na^+/H^+

exchange activity is dependent on both external sodium concentration and external pH of culture medium. Rates of Na^+ influx and of H^+ efflux via the antiporter increase as the external Na^+ concentration is raised, making the cell more alkaline. They also increase due to an increase in pH of the culture medium (Schuldiner and Rozengurt, 1982; Frelin et al., 1988; Tannock and Rotin 1989; Bishof et al., 1996). It has therefore been hypothesized that elevated pH and/or sodium concentrations in the urine stimulate sodium entry into the cell and an increase in pH_i thus resulting in DNA synthesis and cellular proliferation (Ito and Fukushima, 1989; Storer et al. 1996).

To my knowledge, there are no *in vivo* studies directly proving the above hypothesis for urinary bladder epithelium. However, Asamoto et al. (1992) demonstrated hyperpolarisation of the membrane potential (indicative of antiporter exchange activity) of the urinary bladder epithelium, along with an increase in urinary pH and sodium levels, in rats fed 5% sodium saccharin, a well known promotor of bladder carcinogenesis. In this study 1% NaCl failed to increase the membrane potential, but this level may have been relatively low. Therefore, it is plausible that in our studies, the consistently elevated urinary pH and/or sodium concentrations induced by $NaHCO_3$ or $KHCO_3$ have stimulated cell proliferation by enhancing Na^+/H^+ exchange activity. With NaCl and KCl, in the absence of increased urinary pH, sodium ions may have acted as direct stimulants for cell proliferation by elevating the intracellular pH through affecting the Na^+/H^+ antiporter, while potassium ions may have acted by stimulating Na^+/K^+-ATP-ase which also increases cell pH through an integrated strategy involving Na^+/H^+ stimulation (Fukushima, 1991; Cohen 1995; Storer et al., 1996; Harquindey 2002).

From our results and other studies (Shibata et al., 1986, 1992), it is, however, evident that Na^+ and K^+ show only weak proliferative activity in the absence of elevated urinary pH. Raising the urinary sodium concentration under conditions of an equally elevated urinary pH, has been found to enhance the development of carcinomas in the rat urinary bladder (Otoshi et al., 1993). On the other hand, some chemicals such as calcium bicarbonate or magnesium carbonate that raise the urinary pH, apparently without elevating sodium or potassium concentrations, do not have proliferative or tumorigenic effects on the urothelium (Fukushima et al , 1987). On the basis of the above considerations, it may be concluded that high urinary pH and elevated sodium or potassium levels exert a synergistic action on the urothelium.

Human data, and relevance to man of the urinary bladder lesions

Incidences and mortality rates for bladder cancer tend to be higher in developed countries than in developing countries. In the early 1990's most age-adjusted mortality rates for bladder cancer in Europe fell within the range of 1-3 (females) or 5-8 (males) per 100,000. In most of Western Europe, bladder cancer-related mortality has declined in generations born since 1940. Transitional cell carcinoma of the bladder accounts for about 95% of bladder cancer, although squamous cell carcinomas are more common in certain regions such as the Middle East. Cigarette smoking is recognized as the main cause of bladder cancer and accounts for about 50% of cases in most developed countries. A high risk of bladder carcinomas has been observed upon occupational exposure to some aromatic amines (IARC, 1999). A recent epidemiological study showed that there may be an appreciable risk of bladder cancer from occupational and personal use of hair dyes in the USA (Gago-Domingues et al., 2001, 2003). The relevance of these findings for the population in Europe is, however, presently unknown. Heavy consumption of phenacetin-containing analgesics was strongly associated with lower urinary tract and bladder carcinomas. Infectious agents and other diseases of the urinary tract which may cause chronic inflammation, have a major influence on bladder cancer risk. In northern Africa and the Middle East and other areas were Schistosoma haematobium infestation is endemic, there is a consistent relationship between (squamous cell) carcinomas and urinary schistosomiasis (IARC, 1999).

The urinary bladder and the stomach are the main sites within an intact individual that might be subject to significant long-term non-physiological environments. Epidemiological studies have shown that high dietary NaCl intake is closely associated with an elevated risk of gastric cancer development in Japan (Anderson, 1989), and the mitogenic activity of high Na^+ demonstrated both *in vitro* and in animal studies is probably important in this process (Brusick, 1987). Only few epidemiological studies, however, have addressed the issue of increased intake of salts and urinary bladder cancer risk. In a prospective study in US men, no correlation between dietary intake of sodium or potassium and bladder cancer risk was found (Michaud et al., 2000). Higher intake of dietary sodium was, however, associated with significantly increased risk of bladder cancer in a case-control study in the Western New York area (Vena et al., 1992). Thus, available epidemiologic data on salt intake and bladder cancer are inconclusive.

