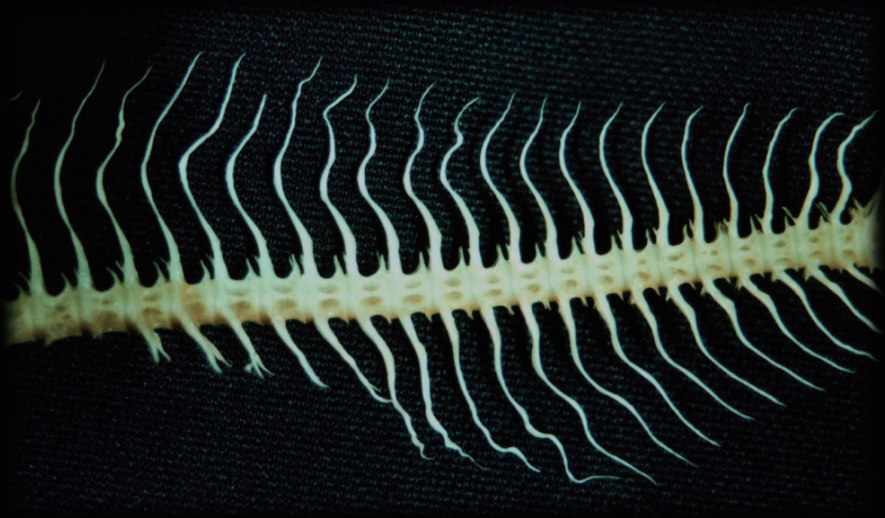
Phosphorus in Fish Nutrition



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Disclaimer:
The present book consists largely of critical reviews and syntheses of published papers. All the comments made herein are based on the author's own interpretation, which by no means shall be construed as absolute or infallible judgment of the papers.

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Conflict of interest:
The author declares that there is no conflict of interest, financial or otherwise, that would prejudice the impartiality of this scientific writing.

Revision history:
The writing of this review began in 1998 as the author's personal research notes. In 2000, the first tentative edition was published, which was revised in 2005. Both these earlier editions were posted only on the author's personal website, and were freely downloadable. The present third edition is the first to be published in a printed form. Approximately half of the present edition has been retained from the previous edition.

Preface

Over the past few decades, a great many things have changed in the lives of scientists. One of the greatest changes to have occurred may be the advent of the internet. In these days of electronic communications, the process of searching for literature has become exceedingly faster and easier than ever before. Internet search engines, dictionaries, and digitalized papers and books now provide us quick access to all sorts of information, ranging from old to new, and from minor to major. The tradition of manually reading of printed pages has increasingly been replaced by keyword searches, which is a much faster method of finding the information one requires. In this IT environment, a mere compendium of knowledge (i.e., literature) can no longer be relied upon to help our research activities. The present book, therefore, is not intended to provide such textbook knowledge, but to provide a critical review of published papers. Consequently, I often find myself disagreeing with the authors' interpretations of their data. In such cases, I have taken the liberty of providing alternative reasoning, hypotheses, or syntheses. While this approach might be unpleasant for the authors, it is important for scientists who use fine-tuned inductive reasoning to seek "true knowledge." This approach must be even more important for students who should know that the same data set can be subject to multiple interpretations.

The present book also provides a historical overview of studies that have contributed to our present knowledge of phosphorus nutrition. This approach might be unique, with most review articles simply covering only recent studies. The advance of science is relentless and new knowledge is being created every day; consequently, looking back into the past may seem like a futile waste of time. Is there any benefit in studying history or old knowledge? In answering this question, consider the words of Clive McCay, a pioneer scientist of fish nutrition and aging: "The fact is useful no matter when it is discovered. There is no doubt that thousands of useful facts have been discovered and lost ... the facts are lost until some bookworm unearths them" (McCay 1973, p. 14). As a scientist, it is disappointing to work hard on something that is already known, is it not? But historical data can also be useful because it can allow for a re-analysis, using deductive logic, in light of newer hypotheses, thus illuminating previously unnoticed facts hidden in an old paradigm.

Since basic physiological functions are well conserved throughout vertebrate evolution, referring to mammalian findings is often rewarding and helpful in order to predict corresponding fish responses. Moreover, phosphorus is a key nutrient for plants; therefore, plant responses to phosphorus deficiency may also be informative. Likewise, the chemical forms of phosphorus in soil and intestinal environments may have some commonalities. In this book, therefore, I have dared to include numerous non-fish research wherever relevant. As we see things from different perspectives and think differently, some alternative interpretations or conclusions may arise. These delicate processes further heighten our inductive capability. "An experiment is never fallacious, only our interpretation of it may be wrong"--Leonardo da Vinci 1452-1519 (Lusk 1933, p. 19).

S. H. Sugiura
December, 2017

Acknowledgments

My first experience with phosphorus may be traced back in 1984. I was then an undergraduate student taking a course in biochemistry. The teacher was Dr. Ogino, a calm awe-inspiring professor of fish nutrition. One day, he brought some research samples into our classroom and remarked, "These are the only samples exist in the world." … I felt, though vaguely, the essence of research and of a scientist. The samples, as I remember, were those of Ogino et al. (1979)— the phosphorus-deficient bent bones. In retrospect, it is so amazing to see that such a small inkling of experience ended up with this book some 30 years later. I do not claim David Hume's causation theory upon this experience, but I can still recollect those early days— it might be one of the earliest billiard balls moved.

I would like to express my sincere thanks to Dr. R.T. Lovell (Auburn University, Alabama, USA), Dr. R.W. Hardy (Hagerman Fish Lab., University of Idaho, USA), and Dr. R.P. Ferraris (Rutgers-New Jersey Medical School, USA) for their invaluable help or mentoring in pursuing my phosphorus research. I also wish to extend my appreciation to all my former professors and colleagues for their help, patience, and encouragement.

About the author:
Shozo H. Sugiura, born in Japan in 1964, began his lifelong hobby of fish feeding at the age of five, and reared over 100 species of fish before entering Tokai University where he learned fish culture and received a Bachelor of Fisheries degree, summa cum laude. While an undergraduate student, he worked at Kanagawa Fisheries Center for seabream fry production and engaged in R&D of microparticulate diets. After graduation, he started to work as a fulltime fish farmer at Japan's largest trout farm to learn about trout and eel farming and feed manufacture. After 3 years of fish farming, he enlisted in the JOCV volunteers (similar to the US Peace Corps), and worked in rural Syria to develop local fish feeds. After 3 years of service in Syria, he started his graduate work and studied for a total of 12 years at Auburn University (M.S.), University of Washington (Ph.D.), University of Idaho, Hagerman (Post doc.), Harvard University (M.Ed.), and New Jersey Medical School, UMDNJ (Res. assoc.). He is currently a professor of fish nutrition and aquaculture at the University of Shiga Prefecture-School of Environmental Sciences, Japan.

Contents

Abbreviations

(abbreviations of infrequent use are defined in the text)

[Ca]: Ca concentration (same for other solutes)
"~" sign: approximately
$1,25(OH)_2D$: calcitriol
ADC: apparent digestibility coefficient, %
ALP: alkaline phosphatase
BBMV: brush-border membrane vesicles
BMD: bone mineral density
BW (bw): body weight (of fish)
Calcitriol: 1,25-dihydroxycholecalciferol (also called 1,25-dihydroxyvitamin D_3, or $1,25(OH)_2D_3$, or $1,25(OH)_2D$)
CAT: catalase
CHO: carbohydrates
CKD: chronic kidney disease
CP: crude protein (content)
d: day(s)
di-Ca phosphate: di-calcium phosphate, or $CaHPO_4$
di-K phosphate: di-potassium phosphate, or K_2HPO_4
di-Na phosphate: di-sodium phosphate, or Na_2HPO_4
DM: dry matter
DO: dissolved oxygen concentration
DRI: Dietary reference intake (for humans)
DXA or DEXA: dual-energy x-ray absorptiometry (measures bone density)
E2: estradiol
Entpd5: ectonucleoside triphosphate/diphosphohydrolase 5
ERs: estrogen receptors
ESRD: end-stage renal disease— Stage 5 CKD (CRF: chronic renal failure)
FAS (Fas): Fatty acid synthase
FCR: feed conversion ratio (wt of feed fed / body wt of fish increased).
FE: feed efficiency, % (body wt of fish increased × 100 / wt of feed fed)
FGF23: fibroblast growth factor 23 (a phosphaturic hormone)
fw: freshwater
GFR: glomerular filtration rate
GI: gastro-intestinal
GPx: Glutathione peroxidase
GR: glucocorticoid receptor
GSI: gonado-somatic index (= gonad wt / body wt)
HSI: hepato-somatic index (= liver wt / body wt)
Ip (IP) injection: intraperitoneal injection
MDA: malondialdehyde
mono-Ca phosphate: mono-calcium phosphate, or $Ca(H_2PO_4)_2$
mono-K phosphate: mono-potassium phosphate, or KH_2PO_4
mono-Na phosphate: mono-sodium phosphate, or NaH_2PO_4
NaPi: sodium phosphate cotransporter
NPT1: NaPi-I, or Type-I NaPi (*Slc17a1*)
NPT2 or NPT2a: NaPi-IIa, or Type-II NaPi (*Slc34a1*)

OXPHOS: oxidative phosphorylation
P: phosphorus (in nutrition, P is not elemental P, but is phosphate, or PO_4).
PCr: Phosphocreatine, or creatine phosphate
PDF: potassium diformate
PHEX: (phosphate regulating gene with homologies to endopeptidases on the X chromosome)
PHOSPHO1: phosphatase orphan1
Pi: inorganic phosphorus, inorganic P
PiT-1: Type-III NaPi (*Slc20a1*); PiT-2: Type-III NaPi (*Slc20a2*)
PL: phospholipids
PPARs: peroxisome proliferator-activated receptors
PTH: parathyroid hormone
RAR: retinoic acid receptor
RDA: Recommended dietary allowance (recommended daily intake of nutrients)
ROS: reactive oxygen species
RXR: retinoid X receptor
SOD: Superoxide dismutase
T3: triiodothyronine; T4: thyroxine
TAN: total ammonia nitrogen
TJ: tight junction
TNAP (TNSALP): tissue non-specific alkaline phosphatase = liver/bone/kidney (L/B/K)-alkaline phosphatase
TR: thyroid receptor
TRAP or TRACP: tartrate-resistant acid phosphatase (bone marker)
tri-Ca phosphate: tri-calcium phosphate, or $Ca_3(PO_4)_2$
UCP: uncoupling protein
VD: vitamin D; VC: vitamin C; VK: vitamin K; VA: vitamin A
VDR: vitamin D receptor
wk: week(s)
wt: weight
WT: water temperature in °C

Definitions

Availability: The fraction (%) of a dietary nutrient that is absorbed in the GI tract. Synonymous with digestibility. However, the term "digestibility" is an esoteric jargon and is also literally incorrect and confusing because: (a) many dietary nutrients are absorbed without complete digestion, and (b) many minerals are already digested, and yet only partially absorbed in the intestine. Also, inorganic elements including P are indigestible. Hence, using the term digestibility is often avoided. Alternatively, more unequivocal terms (e.g., availability, absorption) are used preferentially in inter-scientific communications.

Bioavailability: The amount of a nutrient or chemical substance (e.g., drugs, toxins) in a diet or in water that is absorbed and utilized or affected in some biological functions (usually expressed as % of intake or relative to some standard source). Bioavailability is usually determined based on a physiological function (e.g., bone mineral density, body retention, enzyme activity, blood or tissue concentration, etc.) rather than intestinal absorption.

Digestibility: (cf. Availability)

*Apparent digestibility (%) = (Dietary intake - Fecal excretion) × 100 / Dietary intake

*Apparent digestibility is sometimes called apparent digestibility coefficient, or ADC.

*Apparent digestibility is synonymous or near synonymous to the following: apparent absorption, net absorption, and apparent availability.

*True digestibility (%) = (Dietary intake - (Fecal excretion - Endogenous excretion)) × 100 / Dietary intake

*True digestibility is always higher than the apparent digestibility of the same diet.

*Both apparent digestibility and true digestibility do not account for intakes by water-drinking and by branchial or surface absorption.

Feed conversion (ratio), or FCR = Feed fed (g, dry wt or as-fed wt) / Fish wt gain (g, wet wt).

Feed efficiency (%), or FE = Fish wt gain (g, wet wt) × 100 / Feed used (g, dry wt or as-fed wt). This index is occasionally expressed as the ratio, instead of %.

*In this book, both FE and FCR are used because both are equally common. However, as Baker (1986) noted, the use of FE is preferred, at least in scientific writing.

Net absorption: = Apparent absorption, Apparent digestibility, Net intestinal absorption.

Retention: The amount of a nutrient or chemical substance in the diet or water that is absorbed and retained (for a specified period of time) in the body or in a specific tissue or cell.

Stoichiometry: A branch of science that studies the elemental mass balance between all reactants used and all products formed (in both the kind and amount), which is best known in chemical reactions. Stoichiometry follows the law of conservation of mass (matter or elements). Similar stoichiometric calculations are made in such study areas as plant nutrition and ecological food-web interactions. In nutrition, stoichiometric calculation involves three factors: the ingestion (diet), retention (body), and excretion (fecal, urinary, etc.). These stoichiometric ratios should differ greatly depending on the element (and its bioavailability), nutritional balance, species (organism), individual (age, growth, various physiological state), and numerous environmental factors. By modulating these factors, fish nutrition research basically aims to maximize the body retention/excretion ratio.

Transport: The movement of a solute across the cellular membrane by an active process (primary or secondary) mediated by a transporter or solute carrier. In a broader sense, this also includes passive components, such as simple and facilitated diffusions, and paracellular diffusion.

True absorption: = True digestibility, Intestinal absorption.

Uptake: The amount of a nutrient or chemical substance in a medium that is absorbed by a living cell, tissue, or body. Often measured using radioisotopes such as ^{32}P.

Utilization: The amount of a nutrient in a diet or in water that is absorbed and utilized for some biological function(s).

Introduction to phosphorus

Phosphorus (P) is essential for all life on Earth, including all eukaryotes (i.e., animals, plants, fungi, protists), all prokaryotes (i.e., bacteria, archaea), and even viruses. The primary reason why P is so essential for life is that all these life forms contain nucleic acids (i.e., DNA, RNA) that contain P in their backbone structure.

In animal species, P plays a number of roles in intermediary metabolism, including phosphorylation of numerous proteins (e.g., enzymes, hormones, and signaling proteins) for their activation-deactivation, generation of high-energy carriers (e.g., ATP, creatine phosphate, and other phosphagens), and maintaining (buffering) blood acid-base balance in the kidney. Phosphorus is an essential component of membrane phospholipids, hydroxyapatite of skeletal tissues (i.e., bones, teeth, scales), and erythrocyte 2,3-diphospho-glycerate for oxygen delivery to the peripheral tissues. Phosphorus is also involved in the aging process (via klotho), Alzheimer disease (via tau phosphorylation), and yet many other biochemical functions. Despite its pleiotropic roles in life, biochemical and molecular mechanisms of P deficiency have not been well characterized, not even in mammals. This is probably because deficiencies of dietary P are rare in normal human dietetics.

Phosphorus is also critical for plants, including algae and phytoplankton. Phosphorus limits primary production in most aquatic ecosystems (**Hakason & Carlsson** 1998; **Tyrrell** 1999; **Mainston & Parr** 2002); therefore, an abundance of P in an aquatic ecosystem directly contributes to eutrophication, thus causing algal bloom and the hypoxia of natural waters. In extreme cases, the addition of P can alter or even destroy aquatic habitats and create an azoic environment (**Cullen & Forsberg** 1988; **Chowdhury** et al. 2017).

In animal species, including fish, excess dietary P is excreted in urine and feces, leading to the release of P to the environment. Thus, minimizing the excretion of P is important in both livestock production and aquaculture, particularly the latter. This is because it is difficult to impossible to collect fish fecal and urinary waste, whereas the same is still possible in animal and poultry production systems with their wastes routinely collected for use as agricultural fertilizer either directly or indirectly (after animal waste treatments).

In the context of rapidly growing global aquaculture industry, as well as increasing environmental awareness, environmental regulatory agencies have enacted stringent guidelines to limit the amount of P that the aquaculture industry can discharge into public waters. Of course, this is a proper and necessary decision in order to protect the local environment and ecosystems. Yet, these guidelines not only reduce P pollution but also reduce aquaculture production itself (**Carlberg & Olst** 2001). The continuous increase of aquaculture production is important, both in terms of human nutrition and poverty alleviation around the world (**Tacon** 2001; **Desai** 2004). It is, therefore, imperative to improve/develop technologies that can reduce the environmental burden of aquaculture.

Historically, fish would retain only ~20% of dietary P, with most dietary P being discharged into the aquatic environment (**Ketola** 1982; **Philips & Beveridge** 1986;

Wiesmann et al. 1988; **Ackefors & Enell** 1990; **Holby & Hall** 1991; **Ketola & Harland** 1993; **Enell** 1995). However, over the past few decades, the P content of aquaculture feeds has decreased considerably, thus resulting in a substantial reduction in the excretion of dietary P by fish. This reduction in the P content of aquaculture feeds is a result of environmental regulations requiring aquaculture facilities to reduce P excretion in effluent water. Replacing expensive fish meal with less expensive plant ingredients, which are low in P, also contributed to the reduction of dietary P and, therefore, effluent P concentrations.