In extrapolating findings from rodent studies to humans, an evaluation is required of both the effects of dose and the effect of species extrapolation. Bladder responses to sodium- or potassium salts in rodents are observed only when they are administered at high concentrations in the diet, usually in the range of 1.0-7.5% (Cohen et al., 1995). In chronic studies in rats, these levels are equivalent to approx. 500 - 3750 mg salt/kg body weight/day. Data suggest that humans can survive on salt (NaCl) intakes that range from 0.46 g/day to 13.8 g/day (ILSI, 2003). For a 'standard' human (60 kg) this is between 8 - 230 mg/kg body weight/day. The relevance to man of this high-dose phenomenon in rats is therefore doubtful. The other important factor when extrapolating from rodents to humans is species specificity. There are marked variations in response to treatment between species as well as among strains within species. For instance, sodium ascorbate and the sodium salt of ortho-phenylphenol, which induce urothelial proliferation in rats, are completely without effect on the urinary bladder of mice (Cohen et al., 1995; Cohen, 1999). Following oral treatment with carbonic anhydrase inhibitors, which raise both urinary sodium concentration and pH, urothelial hyperplasia develops in rats and mice, but not in rabbits, dogs and monkeys (Durand-Gavagna et al., 1992). The pH, osmolarity and concentrations of various salts in the urine may be of major importance for the selective nature of the bladder response. In humans, urinary pH tends to be acidic, between 5.0 and 6.0, but is greatly influenced by diet. Values as low as 4.5 and as high as 8.5 can be achieved. Also sodium and potassium levels vary widely in humans depending on diet and on total water intake, and range from 1-300 and 10-150 mMol/l, respectively (Cohen, 1995). In rodents, urinary pH is usually between 5.5 and 7.5 (depending on the type of diet), extending as low as 5.0 and as high as 9.0. Sodium and potassium levels in rats range between 10-450 and 30-600 mMol/l, respectively and also the overall osmolarity of the urine in rodents (2100-3100 mOsmol/kg) is significantly higher than in humans (100-1000 mOsmol/kg) (Cohen, 1995; Anderson, 1989). In our studies, the urinary pH was generally between 6.5 and 7.5 in controls and between 8 and 8.5 in rats fed alkalizing salts. The urinary concentrations of sodium + potassium were about 300 mMol/l in controls, and about 500 mMol/l in rats treated with high levels of sodium and/or potassium salts. Taking into account that in humans the urinary pH generally tends to be acidic and that the concentrations of sodium and potassium are generally lower than those observed with alkalizing salts in our studies in rats, a persistent combination of elevated urinary pH and high sodium and/or potassium levels is unlikely in humans. Nevertheless, certain sub-populations consuming special

(vegetarian) diets might be especially at risk for developing urinary bladder cancer, because such diets are associated with elevated urinary pH. However, in six out of seven epidemiological (case-control or cohort) studies published between 1979 and 1994, a reduced risk of bladder cancer was found with increasing consumption of fruits and vegetables (reviewed by La-Vecchia and Negri, 1996).
In conclusion, the response of the bladder to high pH and high sodium or potassium levels depends on the species. The species specificity of the bladder response and the fact that it is a high-dose phenomenon makes its relevance to man at least doubtful.

Acid-base and tumour development

From the above discussion it is clear that high micro-environmental pH may contribute to development of urothelial tumours. In humans various oncogenetic situations of mucosal epithelia have been associated with local alkalosis. For instance, ureterosigmoidostomy-induced alkalosis and high sodium concentrations is associated with adenomas of the colon, betel quid tobacco with oropharyngeal tumours, and increased bicarbonate elimination with pancreas tumours (for review see Harquindey et al., 1995). Our findings therefore support the hypothesis that a chronically high microenvironmental pH induces the onset of mucosal malignancies. In Chapter 3, however, no indications were found that systemic alkalosis affected tumour incidences in tissues other than the urothelium. Hence, alkalosis may be a factor in tumorigenesis only in those epithelial surfaces where environmental pH can reach much higher values in comparison to the systemic pH.
Studies described in this thesis also addressed the question whether acidosis could favourably affect tumour development. The studies described in Chapters 2 and 3 allow the conclusion that systemic acidosis does not influence the occurrence of spontaneous tumours. We found no inhibitory effect on early or late progression of experimentally-induced urinary bladder tumours, despite the profound decrease in environmental pH that was induced (Chapter 6).
As a result of the observations of Warburg (1930) that tumour cells preferentially convert glucose and other substrates to lactic acid, it was assumed for many decades that cancer cells are acidic. The development of pH electrodes small enough to be inserted in living tissues led to the apparent confirmation of this assumption. It has, however, become clear that these quite large electrodes mainly measured the pH of the extracellular fluid rather than the intracellular pH (Griffiths, 1991). Due to novel techniques which are able to preferentially measure intracellular pH (pH_i) or

extracellular pH (pH_e) in malignancies it has been confirmed that, in contrast to the pH_e, the pH_i in tumours is not acidic but neutral to alkaline (Vaupel and Höckel, 2000; Harquindey 2002). In fact, the cytosolic pH of neoplastic cells has often been reported to be more alkaline than that of normal cells. This abnormally high pH_i has been suggested to be involved in entering the cells into the S-fase cell cycle and also in maintaining them in a status of permanent proliferation (reviewed by Harquindey 2002). A decreased pH_i on the other hand, may be associated with apoptosis (Shrode et al., 1997). Under the acidic extracellular conditions that occur in solid tumours (Wike-Hooley et al., 1984; Vaupel et al., 1989), regulation of pH_i plays an important role in maintaining the viability of tumour cells. Acid extrusion in most mammalian cells is carried out by membrane-based ion transport mechanisms. Principal membrane-based ion transport mechanisms known to contribute to the regulation of pH_i in many cell types are the Na^+/H^+ antiporter, Na^+/K^+ ATP-ase and the Na+-dependent HCO_3^-/Cl^- exchanger (Frelin et al., 1988; Tannock and Rotin, 1989; Harquindey, 2002). The Na^+/H^+ antiporter is probably the major mechanism in counteracting cytoplasmic acidification. If undisturbed by outside interferences, the acid extruding mechanisms in cancer cells are working at full power, to protect them against the damaging, acidic extracellular-intratumoral pH. The steady state abnormal high pH_i keeps cancer cells and tumours within an uninterrupted proliferative and anti-apoptotic state (Boyer et al., 1993; Harquindey, 2002).