More recent figures show that fish retain 30-40% of P in typical commercial feeds (**Green** et al. 2002a, 2002b), or over 50% of P in commercial low-P feeds (**Sugiura** et al. 2005) (**Fig. 1**). The reduction of P in aquaculture feeds and effluent, however, has increased the incidence of clinical P deficiency in cultured fish. A further reduction in effluent P excretion will require a better understanding of P nutrition in fish. In addition, the management of environmentally conscious aquaculture facilities will require frequent monitoring of fish P status, not to mention effluent P concentration.

Phosphorus is a finite natural resource. The exponential increase in the global human population corresponds with a rising demand on food production infrastructure. In many arable areas around the world, P-fertilization is often necessary both to sustain and increase crop production. High-yield, sustainable food production, therefore, depends on minimizing the use (i.e., mining) of P (**Smil** 2000; **Cordell** et al. 2009; **Van Vuuren** et al. 2010). Although most (~80%) mined P is used as agricultural fertilizer, some (~5%) is used for P-supplementation in animal, poultry, and fish feeds (**Smit** et al. 2009). Minimizing the use of inorganic P supplements in aquaculture feeds is in accord with this endeavor.

In this context, the present book has been written to facilitate the sustainable, environmentally friendly development of aquaculture. Emphasis is placed on the requirements and bioavailability of P. Phosphorus requirements (Chapter 1) emphasizes systemic aspects of P nutrition, such as various signs of P deficiency, as well as various biochemical, physiological, and molecular responses to deficiencies of dietary P (**Tables 1 and 2**). Phosphorus bioavailability (Chapter 2) explores various aspects of dietary P, such as feed processing technology, and intestinal P absorption (**Table 3**). Both P requirements and bioavailability constitute the twin pillars of environmentally friendly fish feeds. Consequently, these chapters have been synthesized in Chapter 3 for more practical applications (**Table 4**).

Also, I have intentionally omitted some P-related subjects, giving others only a cursory treatment, where similar or more comprehensive reviews have already been published, thus avoiding redundancy. Readers, therefore, might want to refer to other reviews as complementary sources of information, including **Nose & Arai** (1979), **Ogino** (1980b), **Lall** (1989, 1991, 2002), **NRC** (1993), **Davis & Gatlin** (1996), **Cho & Bureau** (2001), **Hardy & Gatlin** (2002), **Sugiura** et al. (2004), **Vandenberg & Koko** (2006), and **NRC** (2011), among other sources (as indicated in respective sections).

Chapter 1.

Phosphorus deficiency & requirements

§ 1. An emerging concern

Several decades ago, the foremost concern among fish nutritionists was to formulate diets containing all the essential nutrients above the minimum dietary requirements to ensure that aquacultured fish could grow normally or rapidly. Of course, various other factors have also been considered in order to optimize fish performance, including resistance to diseases, toxins, and other stresses under sub-optimal environmental conditions, such as intensive aquaculture ponds and cages. Notwithstanding, excess supplies of essential nutrients in these diets have rarely become a problem, unless the excess becomes economically undesirable or toxic, as in the case of some nutrients.

However, in light of increasing global concerns about environmental change, the effort has been directed to reducing the excess portion of dietary nutrients that are excreted by the fish. Among these nutrients, particular attention has been given to P as a critical pollutant discharged from aquaculture facilities. Consequently, fish nutritionists today should be able to prescribe P in diets not only at the level necessary to satisfy the biological requirements of fish, but also to meet environmental guidelines. In other words, it is imperative to know the minimum dietary P requirements accurately. Any excess P in diets will cost the environment by stimulating the eutrophication of aquatic ecosystems.

The emerging importance of P research is also apparent from published books. For example, in the first edition of *Fish Nutrition* (published in 1972, total 713 pages), there is not a single mention of P or anything P-related (which is quite amazing). However, in the third edition of *Fish Nutrition* (published in 2002, total 824 pages), there can be found many P and P-related pages (e.g., P requirements, P metabolism, P availability, bone disease, phytate, low-polluting feeds, etc.). The importance of P research is well-recognized today.

Traditionally, the dietary requirements of essential nutrients were determined using small juvenile fish. The same is true for pioneering research involving other animal species. As the 19th century was coming to a conclusion, a number of researchers began to introduce mice or rats and purified rations to their nutrition research (**Lunin** 1881; **Socin** 1891; **Pekelharing** 1905; **Hopkins** 1906; **McCollum** 1908; **Osborne & Mendel** 1909). Earlier still, between the 1870s and 1880s, **Weiske**, **Pekelharing**, **Eijkman**, and others introduced rabbits to their research. In 1896, **Eijkman** used chicken. Even earlier, **Mayow** (1674), **Priestley** (1771), **Lavoisier** (1777, 1780), and others used mice or other small animals to study the nature of respiration and heat production using a glass jar, or calorimeter (**Lusk** 1933; **McCollum** 1957; **McCay** 1973). These small animals were

easy to handle and to experiment with. Small animals consume much less feeds than do larger animals, which made the use of purified or expensive diets possible. In addition, young animals grow rapidly and are thus very sensitive to malnutrition. Use of small and young animals and purified diets subsequently became the gold standard of nutrition research, which led to the discovery of essential nutrients and established their dietary requirements for various animal species, including fish.

However, in commercial aquaculture production, large fish consume the most feed and excrete the most waste in the effluent. Consequently, large fish must be the primary target for environmentally friendly feed development and use. Large or old fish require less essential nutrients in their diets than do smaller fish. This is because large fish grow slower than small fish (per diet consumed), and they use increasing portions of dietary nutrients for maintenance, which can be largely recycled. This is comparable to the difference between human children and adults. Indeed, in livestock animals and humans, the dietary requirements or recommended dietary allowances (RDA) of nutrients, including P, for adult or older animals are reported to be lower than those of the young. For example, the dietary available P requirement of young pigs (5-7 kg body wt) is 0.45%, while that of older pigs (100-135 kg body wt) is only 0.21% per dry feed (**NRC** 2012, p. 210). Consequently, applying the data determined with small fish to large fish in commercial aquaculture production, while biologically justified, is economically and environmentally untenable.

Unfortunately, only a few requirement studies have been conducted to date using large fish. This is mainly because it is difficult to study the nutrient requirements of large fish using conventional or established methods. These methods require months of feeding to observe the signs of clinical deficiencies in large fish because they already have substantial body stores of these nutrients, issues pertaining to their specific growth rate, the percentage of feed intake, and feed efficiency that are all low as compared with younger fish. In other words, old fish or animals are resistant to malnutrition for an extended period. Also, large fish consume considerably more feed than do small or young fish, which makes the use of expensive research diets economically unaffordable. A different approach, therefore, is necessary to determine the specific nutrient requirements of large fish. The following sections emphasize the response indicators of large fish to dietary P concentration.

§ 2. Growth magnification and systemic P deficiency

Phosphorus is required for growth (**Fig. 2**). Phosphorus is not required, on a net basis, for non-growth or maintenance (cf. §3 Growth and N:P stoichiometry). Hence, growth is essential to induce P deficiency or to study P requirements. Unfortunately, this simple but important principle has sometimes been overlooked even by contemporary researchers who use sophisticated research techniques and equipment. Such research generates only irrelevant data. **Roloff** (1875) fed dogs with a diet low in Ca, and produced rickets. He noted that the development of rickets depends on the size of the breed, the rapidity of growth, and the degree of deficiency of Ca in the diet. **E. Voit** (1880) fed

puppies a mixture of meat and lard with or without Ca. He noted that those fed the diet without Ca supplement reduced not only Ca but also P in the bones. The animals were well-nourished and developed rickets. He also noted that rickets developed in direct proportion to the growth of the animal.

The renowned German physiologist, Gustav von **Bunge** (undated) noted the relationship between the ash content of milk and the time required to double the weight of newborn animals of various species. For example, the required time for doubling the weight in the following species, man, horse, cow, and dog is 180, 60, 47, and 9 days, respectively. The percentages of ash in the milk of these species in the order named are 0.22, 0.41, 0.80, and 1.31. From this, Bunge concluded that the more rapidly the suckling grows, the greater the needs of the organism for those food stuffs which serve for the building up of the tissues, namely, proteins and salts.

In Wisconsin, **Hart** et al. (1909) reported that pigs fed a ration very low in P made as large gains up to 75-100 pounds, when starting at the weight of 40-50 pounds, as animals receiving the same ration but supplemented with Ca phosphate. After reaching this point loss of weight began, followed by collapse. Pigs on the low-P ration maintained P levels in soft tissues and organs constant and comparable to those of normally fed pigs; however, they drew P from the skeleton, but removed Ca and P in the proportion found in tri-Ca phosphate. **Gregersen** (1911) found in rats that even with an abundant intake of P in assimilable form, no P is retained from a protein-free diet.

Kellner (1913, pp. 96-97) wrote, “In young, growing animals these diseased condition, due to the lack of lime and phosphoric acid, develop more rapidly than with full-grown ones. Puppies, particularly those of the larger breeds, when fed on meat free from bone… and finally the animal is unable to move." **Mellanby** (1919) noted that rickets developed much more readily in the fast-growing dogs than in those growing slowly. So, he characterized rickets "a disease of rapid growth." Later, **Mellanby** (1950) also wrote, “no growth, no rickets" in his memoirs of nutrition research. **McCollum** et al. (1921) found that the addition of butter to a rachitogenic diet, which was low in P and high in Ca, increased the growth of rats and as a result produced more severe rickets (cf. §40 Etiology of rickets).

Kleiber et al. (1936) reported that beef heifers fed low-P rations grew normally for 6 months, compared with animals fed normal-P rations. After this period, however, the low-P animals ceased to grow, but their weights remained constant for about a year. During the same period, normal-P animals continued to grow. After this period, the low-P animals began to lose weight. **Aubel** et al. (1936) reported similar results in pigs.

Day & McCollum (1939) fed weaned rats with a diet containing only 0.017% P but otherwise adequate for growth. They observed that P-restricted rats grew and maintained a fairly good appetite for 2-4 wk, then the animals gradually became inactive and used legs as little as possible, and died in 7-9 wk on the deficient diet. Notably, the authors wrote, "The most striking effect of the P deficiency was on calcium . . . the loss of calcium is so much greater than of phosphorus." (cf. §11 Body Ca content). They also reported spontaneous fractures, and progressive rarefaction of bones by X-ray examination. The lethargic condition of the animals may be related to the low ATP level associated with P deficiency (cf. §16 Biochemical responses).

Gillis et al. (1948) fed chicks a diet containing 0.03% P, but otherwise capable of supporting optimum growth. The chicks ate well for 3 or 4 days and made small initial gains in weight. After this period, the birds rapidly lost appetite and showed general weakness, reluctance to stand or use legs, and lying on their sides. All chicks died between the 5th and 10th day on the diet. These researchers indicated that there is a latent period in P deficiency, during which the animal is apparently, at least externally, normal.

In undernourished human subjects, **Rudman** et al. (1975) found that the retention of P, K, Na, and Cl virtually halted when N (amino acids) was withdrawn from the otherwise complete hyper-alimentation fluid. At all levels of N intake, these five elements, including N, retained in the body at a fixed ratio. The withdrawal of P also halted the retention of the other elements. Interestingly, when N, K, or P was withdrawn from the fluid, infused glucose continued to be utilized completely; however, a larger portion of glucose was used for lipogenesis than during infusion of the complete formula.

These early observations indicate that P is required for N retention (i.e., lean gain, or protein accretion), whereas N (protein) is required for P retention. Consequently, there should be an optimum ratio between P and N intakes. Since the N-retention (protein synthesis) is the fundamental unit of growth (**Brett & Groves** 1979), the P requirement is most aptly expressed as per growth, or N-retention (cf. §30 How to express nutrient requirements). The reduction of growth (N-retention) under severe P deficiency, as exemplified above, could be due, at least partly, to decreased availability of ATP for protein synthesis. The energy (ATP) requirements for protein deposition is much more costly than for the accumulation of fat and glycogen (**Hegsted** 1974; **Hommes** 1980; **Jobling** 1985; **Mommsen** 1998).

Nose & Arai (1979) reported that Japanese eel required 0.27% Ca and 0.29% P in the diet for optimum growth. The highest wt gain of the fish was about 75% of the initial wt in the Ca experiment and only 45% in the P experiment (feeding duration: 6-10 wk). When the growth magnification is this low, the dietary requirements of most nutrients may well be underestimated if fish growth is used as the response criterion, whereas it could be overestimated if the retention or tissue concentration of test nutrients is used as the response criterion. For example, if feeding duration is only a few days, the dietary requirement estimate based on growth will most likely be zero, while the estimate based on retention will be infinity (i.e., no plateau). A certain duration of feeding that allows sufficient multiplication of the initial body size will be required to estimate the dietary requirements accurately. Also important is that when the feed efficiency is low, the dietary requirements should become correspondingly low (cf. §30 How to express nutrient requirements). In their P experiment, the fish gained only 45% during the 10-wk feeding period, suggesting that the basal diet used in the experiment was of poor quality or the rearing method was inadequate. In many studies dealing with large fish, the growth magnification tends to be small, leading to the same problem (cf. §23 P requirements of large fish).

Hardy et al. (1993) fed juvenile rainbow trout for 8 wk with a P-deficient diet, a P-adequate diet, or the mixture of these two diets at various ratios. Fish fed the P-deficient diet showed clinical P-deficiency signs, including anorexia, transient lethargy, reduced growth, and dark coloration in 5 wk, while fish fed the mixture of the P-deficient and P-

adequate diets at a 9:1 ratio showed these signs in 7 wk. Subclinical P deficiency did not affect fish growth until after the body P store was reduced below a certain threshold level. **Storebakken** et al. (2000) could reduce both the fecal and metabolic P excretion of Atlantic salmon by replacing fish meal in the diet with soy protein concentrate. The total P content of the soy protein diet and the fish meal diet was 1.2% and 1.8%, respectively. The fish growth did not differ at the end of the 84-day feeding period; however, the fish fed the soy protein diet had markedly lower P and Ca content as well as the Ca/P ratio of the whole body. The fish (initial body wt ~200 g) only doubled their wt during the experiment. **Uyan** et al. (2007) noted that juvenile flounder (bw 1 g) fed a P-deficient diet grew well during the first 20 days without any P-deficiency signs compared with fish fed P-sufficient diets.

Indeed, a similar relationship between the body store and the growth magnification (feeding duration) has been known in several essential nutrients. For example, **Dupree** (1966) fed channel catfish with a vitamin B_{12}-free diet. The fish grew normally for many months, compared with fish fed a VB_{12}-supplemented diet. However, after this latent period, the vitamin-deficient fish started to decrease their growth rate quite obviously. These results indicate that the growth rate does not respond until after the body store has been reduced below a certain threshold level. The risk is that the initial body store (pool size) can be variable depending on the diet history of the fish or animals (**Baker** 1986). The growth reduction can be immediate if a deficient diet is fed to fish that do not have enough savings in the body.

§ 3. Growth and N:P stoichiometry

Schindler & Eby (1997) showed the relationship among fish growth rate, dietary P content, and the P-excretion rate (Fig. 3 in their paper). At zero growth (i.e., zero N-balance, or maintenance level), the P excretion of fish is directly proportional to the dietary P content (as it should be). As the growth rate of fish increases, the fish fed a low-P diet gradually becomes P-deficient. This indicates that P deficiency does not occur at low fish growth (caused by low feed intake, low water temperature, poor feed efficiency, and poor rearing condition, etc.) even when the dietary P content is much lower than the dietary requirement level.

Jahan et al. (2003e) reported that the excretion of P by fish increased as the fish size increased over ~600 g body weight. The authors noted that this was due to the slow growth of larger carp (hence, due to fish size). However, when the fish grew up to the said size, the water temperature was considerably low (below 15°C) during which fish did not grow at all, but were still consuming feed. This is the maintenance feed intake, as shown in **Fig. 3-1**. Hence, all the P in the diet was excreted (on a net basis), which is analogous to adult humans that consume P-containing food daily, but do not grow; and, therefore, excrete all the P in their food (on a net basis).