It has been postulated that manipulation of extracellular or intracellular acidity may be exploited for tumour treatment. Inhibition of the Na^+/H^+ exchange through the plasma membrane with amiloride or its analogues has been reported to induce cell death *in vitro* as well as *in vivo* through intracellular acidification (Horvat et al., 1993; Luo and Tannock, 1994; Harquindey et al., 1995; Park et al., 1996; Harquindey, 2002). Interactions of hypoxia and acid extracellulair pH may contribute to cell death and necrosis in solid tumours (Tannock and Rotin, 1989, Harquindey, 2002), but can alternatively lead to malignant progression (Höckel and Vaupel 2001). Hyperthermia in tumour cells cultured in acid media has been shown to increase their thermal sensitivity (Harquindey et al., 1995). Also, the cytotoxic effects of hyperthermia are enhanced by a reduction in the pH_i through intracellular acidifiers such as amiloride (Boyer et al., 1993). The growth of implanted tumours could be inhibited by amplification of tissue acidosis through a combination of hyperlactacidaemia, hyperglycaemia en hyperthermia. Each individual treatment, however, had little effect (Mueller-Klieser et al., 1996). It has been postulated that spontaneous regression of cancer may occur only

in cases of severe systemic acidosis, a condition which is practically never observed because of the poor tolerance to uncorrected systemic acidosis. (Harquindey and Gragoe, 1992). Our study, therefore, focused on the urinary bladder because, in contrast to the systemic pH, the urinary pH can considerably be lowered.
We could, however, not demonstrate a tumour inhibiting effect of acidification, despite the pronounced decrease in environmental pH. Apparently the cancer cells were able, through acid extrusion mechanisms, to functionally shield themselves from the extracellular acidic environment. In fact, both acidification and prolonged alkalinization tended to aggravate the malignant appearance of the bladder tumours. This observation is supported by the results of Rotstein and Slaga (1988) who reported that acetic acid, a very weak tumour promoter in the multistage mouse skin model, was found to be very effective at enhancing cancer development when applied during the progression phase of the model. It is also in line with clinical evidence suggesting that hostile micro-environmental factors such as hypoxia, acidosis, energy depletion and accumulation of lactic acid are a cause of genetic instability in tumour cells, providing a selective pressure and allowing expansion of cell variants of a more aggressive phenotype, a process termed malignant progression (Yuan and Glazer, 1998; Rofstad, 2000; Vaupel and Höckel, 2000; Höckel and Vaupel 2001).
Summarizing, the above studies show that alkalosis may be a factor in tumorigenesis, but only in the epithelial cells of the urinary bladder where environmental pH can reach very high values in comparison to the systemic pH. No indication was found for a protective effect of chronic acidosis on spontaneous tumour development, or an inhibitory effect of acid environmental pH on tumour progression.

In brief, the studies presented in this thesis allow the following conclusions:

- Short chain fatty acids, the acid end products of carbohydrate fermentation in the intestines, did not constitute an acid load for the body, and thus the effects of lactose on kidneys, adrenals and testes of rats were not due to a systemic acid load.
- Rats adapted relatively easily to life-long feeding of acidifying or alkalizing diets. Most of the changes induced by the feeding of acid- or base-forming salts may be regarded as physiological adaptations. However, dietary $KHCO_3$ induced oncocytic tubules in the kidneys and neoplastic lesions in the urinary bladder.

- Metabolic acidosis did not induce bone loss in rats.
- Both potassium and sodium ions were strong mitogenic bladder tumour promotors in rats under conditions of elevated urinary pH, and both ions exerted weak tumour promoting activity when the urine was neutral.
- An effect of alkalosis as enhancing factor in tumorigenesis was demonstrated in rats only in the epithelial cells of the urinary bladder where environmental pH can reach very high values in comparison to the systemic pH. This high-dose phenomenon probably carries no relevance to man.
- No indications were obtained for a protective effect of chronic systemic acidosis on spontaneous tumour development in rats.
- Low environmental pH did not inhibit the progression of experimentally-induced urinary bladder tumours in rats.

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Summary

Summary

The composition of the diet can strongly affect the acid-base balance of the body. Many diet manuals including 'acid-forming', 'base-forming' and 'neutral' foods have been published since the early 20th century, and it is well-established that vegetarian diets are alkalogenic in comparison to the acidifying typical Western diets containing high levels of animal protein. Although diet pH and diet acidogenicity are often confused, the pH is not a proper indicator of diet acidogenicity or alkalogenicity. The balance of fixed (non-metabolizable) inorganic anions (Cl, P, S) and fixed cations (Na, K, Mg, Ca) indirectly refers to the metabolic fate of metabolizable anions and cations, and can be used as an indication of the acid or base forming properties of a diet. Foods containing an excess of fixed inorganic anions over inorganic cations have acidifying properties, and conversely foods containing an excess of inorganic cations over inorganic anions result in a relative increase in alkalinity.

The studies described in this thesis deal with toxicological implications of dietary modulation of the acid-base balance in rats. In Chapter 1, an introduction is given into the basic concepts of acid-base physiology. Further this chapter explains the scope and objectives of the work described in this thesis. Findings in chronic studies conducted at our Institute with a number of substances belonging to a chemically heterogeneous family of products, such as disaccharides (lactose, maltitol, isomalt), sugar alcohols (xylitol, sorbitol), resistant starches and various oligosaccharides were the immediate cause for starting the acid-base research project (Chapter 2). These 'slow digestible carbohydrates' share as a common property the increased production in the large intestine of short chain fatty acids (SCFA) resulting from fermentation of carbohydrate residues. Changes observed after feeding high dietary levels of slow digestible carbohydrates to rats or mice in chronic studies are diarrhoea, caecal enlargement, (transient) aciduria, hypercalciuria, renal pelvic calcification and hyperplasia of the renal pelvic epithelium, and increased incidences of neoplastic changes in the adrenal medulla and in the testes. The investigation described in Chapter 2 aimed to test the hypothesis that the continuous supply of SCFA from fermentation may act as an acid load on the body and that the increased production of SCFA is of fundamental significance for the development of the adverse effects found in the kidneys, adrenals and testes. For this purpose, the modifying effects of an acidifying (NH_4Cl) or an alkalizing ($KHCO_3$) diet supplement on lactose-induced changes in rats were studied in a long-term feeding study. Typical effects induced by lactose were soft faeces, reduced