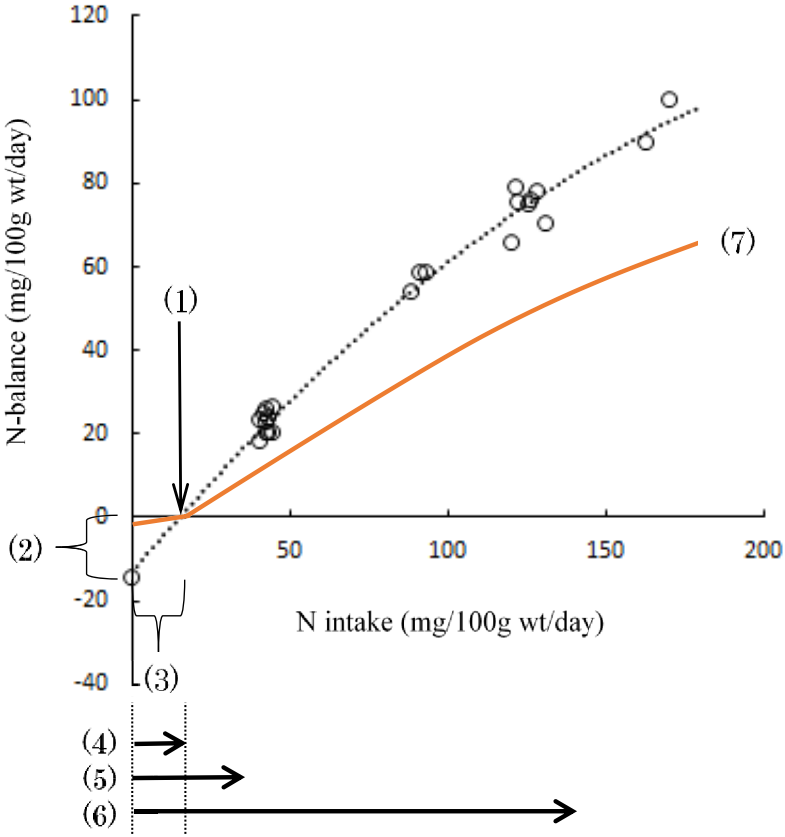

Fig. 3-1. Theoretical parallel relationship between body P and body N retention.
In the figure, numbers in parentheses indicate the following. The N data is from **Ogino** (1980a).
(1) Maintenance N intake. Up to this level, all dietary P will be excreted on a net basis.
(2) Endogenous obligatory loss of N when fed an N-free diet. The loss of N (from soft tissues) accompanies a corresponding amount of P excretion. In this figure, the Y-intercept is -14.6 mg N (11.9 mg urinary +2.7 mg fecal; **Ogino** 1980a). This is approximately 0.456 g of muscle tissues (as muscle contains 20% protein, or 3.2% N; **MEXT** 2015). The same amount of muscle contains approximately 1.127 mg of P (as the muscle P content is 2472 ppm; **Shearer** 1984). Hence, the obligatory N loss (-14.6 mg N) is accompanied by 1.127 mg of P excretion. This is the fed state with an N-free diet. In starvation, however, fish loose ~0.4% of body wt/day (**Hepher** 1988, p. 165). This is slightly lower but close to the value (0.456 g) mentioned above. The difference may be due to the lower fecal loss in starved fish. Hence, in this figure, the N intake (X-axis) can approximate the feeding rate, or feed intake of fish. In prolonged starvation, fish might excrete substantially more N in order to supply the energy required for maintenance using waning muscle tissues. In this state, the obligatory P excretion

also increases. The stoichiometric relationship in this negative N-balance (catabolic state), the excreted N:P ratio follows the same ratio in the waning soft tissues since bone resorption is negligible (cf. §6, §10). However, in the positive N-balance (anabolic state), the stoichiometric N:P ratio follows that of the whole body (including hard tissues). Hence, unlike N and energy balances, the P-balance shows a biphasic response as indicated by the red line in the figure.
(3) Up to the maintenance intake (of N or feed), there is no net requirement of P (cf. §59 Endogenous P-excretion).
(4) Dietary P-requirement varies depending on the intake of N or feed (and other factors that affect fish growth). Up to the maintenance level (no somatic growth), no P is required on a net basis. At or below the maintenance dietary intake (either in feed intake or protein intake), fish will need to excrete all dietary P in order to maintain P-balance.
(5) At low feed intakes with only a slight somatic growth (e.g., aquarium fish, winter feeding), the dietary requirement of P can be low.
(6) At high feeding rates, the dietary P requirement will increase in order to increase the whole body mass (an amount approaching the standard body P content given in Table 2).
(7) Shown by the red line: P retention (above maintenance) and loss (below maintenance). Note different N:P stoichiometry.

Bureau et al. (2006) demonstrated the relationship between fish growth rate and the progression of P deficiency. The authors fed rainbow trout with the same diet, but at four different feeding levels (i.e., 25%, 50%, 75%, and 100% of satiation). The experimental design is similar to **Fig. 3-1**. Fish (initial bw 158 g, final max 621 g) were fed for 24 wk at 8.5°C with a practical-type diet (total P 1.1%, containing fish meal, corn gluten, soybean meal, etc.). The feed efficiency (gain/fed) was nearly 1 at or below 75% feeding levels, but was low (0.83) at satiation feeding. The available P content of the diet was about 0.48% (when calculated based on the P content and P digestibility of respective ingredients, using literature values), whereas it was about 0.80% (when calculated based on the total P × ADC that the authors reported: 73%). These two values are very different, and, therefore, either is incorrect. They presented P-retained by fish (g) and the wt gain of fish (g). Using these data, the fractional (partial) body P% per body wt gain can be calculated, which will be 0.53% (at 25% feeding, near the maintenance level), 0.44% (at 50% feeding), 0.39% (at 75% feeding), and 0.37% (at 100%, or satiation feeding). These values indicate that fish were apparently P-sufficient at a 25% feeding level, slightly P-deficient at a 50% feeding level, and clearly P-deficient at 75% and 100% feeding levels (cf. **Table 2** for normal body P content). At high feeding levels, fish showed typical P-deficiency signs, including the decreases in body ash, body P, moisture, and feed efficiency, and the increase in body fat (cf. **Table 1**). The increase in body fat deposition, in turn, decreased the feed efficiency due to the shift of energy use from growth (N-gain) to a more energy dense fat gain. The almost-linear but with slightly decreasing rate (curvilinear) relationship between ME (metabolizable energy) intake and RE (recovered energy, in both protein and fat) suggests a possible onset of futile cycles or thermogenesis (proton leak) under P deficiency, which also decreases feed efficiency. At high feeding levels, the feed efficiency typically decreases, which has been clearly shown by calculating the marginal feed efficiency (**Hepher** 1988, pp. 302-303). The high carcass P content in feed-restricted fish was explained (by authors) as due to the decrease in soft

tissue mass relative to bone mass (i.e., marasmic), which, however, is not supported by their data of body protein content that was similar regardless of the feeding levels. The body protein (N) content is known to be relatively constant across life stages and dietary factors in fish (**Shearer** 1994) and in mammals (**Kleiber** 1975, p. 58). In starved mice, the whole-body fat content decreased, water content increased, and ash and protein content unchanged (**Kleiber** 1975, p. 58).

Glencross et al. (2008) fed rainbow trout for 28 days with three different diets and at three different feeding levels. A starved group of fish for the same duration was also included to establish the intake-gain relationships for energy, N, and P. In starved fish, the energy balance was largely negative, the N-balance was less negative, and the P-balance was only slightly negative. This difference indicates that the starved fish use body fat as the initial energy source. As fats contain little N and P, these excretions increase little. However, for N, the daily obligatory loss was excreted, and the corresponding amount of P (following the N:P ratio in muscle) was excreted. Hence, during early stages of starvation, the P loss is relatively small. However, for the fed fish, the N:P stoichiometry follows that of the whole body (both soft and hard tissues). Consequently, the slope shows a more steep response, which is obviously seen in the reported data (Fig. 3 in their paper). In this study, the P retention was considerably lower at a low feeding rate than higher feeding rates. The P retention, however, tended to decrease at the highest (satiation) feeding rate, probably due to increased lipogenesis as indicated by the reduced N-retention and increased energy retention at satiation feeding.

§ 4. Concepts of response criteria

Various overt deficiency signs or clinical signs have been used in order to assess the adequacy of nutrition or to establish dietary requirements for essential nutrients. The mortality rate is the most definitive sign, and this too can be used to establish dietary requirements for some nutrients, especially during early ontogeny. Various deficiency signs have been reported for each essential nutrient for various animal species. In fish nutrition research, **McCay** et al. (1927) once wrote, "In evaluating the effectiveness of the diets we have employed two criteria, the rate of growth and the rate of death." This is a rational approach to establishing nutrient requirements since both criteria are practically important. Other responses, such as feed efficiency, economic efficiency, disease resistance, fish (fillet) quality, and environmental effects, are also self-explanatory. However, one wonders upon what grounds maximizing the bone density, tissue accumulation (saturation), blood levels, enzyme levels, and gene expressions should be justified as the basis to establish dietary requirements. Animals could be simply adapting to the changes in dietary intake levels by responding physiologically in order to compensate for the decreased or increased intakes, which may not indicate or predict the clinical deficiency. A dietary level that can maximize the tissue saturation of a certain mineral or vitamin does not indicate that the animal requires that much nutrient intake to maintain optimum health. Further, at the body saturation level of nutrient intake, fish could well excrete considerable portions of dietary intake via the urine or gills, which will be environmentally undesirable. The response criteria, therefore, cannot be chosen based solely on the sensitivity or responsiveness. The criteria should be chosen primarily

based on the importance. When physiological responses are used, instead of practically important responses, to establish dietary requirements, the rational basis for the use of such responses should be given.

Also, it has been reported in some essential nutrients that the oral dose to maximize certain responses (e.g., disease resistance, enzyme activity) is often a pharmacological level far exceeding the minimum requirement that can furnish normal performance of animals and humans. Using such response criteria to establish the requirements of dietary nutrients can be justified, but it should be categorized separately (not as an essential nutrient, but as a drug or immuno-stimulant). For P, such pharmacological effects may or may not exist, as will be discussed in the following sections.