faecal pH and increased caecal weight. In contrast to previous studies, lactose did not increase the incidence of neoplastic changes in the adrenal medulla. Lactose did enhance Leydig cell hyperplasia and neoplasia in the testes as observed previously, but these findings were only significant if the preneoplastic and neoplastic changes were combined. None of the above findings were significantly modified by NH_4Cl or $KHCO_3$, while blood gas values and urinary measurements related to acid-base status were unaffected by lactose. Some of the lactose effects were, however, affected by the dietary load of acid or base. The lactose-induced increase in urinary calcium excretion was further elevated by NH_4Cl, whereas $KHCO_3$ diminished this effect. It was, however, argued that the effects of lactose, NH_4Cl and $KHCO_3$ on urinary calcium excretion resulted from separate mechanisms. As in previous studies, lactose-fed rats showed less severe nephrosis but a higher incidence of mineralization of the renal pelvic epithelium than controls. The incidences of pelvic mineralization and urothelial hyperplasia were significantly decreased by NH_4Cl. $KHCO_3$ also diminished the lactose-induced mineralization of the renal pelvic epithelium. The results of the studies described in Chapter 2 and the subsequent discussion in Chapter 7 allow the conclusion that SCFA, the acid end products of carbohydrate fermentation in the intestines, do not act as an acid load on the body, and, thus, that the effects of lactose on kidneys, adrenals and testes are not due to a systemic acid load.

Chapter 3 deals with studies addressing systematically the effects of disturbances of the acid-base balance on standard toxicity parameters. These studies included 4-wk, 13-wk and 18-month toxicity studies, and a 30-month carcinogenicity study. Rats were fed a natural ingredient diet (controls), base forming diets (containing 2% or 4% $KHCO_3$), or acid-forming diets (containing 1% or 2.1% NH_4Cl). Additional controls were fed 3% KCl (a neutral diet providing K^+ and Cl^- in amounts equimolar to those in the 4% $KHCO_3$ diet and the 2.1% NH_4Cl diet, respectively). NH_4Cl induced the expected metabolic acidosis, as shown by decreased base excess in blood, decreased urinary pH and increased urinary net acid excretion. $KHCO_3$ induced the opposite effects. KCl did not affect the acid-base balance. Clinical condition and death rate were not affected. The feeding of high levels of each salt resulted in growth retardation and increased water intake and urinary volume. Plasma potassium and urinary potassium excretion were increased with $KHCO_3$ and KCl. Plasma chloride was increased with NH_4Cl, but not with KCl. Urinary calcium and phosphate excretion were increased with NH_4Cl, but there were no indications that bone minerals were involved (weight, calcium content and fat free solid of the femur were not affected). Standard haematological and

clinical chemistry parameters were not affected. The administration of NH_4Cl was associated with an increase in kidney weight, which was ascribed to acidosis-induced stimulation of renal ammoniagenesis. This increased weight was not associated with adverse histopathological renal changes. On the contrary, the severity of nephrosis and the incidence of oncocytic tubules were decreased with NH_4Cl, suggesting a beneficial effect of acidosis. In rats fed $KHCO_3$, an early onset (from week 13) of oncocytic tubules was noted in the kidneys, and, after 30 months, the incidence of this lesion was much higher than the background incidence in ageing controls. No progression to oncocytomas was noted. KCl only slightly accelerated the onset of oncocytic tubules (from 18 months). Due to chronic stimulation of the adrenal cortex by either K^+ or by NH_4Cl-induced acidosis, hypertrophy of the adrenal zona glomerulosa occurred with $KHCO_3$, KCl and NH_4Cl. The feeding of $KHCO_3$ resulted in urothelial hyperplasia, papillomas and carcinomas of the urinary bladder. With KCl only a slight increase in proliferative urothelial lesions was noted. Apart from these (pre-)neoplastic lesions in the urinary bladder, which were further described in Chapter 5, there were no treatment-related differences in tumour response among the groups. We concluded that the rats showed a remarkable adaptive capacity to life-long exposure to a considerable acid or base load, and that most of the changes induced by the feeding of acid- or base-forming salts may be regarded as physiological adaptations. However, dietary $KHCO_3$ induced oncocytic tubules in the kidneys and neoplastic lesions in the urinary bladder. NH_4Cl-induced chronic metabolic acidosis was not associated with dissolution of alkaline bone salts in rats. Finally, a protective effect of chronic acidosis on tumour development was not found.

Chapter 4 describes the promoting activities of elevated urinary pH and different urinary sodium or potassium ion concentrations in urinary bladder carcinogenesis studied in an initiation-promotion assay, using N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) as tumour initiating agent. Following initiation with BBN, male rats were kept on diets containing equimolar amounts of NaCl (2.3%), KCl (3%), $NaHCO_3$ (3.4%) or $KHCO_3$ (4%). The alkalizing salts $NaHCO_3$ and $KHCO_3$ induced similar increases in urinary pH, and elevated the urinary sodium and potassium ion concentration, respectively. In the groups fed $NaHCO_3$ and $KHCO_3$, the incidences of preneoplastic lesions (papillary/nodular hyperplasia) and neoplastic lesions (papilloma and carcinoma) in the urinary bladder were higher than in the control group. NaCl and KCl also induced high urinary sodium or potassium ion concentrations but without altering the urinary pH. This was accompanied by increased incidences of hyperplasia,

papillary/nodular hyperplasia and papillomas, but no carcinomas. We concluded that both potassium and sodium ions are strong mitogenic bladder tumour promotors under conditions of elevated urinary pH, and that both ions may exert weak tumour promoting activity when the urine is neutral.