In pigs and chickens, it has been well established that the dietary requirements of P for maximum bone strength and bone-ash content are higher than the requirements for maximum wt gain (**Sauveur & Perez** 1987; **NRC** 1998). **Ogino & Takeda** (1976) reported that the dietary requirement of available P for maximum growth of juvenile carp (initial bw ~4 g, final 7-12 g) was 0.6-0.7%, and that for maximum bone mineralization was higher (~1.5%) than that for optimum growth. **Bureau & Cho** (1999) reported that increasing dietary P intake had no significant effect on growth and feed efficiency, but significantly increased the P content of the whole carcass, vertebrae, plasma, and urine. **Rodehutscord** (1996) noted in rainbow trout (initial bw 53 g, final max 200 g) that the P requirement for maximum gain (3.7 g/kg diet) was lower than that for maximum P deposition or bone calcification (5.6 g/kg diet). The author determined the dietary P requirements (?) based on various (nine) response indicators, which were all different. Such differences may be due to (1) different sensitivities of the response indicators and (2) different concepts of the requirements (i.e., requirement vs. saturation). **Dougall** et al. (1996) studied Ca and P levels in scales, vertebrae, dorsal fin, and serum of striped bass. They noted that ash, Ca, and P in bones and scales were sensitive, while serum P was not. The authors took an average of the requirements determined based on different response variables and from different trials (fish size, feeding period, etc. were different). **Skonberg** et al. (1997) reported that fish growth and feed efficiency were unaffected by dietary P (0.23-1.16% P) in an 8-wk feeding trial with juvenile rainbow trout; however, ash, P, and Ca levels in the fish skin (with scales) and whole body were highly responsive to dietary P. The authors also noted that the plasma P and Ca levels and intestinal alkaline phosphatase (ALP) levels were quite insensitive, while plasma ALP and body lipid levels showed some responses to dietary P levels.

Eya & Lovell (1997) noted that channel catfish (initial bw 61 g, final 569-634 g) fed five different diets of varying available P content (from 0.2 to 0.6%) did not show any significant differences in wt gain, feed conversion, and dressing percentage in a 140-day feeding trial in earthen ponds. The diet had feed conversions between 1.7 and 2.0. Serum P, bone ash, and bone P increased linearly, while muscle fat and visceral fat decreased linearly as dietary P increased. The dietary available P requirements to maximize serum ALP activity and bone-breaking strength were 0.25 and 0.31%, respectively. The authors also suggested a possibility of feeding more P than the minimum requirement to reduce fish body fat if there is an economic benefit. **Lewis-McCrea & Lall** (2010) wrote, "The nutritional status of phosphorus in fish is best represented by bone ash and phosphorus concentrations as they are sensitive indicators of dietary phosphorus intake." **Prabhu** et al. (2013) reviewed 64 studies on P requirements of various fish species. The authors

reanalyzed the reported available P requirement data (%, dry diet) based on a meta-analysis and broken-line regression with a linear-plateau model, which varied greatly depending on the response criterion used: 0.47% based on whole-body P content, 0.52% based on vertebral P, 0.35% based on maximum wt gain, and 0.28% based on plasma P. Among these, the authors indicated that the wt gain of fish was the "most reliable", while the whole-body P content was the "most stringent." **Prabhu** et al. (2016) wrote, "In general, weight gain as the criterion resulted in a lower estimate (by 18–42%) than those obtained using whole-body or vertebral mineral concentrations as response criteria."

Since nutrients are supplied not only from diets, but also from the body pool (body reserves) that the fish initially have, the feeding duration has to be sufficiently long to minimize the effect of the latter (**Baker** 1986). Alternatively, the dietary requirement of a nutrient can be better determined at various time intervals until the estimated requirement values stabilize. In trout, **Sugiura** et al. (2007) noted that the serum P responded quickly to dietary P concentrations, but it took several weeks to give a stable response even under well-controlled experimental conditions. Also, the authors reported that bone P responded slowly to dietary P levels and it could take many weeks of feeding before reaching a stable response. They further indicated that molecular markers (mRNA) generally did not show any stable responses (i.e., for many genes, the response to dietary P was clear during the acute phase, but unclear during the chronic phase). Traditionally, the P status of fish has been estimated based on such response criteria as growth rate, bone P, bone ash, and blood P levels (**Lall** 1991). However, the sensitivity and accuracy of these indicators are insufficient, especially in the early phase of P deficiency or excess. Thus, alternative indicators that can determine or even predict forthcoming clinical P deficiency or excess with greater sensitivity and precision are needed.

In medicine, because of the prime importance of "early detection, early treatment," various early detection methods have been studied and developed, especially in the past few decades with the advent of molecular technology. Such early detection/diagnostic tools, including biochemical markers, are all non-invasive methods (e.g., analyzing blood or urine samples, X-ray, CT, MRI, etc.). In fish, however, invasive methods can be used (except perhaps for endangered species, expensive Koi, etc.). Due to this difference between humans and fish, diagnostic methods to be used in fish can be direct (e.g., analyzing the bone P content) rather than using medical instruments that are often more expensive, more technical, and yet less accurate and reliable. Advanced high-tech methods that are used in humans may not be suitable as a routine diagnostic method in aquacultured fish. This difference must be recognized when choosing "practical" P-response indicators and diagnostic methods. In the following sections, we discuss various conventional and potential indicators of the P-status of fish.

§ 5. P deficiency & Growth, feed intake, feed efficiency

Using growth as the response indicator to establish nutrient requirements of fish has two major reasons. First, it is the most important economic or practical trait in aquaculture management. Second, it estimates the adequacy of nutrient intakes on an overall and conclusive basis, and, therefore, it will be the final proof of the nutritional

adequacy (with some exceptions). **Knauthe** (1898) reported that carp increased both wt gain and N-retention when meat ash was added to rations of meat meal and corn meal or meat meal and rice meal. Meat ash, which is low in Ca and high in P, is highly bioavailable to agastric fish like carp. **Knauthe** also reported that when meat ash was withdrawn from a meat meal-rice meal diet, digestibility of protein by carp decreased from 91.2% (with meat ash) to 89.6% in the first 5 days and to 83.2% in the next 5 days. Digestibilities of fat and carbohydrates decreased likewise. He also reported that the fish reduced appetite, reduced body protein, and reduced fat deposition and fat synthesis from carbohydrates. **Knauthe** formulated a diet for mature carp, which contained more carbohydrates (as corn) and less protein (as meat meal) than a diet for young fish. The author suggested fortifying the former with basic Ca phosphate, probably because increasing carbohydrates resulted in a decrease in the P content of the diet.

McCay et al. (1927) noted that brook trout (initial bw ~2 g) fed a diet containing casein, starch, cod-liver oil and yeast for 12 wk were very listless and markedly abnormal compared with those fed the same diet but supplemented with Osborne & Mendel's salt mixture. The growth, mortality, and the body ash content were, however, not different. In this case, the primary response of fish to the dietary treatment is the behavior or appearance. **Sekine** et al. (1929) reported the result of a 63-day feeding trial conducted in 1927. They noted that the ash content of rainbow trout fry (initial bw 0.18 g) was higher when the fish were fed a silkworm pupae-based diet supplemented with Osborne's salt mixture than when the fish were fed the same diet but without the salt mixture. **Sekine & Kakizaki** (1929) reported the results of a study conducted in 1926-27 in which they fed salmon fry for 38 days with either cooked rice, shark meal, sardine meal, or raw sardine as a sole diet. One group of fish was starved during the same period. A group of fish fed raw sardine showed the highest survival (97%) and growth. The fish fed rice did not grow, and the survival rate was lower than the starved group. However, both starved fish and the fish fed on rice increased their body Ca content more than twice, while the P content increased only slightly and the Mg content decreased markedly during the period. The fish fed shark meal, sardine meal, or raw sardine increased the weight and the retention of Ca, P, Mg, N, and lipids.

Sekine & Sato (1933) reported the results of a feeding trial conducted in 1930-31 with sockeye salmon (initial bw 0.17 g, final ~40 g, fed 391 days). The authors studied the supplemental effect of tri-Ca phosphate (and Fe-citrate) using a diet containing fish meat [sic], silkworm pupae, rice bran, flour and a small amount of cod-liver oil. The basal non-supplemented diet contained 23% protein, 55% carbohydrate, 13% lipids, 0.45% Ca, 0.85% P, and 0.035% Fe (dry basis). Five grams of $Ca_3(PO_4)_2$ and 0.2 g of Fe-citrate were added to 700 g (dry wt) of the basal diet. The fish fed the P and Fe supplemented diet grew markedly better than those fed the basal diet; however, the survival rates (mortality) did not differ. The fish fed the P and Fe supplemented diet had higher percentages of ash, Ca, P, Fe and Mg in the body (dry basis) than the fish fed the basal diet, whereas the percentages of protein and fat did not differ. **Krockert** (1938) fed brook trout with a diet containing 95% dried livestock blood, 4% dried potatoes, and 1% Ca phosphate. This diet was proven to support good growth of the fish, easily obtainable, and cheap. Increasing the amounts of potatoes (to 17.5%) and Ca phosphate (to 2.5%), and supplementing the diet with vitamins were suggested to increase the growth of the fish.