Since it is widely accepted that mitogenic tumour promotors (inducing sustained hyperplasia) by themselves may induce tumours without prior initiation, the effects of administration of a strong bladder tumour promotor (the alkalizing potassium salt $KHCO_3$) and a weak tumour promotor (the neutral potassium salt KCl) on the urinary bladder were examined in rats not treated with a bladder tumour initiator (Chapter 5). Equimolar dietary amounts of potassium ion, provided by either $KHCO_3$ (4%) or KCl (3%) were administered to male and female rats for periods up to 130 weeks. Comparable increases in urinary volume and potassium levels were found with both $KHCO_3$ and KCl, but only $KHCO_3$ elevated the urinary pH. The feeding of $KHCO_3$ resulted in simple epithelial hyperplasia and, after prolonged exposure, in papillary/nodular hyperplasia, papillomas and transitional cell carcinomas of the urinary bladder, whereas KCl induced only a slight increase in proliferative urothelial lesions and no carcinomas. It was concluded that $KHCO_3$, a strong promotor of urinary bladder carcinogenesis, is capable of inducing urinary bladder cancer in rats without prior application of an initiator, whereas KCl, a weak tumour promotor, is not. The relevance for humans of the findings in Chapters 4 and 5 was discussed in Chapter 7. It was concluded that the species specificity of the bladder response and the fact that it is a high-dose phenomenon makes its relevance to man very doubtful.

Systemic alkalosis has been postulated to enhance carcinogenesis, whereas it has been suggested that metabolic acidosis may inhibit tumour growth and may lead to tumour regression. While in Chapter 3 the influence of systemic acidosis or alkalosis on the occurrence of spontaneous tumours was investigated, Chapter 6 describes the effect of pH on the early and late progression of experimentally-induced tumours. The study focused on the urinary bladder because, in contrast to the systemic pH, the urinary pH can be manipulated to a great extent. Bladder lesions were initiated by BBN and promoted by $NaHCO_3$ in the diet. After short (15 wk) and long-term (25 wk) promotion with $NaHCO_3$, groups of rats were fed a diet containing the acidifying salt NH_4Cl or control diet. All surviving rats were killed after a total study duration of 52 weeks. Additional control groups were, after initiation, maintained on diets containing $NaHCO_3$ and killed after 15 wk or 25 wk of promotion, or at the end of the study. Initiation by BBN followed by treatment with $NaHCO_3$ caused a high incidence of

papilla ry/nodular hyperplasia, papillomas and carcinomas of the bladder epithelium. These lesions progressed with time or longer duration of $NaHCO_3$ promotion. A tumour protective effect of urinary acidification by NH_4Cl was not found. In fact, both acidification and prolonged alkalinization tended to aggravate the malignancy of bladder tumours.

The studies presented in this thesis show that alkalosis may be a modulating factor in carcinogenesis, but only for the urinary bladder epithelium where environmental pH can reach much higher values than the systemic pH. No indication was obtained for a protective effect of chronic acidosis on spontaneous tumour development, or for an inhibitory effect of an acid environment on urinary bladder tumour progression.

In brief, the studies presented in this thesis allow the following conclusions:

- Short chain fatty acids, the acid end products of carbohydrate fermentation in the intestines, did not constitute an acid load for the body, and thus the effects of lactose on kidneys, adrenals and testes of rats were not due to a systemic acid load.
- Rats adapted relatively easily to life-long feeding of acidifying or alkalizing diets. Most of the changes induced by the feeding of acid- or base-forming salts may be regarded as physiological adaptations. However, dietary $KHCO_3$ induced oncocytic tubules in the kidneys and neoplastic lesions in the urinary bladder.
- Metabolic acidosis did not induce bone loss in rats
- Both potassium and sodium ions were strong mitogenic bladder tumour promotors in rats under conditions of elevated urinary pH, and both ions exerted weak tumour promoting activity when the urine was neutral.
- An effect of alkalosis as enhancing factor in tumorigenesis was demonstrated in rats only in the epithelial cells of the urinary bladder where environmental pH can reach very high values in comparison to the systemic pH. This high-dose phenomenon probably carries no relevance to man.
- No indications were obtained for a protective effect of chronic systemic acidosis on spontaneous tumour development in rats.
- Low environmental pH did not inhibit the progression of experimentally-induced urinary bladder tumours in rats.

Samenvatting

Samenvatting

De belasting van het lichaam met zuur of base kan sterk worden beïnvloed door onze voeding. Al sinds het begin van de 20^{e} eeuw is onderzoek gedaan naar de mate waarin verschillende voedselcomponenten verzurend zijn of juist een alkalische of neutrale reactie geven in het lichaam. Zo is bijvoorbeeld de, veel dierlijke eiwitten bevattende, Westerse voeding verzurend, terwijl vegetarische voeding een base overschot vertegenwoordigt. Of voedsel een zuur- of base-belasting oplevert kan niet worden voorspeld door het meten van de zuurgraad (pH). De zuur- of base-vormende eigenschappen van een voedingsmiddel zijn afhankelijk van het metabolisme van organische stoffen in het lichaam. De balans tussen niet-metaboliseerbare kationen (natrium en kalium) en anionen (chloor) kan worden gebruikt als een indirecte indicatie voor de aanwezigheid van metaboliseerbare kationen en anionen. Een overmaat aan niet-metaboliseerbare anionen betekent een zuurbelasting voor het lichaam, terwijl een overmaat aan niet-metaboliseerbare kationen een alkalische reactie geeft.