In livestock animals, especially in cattle, general symptoms of dietary P deficiency, such as growth depression, loss of appetite, and weakness, were formally called aphosphorosis. Aphosphorosis is also accompanied by the reduction in feed efficiency. **Eckles & Gullickson** (1928) reported that a dietary P deficiency decreased the utilization of feed in cattle. The cattle fed a low-P feed required 20% more feed in order to give a comparable growth to the normal-P animals. **Theiler** (1933) reported that ruminant animals given sufficient P utilize their feed better and gain more per unit of feed consumed than do animals on a low-P diet. **Riddell** et al. (1934) and **Kleiber** et al. (1936) also reported in cattle that the P deficiency lowers the efficiency of food utilization for wt gain. Lack of appetite, they noted, was one of the most conspicuous symptoms in P-deficient animals. **Aubel** et al. (1936) reported similar results in pigs. Loss of appetite is common in P-deficient animals (**Morrison** 1959, p. 98). Interestingly, **Aubel** et al. (1936) noted that P-deficient pigs greatly increased the consumption of water (drinking), while the volume of urine also increased. Thus, the net water retention by the animals was less in P-deficient pigs than P-normal pigs. The authors did not discuss why P deficiency increased the drinking rate. In animals, dietary P deficiency does not affect digestibility of dietary nutrients, but the digested nutrients are utilized inefficiently (**Riddell** et al. 1934; **Kleiber** et al. 1936; **Morrison** 1959, p. 98). In humans, however, a moderate dietary P fortification (above the normal or RDA level) decreases food intake and appetite (**Ayoub** et al. 2015; **Bassil & Obeid** 2016) — i.e., dietary P deficiency increases appetite and, therefore, obesity.

The growth rate is a definitive measure of sufficiency or deficiency of essential dietary nutrients. However, the poor growth is not an early sign of dietary P deficiency because fish have body P stores that can support normal growth for a certain period (**Hardy & Barrows** 2002; cf. §23 P requirements of large or adult fish). Upon depletion of body P stores below a certain threshold level, the P-deficient fish start to decrease the growth rate as reported by numerous studies with various fish species (**Table 1**). The major disadvantage of this measurement (i.e., weighing fish) is its slow response to dietary perturbations. Hence, the growth response of fish has been reported to be insensitive to dietary P restriction by some researchers (e.g., **Hardy** et al. 1993; **Eya & Lovell** 1997). When using the growth or other slow response indicators, it is critical to estimate the dietary P requirement at several time points, and to continue the feeding trial until the estimated requirement values stabilize. This is important in order to avoid reporting misleading requirement values due to insufficient feeding duration. Alternatively, the growth depression of fish can be more sensibly detected by measuring the N-excretion of fish, especially large fish (**Sugiura** 1998). As the N-retention indicates the protein accretion, or lean growth of fish, the increase in N-excretion means the growth depression. **Yang** et al. (2006) showed that the growth data (wt gain) of silver perch obtained from an 8-wk feeding trial agreed quite well with the data of 24-h ammonia excretion by the fish.

§ 6. P deficiency & Bone mineralization

Unlike growth and mortality, no practical meaning is associated with maximizing the P or Ca content of bones, scales, other tissues, or whole body. However, when dietary P

intake is lower than required, the concentration of P in certain tissues (body stores) decreases well before the growth decreases. It is, therefore, useful to measure the tissue P concentration to estimate/diagnose the adequacy of P intake. However, when dietary P intake decreases, fish compensatory increase the efficiency of P absorption by changing some enzyme and hormonal concentrations in the body or upregulating P transporters at the absorption sites (i.e., intestine, kidney, and possibly other tissues). These physiological mechanisms help fish cope with suboptimal P intakes, but to a limited extent. Hence, slight or moderate decreases in P concentrations in certain tissues do not necessarily indicate or predict a clinical P deficiency. Also, "maximizing" the bone ash or body P content may involve some toxic or adverse effects of excessive dietary P intake (cf. §47 Toxicity of excess P).

Fordyce (1791) reported that when his canary hens were fed seeds, many of the birds died, but when they received the same seeds and a piece of old plaster they were in good health. Fordyce concluded that canaries require a calcareous supplement to the seed diet. His experiment with fish, however, showed that fish were independent of a source of bone-forming materials. The fish were not given any food for months, but they grew rapidly and were healthy. **Gahn** (1769), **Scheele** (1771), and others conducted the first quantitative analysis of P in several fishes and their bones, and discovered that fish bone consists of Ca salts of phosphoric acid (cited in **Vinogradov** 1953). In 1790, **Lavoisier** wrote, "Phosphorus is found in almost all animal substances, and in some plants which give a kind of animal analysis."

Bobba (1801) presented his theory on the cause of rickets in humans. He thought, "by a derivation of the phosphat [sic] of lime from the bones to the joints (in rickets), symptoms of gout are produced, at the same time a mollification of the bones, which complication is named *arthritis rachitica*." He thought, "bad quality of the milk with which children are nourished is likely to be a frequent remote cause of the rickets." **Johnson** (1803) reported that chickens fed Ca phosphate had harder bones, and that Ca phosphate had also been fed profitably to children and pregnant women as a means of improving soft bones and healing fractures. **Lawrence** (1829-30) wrote, "In cases of rachitis, . . . we find less earthy matter and a greater proportion of animal substance than is natural. We find that the bones, in rickets, often admit of being cut with the knife." **Brodhurst** (1868) wrote a similar account. Also, May **Mellanby** (1918) made a similar comment on rachitic puppies, "the deficiency in calcium salts (in teeth) may result in the teeth being so soft that they can be cut with a scalpel."

Guerin (1839), a French surgeon, fed some puppies on meat, and reported that they developed rickets; whereas the control animals, which were suckled, did not develop rickets. **Chossat** (1842, 1843) found that pigeons fed on wheat alone died in 10 months, during which period salts were gradually withdrawn from the bones which thereby became fragile, and that this was prevented by giving a supplement of Ca carbonate. He mentioned that P in the wheat was not utilized because of deficiency of Ca. **Bibra** (1844) published a book of 430 pages devoted to chemical analyses of bones of mammals, birds, reptiles, and fishes. He showed deviations of ricketic, osteoporotic, and osteomalacic bones from the composition of normal bones in the proportion of organic to inorganic constituents. **Bishop** (1848) wrote, "During the period of the incubation of this disease (i.e., rickets) all the bones of the skeleton are more or less affected: they not only become soft and pliable, but their chemical and mechanical structure also undergoes a change."

And further, "They are lighter than natural, . . . being porous, soft, spongy, and compressible."

Lehmann (1851, p. 413) wrote, "In healthy human bones the phosphate of lime ranges from 48 to 59%; in softening of the bones it may sink to 30%. It is, however, singular that in almost all diseases of the bones, whether the results of osteoporosis, osteomalacia, or osteopsathyrosis, we find a diminution of the phosphate of lime." **Anderson** (1878, p. 122) wrote, "rickets, which clearly shows, on chemical examination, is a deficiency disease of the inorganic matter . . . either the food is wanting in phosphate of lime, or there is a defect in its assimilation." And, "In rickets, bone becomes soft and pliable, yielding to any weight or strain put upon it, so that the lower limbs become bowed, the spine curved, and the cranium enlarged; the skeleton, from its imperfect construction, fails to fulfil the duties which properly belong to it. In rickets the inorganic deficiency is recognized, as productive of the disease, because the deficiency is obvious. The inorganic material bears a large proportion to the organic, and as the construction of bone is known, any great alteration in the relative proportion of organic and inorganic matter, is readily apparent; but in structures which show a small proportion of inorganic matter, deficiency of this may readily be overlooked . . . ." **Anderson** (1878) also presented numerous data on the P content (and major bases) of various tissues (tendon, skin, kidney, lung, brain, heart, aorta, and spleen) in various animal species (ox, pig, sheep, human) under normal and diseased states. The author compared the differences in the P content and P/base balances among different organs and species. He also presented data on the P content of healthy urine and feces of humans on different diets. The author did not, however, directly study the effect of different P intakes.

According to Fernandes de **Barros** (undated), the ratio in which the carbonate of lime stands to the phosphate in the bones is 1:3.8 in the lion, 1:4.15 in the sheep, 1:8.4 in the hen, 1:3.9 in the frog, and 1:1.7 in a fish. **Weiske** (1883) reported that the vertebrae of carp and pike contained about 34% organic matter and 66% inorganic matter. The bone ash contained ~54% CaO and 46% P_2O_5 and a trace amount of Mg. **Heubner** (1909) noted that rickets could be produced in dogs by feeding diets very low in P. He used egg-albumin as a source of protein. **Hart** et al. (1909) fed pigs for 3-4 months with diets of a low-P content or with one of the following P supplements: precipitated Ca phosphate, bone ash, rock phosphate, and phytin. They estimated the biological value of these P sources and the approximate P requirement based on various responses, such as wt gain, bone-breaking strength, bone ash content, bone density, bone wall thickness, bone diameter, and Ca and P content in various bones, blood, muscle, liver, and other tissues of the body. They also estimated the dietary P requirement based on P balance (i.e., intake minus excretions via feces and urine). **Burnett** (1906, 1910), **Alway & Hadlock** (1909), and **Forbes** (1909) conducted similar studies. **Kellner** (1913, p. 97) wrote, “On examining bones of such miserably grown animals (i.e., osteomalacia) it is seen that the parts, notably on the ends of the joints, are composed of soft cartilage in which lime and phosphoric acid are only slightly deposited."

Embody & Gordon (1924) wrote, "Calcium and phosphorus are used in the building of the skeleton and especially important is it that the intake of these two constituents of the food be adequate for use in the case of rapidly growing young trout (in the feeding of hatchery trout)." In rickets, **Hess** (1929, p. 147) wrote, "No difference has been found in the potassium or sodium content of the bones in rickets. However, numerous

investigators have reported an increase in magnesium. **Gassmann**, for example, gives the figure of 0.1 per cent for normal bone and 0.53 per cent for rachitic bone, and states that there is a similar increase in the teeth." **Hess** (1929) also reported that the ash to organic matter in the normal bone is about 3 to 2; however, in rachitic bones, the ratio may be reduced to 1 to 4 with corresponding decreases in Ca and P.

Hara (1930) studied the N, ash, Ca, and P content in defatted-ground bones of 4 yr-old mackerel, 2 yr-old trout, sardine, pigs, rabbits, and dogs. The bones contained 3.45-5.30% P (air-dry basis) in mackerel (n = 6), 3.74-4.89% P in trout (n = 2), and 2.61-4.71% P (n = 6) in sardine. The author noted that the bone Ca content tended to be higher in females than males, while the bone P content was higher in males than females. **Morgulis** (1931) analyzed bone ash of various animal species for Ca, Mg, K, PO_4, and CO_2. He reported that the main difference in the chemical composition of the bone ash between marine fishes and a variety of higher vertebrates was in the proportion of $CaCO_3$, which was only about one-half in the former than the latter. The author also mentioned that the PO_4/CO_3 ratio was variable, being markedly lowered by rickets and P deficiency, and that the principal component of bone ash was $Ca[\{Ca_3(PO_4)_2\}_6](OH)_2$. **Shimada & Kaneda** (1937) reported that the bones of carp contained less ash, Ca, and P than those of seabass, cod, and seabream.

According to a treatise of **Vinogradov** (1953), fish bone contains from 35 to 58% of protein and up to 65% of mineral residue. Fish teeth contain somewhat less organic matter. In fishes with hard bones (e.g., seabream, pollock, seabass, etc.), there is more mineral residue than in those with soft bones (e.g., carp) whose scales show relatively higher Ca than P. Unlike other hard tissues, the mineral constituents of otoliths are mostly $CaCO_3$ with trace amounts of Mg, P, and other elements (in the case of cod, 90-96% is $CaCO_3$). **Vinogradov** (1953) tabulated the mineral composition of various fish species reported by that time. Citing some from his Table 323, the ash content (%) of dry bone is 45 (sardine), 42 (carp), 64 (cod), 53 (seabass), 65 (seabream). The bone $Ca_3(PO_4)_2$ content (%/ash) is 80 (shark), 94 (shad), 93 (sardine), 75 (carp), 74 (cod), 77 (seabass), 82 (drum), 78 (seabream), 84 (mackerel), 83 (anglerfish). The bone $CaCO_3$ content is 7 (shark), 3 (shad), 6 (sardine), 24 (carp), 24 (cod), 22 (seabass), 7 (drum), 21 (seabream), 6 (mackerel), 7 (anglerfish). Compared with bones, scales tend to contain more Ca and less P in ash (cf. §9 Scales, fins).

Launer et al. (1978) used neutron activation analysis to determine the P and Ca content in fish samples, and reported that the P content of eviscerated channel catfish and its fat-free skeleton were highly variable depending on the sampling season. Bone P increased during the wintering (non-feeding) period, but bone Ca decreased during the same period. **Weiss & Watabe** (1978) noted that the bone resorption in fish causes the loss of Ca-phosphate from the bone, but Ca-carbonate remains in the bone, resulting in an increase in bone carbonate. **Urasa** et al. (1985) reported that the maturing female tilapia fed a P-free diet for 6 wk had a markedly low P content (~44% decrease) in the opercula bone compared with female controls, but the Ca content was similar, indicating that P was selectively extracted from the bone. This resulted in an increase in the Ca/P molar ratio from 1.6 in the control fish to 2.6 after 6 wk on the P-deficient diet. The authors also noted a similar selective reduction of P in scales.

Takeuchi & Watanabe (1982) reported that the ash, Ca, Mg, and P content and the Ca/P ratio in the vertebrae of carp (initial bw 13.2 g, final 8.6 g) did not change during

86 days of starvation at 25°C, although the body protein content decreased from 14.5 to 6.8% and the body lipid content from 4.8 to 0.7% (wet basis) during this period. Percentages of ash and water (wet whole body) steadily increased from 3.2 to 4.2% and from 79 to 90%, respectively. These results suggest that starvation per se does not reduce bone minerals, while minerals in muscles, including a certain amount of P, will be lost during prolonged starvation (cf. §10 P deficiency & Body P content; §59 Endogenous P excretion).

Hamada et al. (1995) studied bone ash of 15 fish species plus cattle, swine, and fowl. They used both X-ray diffraction analysis to determine the crystal structure of the bone ash, and elemental analysis (13 elements analyzed) to determine the composition. The results showed that some fish had hydroxyapatite-type bones, while others had tri-Ca-type bones or intermediate of these two types. These authors suggested that since Ca of hydroxyapatite can easily be substituted by Mg and other elements, (Ca+Mg)/P ratio rather than Ca/P ratio may give accurate estimates for the theoretical value: i.e., Ca/P molar ratio in hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, which is 1.67 or that in tri-Ca phosphate, which is 1.50 (**Irving** 1973, pp. 21-28). The (Ca+Mg)/P molar ratio that the author determined ranged 1.47-1.63. **Hendrixson** et al. (2007) analyzed skeletons of eight fish species. The bones contained 25.3% Ca and 11.8% P on average. The Ca:P ratio of bones was 2.14 on average (by mass) — close to the 2.15 ratio of hydroxyapatite.

Toppe et al. (2007) reported the composition of bones of nine fish species. The bone ash content (whole bones, fat-free dry basis) was 42% (salmon), 44% (trout, mackerel), 48% (small herring), 52% (large herring), 50% (blue whiting), 58-59% (small and large pollock), 58% (cod), and 62% (horse mackerel). The Ca/P ratio of bones was 1.66 (mackerel), 1.67 (salmon), 1.68 (cod), 1.69 (trout), 1.71 (small herring), 2.07 (large herring), 1.84 (Pollock), 1.95 (blue whiting), and 2.10 (horse mackerel). **Albrektsen** et al. (2009) noted that seawater Atlantic salmon fed P-deficient diets did not alter the vertebral Ca/P ratio (1.82-1.85), whereas the vertebral P and Ca content were greatly decreased. However, these values, as well as other values shown in **Table 2**, are considerably variable and generally lower than the theoretical values mentioned above. According to **Lall** (1991, 2002), the Ca/P ratio in scales and bones of various fish species ranges from 1.5 to 2.1, and the ratio of Ca/P in the whole body ranges from 0.7 to 1.6. About 86-88% of the total body P is found in fish bones, but fish scales also contain large amounts of Ca and P (cf. §9 Scales, fins; §10 Body P content).

For humans, techniques of measuring bone density must be non-invasive, low-toxicity, and reasonably accurate. Various techniques have been used/tested depending on the purpose (i.e., diagnostic tools, fracture prediction, and osteoporotic healing), including digital X-ray radiogrammetry (DXR), single- or dual-photon absorptiometry (SPA, DPA), single- or dual-energy x-ray absorptiometry (SXA, DXA, or DEXA), quantitative computed tomography (QCT), micro-CT, microdensitometry (MD), quantitative ultrasound (QUS), and MR imaging (for detail, cf. **Johnston** et al. 1996; **Patel** et al. 2015). Among these, DXA is the most common method to assess bone mineral density (BMD) due to its accuracy, rapidity, low cost, and low radiation exposure (**Schaafsma** 1997; **Patel** et al. 2015). However, as DXA measures BMD in terms of area, DXA tends to overestimate the areal BMD in larger bones (**Patel** et al. 2015). BMD is measured in the lumbar spine, hip, or forearm, and is expressed in g/cm^2. Whereas DXA measures BMD, various bone markers, in contrast, indicate the rate of bone remodeling.