Dit proefschrift richt zich op de toxicologische implicaties van het beïnvloeden van de zuur-base balans van de rat via de voeding. In hoofdstuk 1 worden de basisconcepten van de zuur-base fysiologie beschreven en wordt ingegaan op de achtergronden en de doelstellingen van de experimenten beschreven in dit proefschrift. Een belangrijke aanleiding voor de start van het zuur-base project vormde het onderzoek aan slecht verteerbare koolhydraten dat op ons Instituut werd uitgevoerd. Hoewel deze slecht verteerbare koolhydraten tot een heterogene groep van verbindingen behoren, zoals disacchariden (lactose, maltitol, isomalt), polyolen (xylitol, sorbitol), oligosacchariden en gemodificeerde zetmelen, leidde het voeren van hoge doses van al deze stoffen aan ratten tot fermentatie van koolhydraatresiduen in de blinde- en de dikke darm, gepaard gaand met de productie van vluchtige vetzuren. Tevens veroorzaakte de chronische blootstelling van ratten en muizen aan hoge doses van deze stoffen diarree, vergroting van de blinde darm, verhoogde uitscheiding van calcium in de urine, mineralisatie van de nierpapil en hyperplasie (toename van cellen) van het epitheel van het nierbekken, tijdelijke verzuring van de urine, en tot tumoren in het bijniermerg en de testikels. Verondersteld werd dat deze effecten in verband staan met een verzuring van het lichaam door de voortdurende aanvoer van vluchtige vetzuren uit de darm. Om deze hypothese te testen werd chronisch onderzoek gedaan bij ratten waarbij werd getracht de effecten van lactose te beïnvloeden door toevoeging van het basevormende zout kaliumbicarbonaat ($KHCO_3$) of het verzurende zout ammoniumchloride (NH_4Cl) aan

een hoog-lactose dieet. Uit dit onderzoek, beschreven in hoofdstuk 2, bleek dat enkele effecten van lactose werden beïnvloed door het toegevoegde verzurende of het basevormende zout. Zo bleek NH_4Cl de door lactose geïnduceerde hogere calcium uitscheiding in de urine verder te verhogen terwijl $KHCO_3$ deze juist verlaagde. Er kon echter worden beargumenteerd dat aan de verhoogde calcium uitscheiding door lactose of NH_4Cl verschillende mechanismen ten grondslag lagen. De door lactose veroorzaakte toename van mineralisatie van de nierpapil en hyperplasie van het nierbekkenepitheel werden zowel door $KHCO_3$ als door NH_4Cl sterk verminderd. Andere typische lactose effecten, zoals de inductie van testikel tumoren, werden niet beïnvloed door $KHCO_3$ of NH_4Cl. Bovendien bleek uit bloedgasanalyse en bepaling van de zuur uitscheiding in de urine dat lactose geen blijvende effecten op de zuur-base balans van het lichaam uitoefende. Uit dit onderzoek kon worden geconcludeerd dat, door fermentatie van slecht verteerbare koolhydraten ontstane, vluchtige vetzuren geen zuurbelasting voor het lichaam vertegenwoordigen, en dat effecten van lactose niet kunnen worden toegeschreven aan verzuring.

Hoofdstuk 3 beschrijft onderzoek waarin de invloed van de zuur-base balans op gangbare toxiciteitsparameters en tumorvorming bij de rat op een systematische wijze werd bestudeerd in 4 weken, 13 weken en 18 maanden durende toxiciteitstudies, en in een 30 maanden durende carcinogeniteitstudie. In deze studies werd aan de ratten controle voer, base-vormende voeders (2% en 4% $KHCO_3$ supplement), of zuur-vormende voeders (1% of 2.1% NH_4Cl supplement) verstrekt. Bovendien kreeg een extra controle groep een voeder waaraan 3% kaliumchloride (KCl) was toegevoegd (een neutral voer, met daarin evenveel kalium en chloor als in respectievelijk het 4% $KHCO_3$ voer en het 2.1% NH_4Cl voer). Uit bloedgasanalyse en de mate van zuuruitscheiding via de urine bleek dat NH_4Cl de verwachte metabole acidose induceerde en $KHCO_3$ de verwachte metabole alkalose, terwijl het KCl voer het zuur-base evenwicht niet beïnvloedde. Blootsteling aan hoge concentraties van al deze zouten resulteerde in groeivertraging, grotere wateropname en verhoogd urine volume. De kalium concentratie in plasma en de kalium excretie in de urine werden verhoogd door $KHCO_3$ en KCl, terwijl NH_4Cl, maar niet KCl, het plasma chloride gehalte verhoogde. Het verzurende (NH_4Cl) voer deed de excretie van calcium en fosfaat in de urine stijgen, maar had geen effect op het calcium gehalte van het bot. Standaard hematologische of klinisch chemische parameters werden niet beïnvloed door de belasting met zuur of base. De door acidose gestimuleerde ammoniumproductie in de nier leidde in de NH_4Cl groepen tot een verhoogd niergewicht. Dit ging echter niet

gepaard met nadelige histopathologische veranderingen in de nier. Integendeel, met NH_4Cl werd een verminderde mate van “nephrose” en een lagere incidentie van “oncocytic tubules” gevonden, wat wijst op een gunstig effect van acidose op de nier. $KHCO_3$ verstrekking resulteerde in het vervoegd (vanaf week 13) ontstaan van “oncocytic tubules” in de nier, en na 30 maanden was de incidentie van deze afwijking in de $KHCO_3$ groep veel hoger dan die in controle ratten, en dan de achtergrondincidentie in oude dieren. Er werd echter geen progressie tot “oncocytomas” waargenomen. Tengevolge van chronische stimulatie van de bijnierschors door kalium of door de geïnduceerde acidose, trad er verdikking op van de zona glomerulosa van de bijnier met zowel $KHCO_3$, KCl als NH_4Cl. $KHCO_3$ veroorzaakte hyperplasie van het urineblaasepitheel en, in een later stadium, papilloma’s and carcinoma’s in de urineblaas. Met KCl werd slechts een geringe toename van proliferatieve veranderingen in het urineblaasepitheel waargenomen. Behalve deze (pre-) neoplastische veranderingen in de urineblaas door $KHCO_3$, die in hoofdstuk 5 uitvoerig worden beschreven, waren er geen behandelings-gerelateerde verschillen in tumor response. Uit dit onderzoek bleek dat ratten zich opmerkelijk goed kunnen aanpassen aan levenslange belasting met zuur of base. De meeste van de waargenomen effecten kunnen worden beschouwd als fysiologisch aanpassing aan zuur- of basevormende zouten. $KHCO_3$ induceerde echter ook “oncocytic tubules” in de nieren en neoplastische veranderingen in de urineblaas. Verder bleek uit dit onderzoek dat de door NH_4Cl-geinduceerde metabole acidose niet gepaard ging met het oplossen van alkalische calciumzouten uit het bot, en dat chronische acidose geen beschermende werking uitoefende op het ontstaan van ouderdomsgerelateerde tumoren. In hoofdstuk 4 wordt een initiatie-promotie model beschreven waarin de promoverende werking van alkalische urine (hoge urine pH) en de rol van natrium en kalium op het ontstaan van urineblaas tumoren werd bestudeerd. Ratten kregen gedurende 4 weken N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) als tumor initiator toegediend via het drinkwater, en vervolgens via het voer equimoliare hoeveelheden natrium chloride (2.3% NaCl), natriumbicarbonaat (3.4% $NaHCO_3$), kaliumbicarbonaat (4% $KHCO_3$) of kaliumchloride (3% KCl) gedurende 32 weken. De basevormende zouten $NaHCO_3$ and $KHCO_3$ veroorzaakten een alkalische urine en hoge concentraties van respectievelijk natrium en kalium in de urine. Met beide zouten werd een verhoogde incidentie van preneoplastische veranderingen (“papillaire” en “nodulaire” hyperplasie van het epitheel) en neoplastische veranderingen (papilloma en carcinoma) in de urineblaas waargenomen. De neutrale zouten NaCl en KCl verhoogden ook respectievelijk de

natrium en kalium concentratie in de urine, zonder echter de urine pH te beïnvloeden. Dit ging gepaard met verhoogde incidenties van “papillaire” of “nodulaire” hyperplasie en papilloma’s maar er ontstonden geen carcinoma’s. Uit dit onderzoek werd geconcludeerd dat verhoogde concentraties van zowel natrium- als kaliumionen in de urineblaas een sterke tumor promoverende werking uitoefenen onder alkalische omstandigheden, en dat deze ionen een zwakke promoverende werking hebben wanneer de urine pH neutraal is.

Het is bekend dat een tumorpromotor op zichzelf, dus zonder voorafgaande initiatie, tumoren kan induceren door het voortdurend aanzetten tot celdeling. In hoofdstuk 5 worden de effecten beschreven van het basevormende $KHCO_3$ (een sterke tumorpromotor) en het neutrale KCl (een zwakke tumorpromotor) op het ontstaan van urineblaastumoren bij ratten zonder voorafgaande blootstelling aan een initiator. Equimolaire hoeveelheden kalium werden via het voer aan ratten verstrekt als $KHCO_3$ (4%) of KCl (3%) in proeven die 2,5 jaar werden voortgezet. Beide zouten veroorzaakten volgens verwachting vergelijkbare verhogingen van het urine volume en de kalium concentratie, maar alleen $KHCO_3$ induceerde een alkalische urine. $KHCO_3$ veroorzaakte “simpele” hyperplasie van het urineblaasepitheel en, na langere blootstelling, “papillaire” en “nodulaire” hyperplasie van het epitheel en papilloma’s and carcinoma’s in de urineblaas, terwijl KCl alleen geringe toename van proliferatieve veranderingen in het urineblaasepitheel veroorzaakte zonder dat er carcinoma’s ontstonden. Geconcludeerd werd dat $KHCO_3$, een sterke tumorpromotor, zonder voorafgaand initiatie tumoren kan induceren in de urineblaas van de rat, terwijl de zwakke tumorpromotor KCl hiertoe niet in staat is. In hoofdstuk 7 wordt beargumenteerd dat de relevantie voor de mens van deze bevindingen bij de rat zeer twijfelachtig is door de verschillen tussen deze soorten en het feit dat deze effecten alleen optreden bij zeer hoge blootstellingsniveaus.

In de literatuur wordt vaak verondersteld dat metabole alkalose het ontstaan van tumoren kan bevorderen, terwijl metabole acidose juist tumorgroei zou tegengaan en zelfs zou kunnen leiden tot tumorregressie. De effecten van systemische acidose of alkalose op het ontstaan van spontane tumoren zijn beschreven in hoofdstuk 3. Hoofdstuk 6 betreft onderzoek naar de effecten van de omgevings pH op de vroege en late progressie van experimenteel opgewekte tumoren. Dit onderzoek richtte zich speciaal op de urineblaas omdat de urine pH, in tegenstelling tot de zuurgraad in het lichaam, in sterke mate kan worden beïnvloed. In de urineblaas van de rat werden tumoren opgewekt door initiatie met BBN en vervolgens door kortdurende (15 weken)

of langdurige (25 weken) promotie met $NaHCO_3$ via het voeder. Na deze perioden kregen de dieren een zuurvormend (NH_4Cl) voer of een neutraal controle voer, en de effecten van deze voeren werden vergeleken na een totale studieduur van 52 weken. In extra, met BBN behandelde, controle dieren werden tevens de effecten van promotie met $NaHCO_3$ gedurende 15 weken, 25 weken of de gehele studieduur onderzocht. De behandeling met $NaHCO_3$ resulteerde in hoge incidenties van “papillaire” en “nodulaire” hyperplasie van het epitheel, en van papilloma’s en carcinoma’s in de urineblaas. De ernst van deze afwijkingen nam toe naarmate de behandeling met $NaHCO_3$ langer duurde. Het verlagen van de urine pH door middel van NH_4Cl had echter geen beschermende werking op de vroege- of late progressie van tumoren. Integendeel, zowel de belasting met zuur als voortdurende blootstelling aan base, leek de agressiviteit van de tumoren eerder te doen toenemen. Uit het onderzoek beschreven in hoofdstukken 3 en 6 werd geconcludeerd dat, althans in de urineblaas waar een zeer hoge pH kan worden bereikt, alkalische omstandigheden een rol kunnen spelen bij het ontstaan van kanker. Er werden echter geen aanwijzingen gevonden voor een beschermend effect van chronische acidose op het ontstaan van spontane tumoren in het lichaam, of voor een remmend effect van een lage omgeving pH op de progressie van geïnduceerde tumoren in de urineblaas.

Curriculum vitae

Ben Lina werd geboren op 4 januari 1954 te Akersloot. Na het voltooien van de middelbare schoolopleiding (HBS-B) aan het Lyceum Oranje Nassau te Harderwijk, begon hij in 1971 aan zijn studie Biologie aan de Rijksuniversiteit Utrecht. In de doctoraalfase bewerkte hij het hoofdvak farmacologie, deels bij het Rudolf Magnus Instituut voor Farmacologie te Utrecht en deels bij de "Addiction Research Foundation, University of Toronto", het hoofdvak endocrinologie bij de Rijksuniversiteit Utrecht, en de bijvakken hydrobiologie en algemene didactiek eveneens aan de Rijksuniversiteit Utrecht. De studie werd in het voorjaar van 1979 afgerond. Van juli 1979 tot december 1980 vervulde hij zijn militaire dienstplicht; in 2003 werd hem eervol ontslag verleend als reserve kapitein der Geneeskundige Troepen. Sinds januari 1981 is hij als toxicoloog ("study director") werkzaam bij TNO Voeding te Zeist. In 1993 is hij geregistreerd als Medisch Biologisch Wetenschappelijk Onderzoeker, richting Toxicologie (SMBWO erkenning) en sinds 1997 is hij ingeschreven in het register van erkende toxicologen van de Nederlandse Vereniging voor Toxicologie. Hij heeft deel uitgemaakt van diverse wetenschappelijke commissies, waaronder de Gezondheidsraadcommisie Toxicologische Aspecten van Biotechnologisch Bereide Producten, 1990 -1992, en de Europese "Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers" (SCCNFP), 1990 - 2004.

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Dankwoord

Binnen de multidisciplinaire omgeving van TNO hebben veel mensen meegewerkt aan dit onderzoek, zoals biototechnici, voederbereiders, clinisch-chemisch analisten, histotechnici, pathologen, administratieve medewerkers, computer technici en statistici. Ik ga al deze collega's hier niet met naam en toenaam noemen. Het allerbelangrijkste dat ik hier wil zeggen is dat ik jullie dankbaar ben voor de collegiale samenwerking binnen wat vroeger de afdeling BioTox van het CIVO was. Ondanks regelmatige naamswisselingen, wordt deze afdeling gelukkig nog altijd gekenmerkt door plezierige onderlinge verhoudingen en grote saamhorigheid. Voor mij zijn het als team knokken voor een gezamenlijk resultaat, de onderlinge verbondenheid, en het elkaar opvangen en aanvullen de meest essentiële factoren van mijn werk bij TNO.

Een aantal mensen wil ik toch in het bijzonder bedanken. Allereerst mijn promotoren Prof. Dr V.J. Feron en Dr R. Kroes. Vic, hoewel jouw werkterrein de hele wereld omvat, vertegenwoordig je voor mij toch vooral de ideale TNO-er. Het op logische en pragmatische wijze toepassen van kennis doe je af met "gewoon je boerenverstand gebruiken". Je hebt het "wij-gevoel" hoog in het vaandel staan, bent altijd enthousiast bezig anderen te betrekken bij jouw projecten en laat ze daarbij volop meedelen in de credits. Ik dank je voor je stimulerende begeleiding tijdens het schrijven van mijn proefschrift en ben erg blij dat je bereid was om op te treden als mijn promotor.

Bob, het bestuderen van slechtverteerbare koolhydraten is in de grijze oudheid gestart onder jouw leiding als directeur van het CIVO. Het is typerend dat het onderzoek aan dit soort stoffen nog steeds uiterst actueel is. Ik blijf verbijsterd over de bijna nonchalante wijze waarop je zeer doeltreffend regelde dat Vic ook na het verstrijken van de officiële termijn nog op kon treden als eerste promotor. Jouw charme blijkt zelfs door de telefoon vanuit Frankrijk uitstekend te werken.

En dan mijn copromotor Dr R.A. Woutersen. Ruud, ik heb me altijd afgevraagd hoe jij zoveel dingen tegelijk kunt doen, zowel binnen als buiten TNO. Ik heb grote bewondering voor de manier waarop jij altijd achter jouw mensen hebt gestaan als "no-nonsense" afdelingshoofd. Ik bedank je voor het kritisch lezen van elk concept hoofdstuk; nota bene tijdens jouw vakantie was je nog bereid om mijn tekst te controleren op typefouten.

Voor mijn opleiding in de toxicologie heb ik veel te danken aan Dr H.P. Til en Dr A.P. de Groot. Heero, jij bent voor mij het prototype van een betrokken en integere collega. Dr de Groot was de grondlegger van het zuur-base onderzoek. Door het grote respect

dat ik voor hem koesterde, durfde ik slechts heel voorzichtig met hem van mening te verschillen.
Met name wil ik ook twee collega's noemen met wie ik dagelijks zeer nauw samenwerk. Marian, of je het plannen, het maken van datatox-bestanden en het archiveren nu wel of niet aan mannen kunt overlaten, zonder jou zou het een stuk minder flitsend gaan. Diana, ons regelmatig overleg en jouw mening zijn voor mij onmisbaar, ook al moet ik helaas verontrustend vaak toegeven dat je "eigenlijk wel gelijk hebt".
En, tenslotte, wil ik natuurlijk Wilma bedanken. Jij hebt alle perikelen van nabij mee moeten maken. Ik denk dat we nu eerst maar op vakantie gaan.