HANDBOOK OF NUTRITIONALLY ESSENTIAL MINERAL ELEMENTS

edited by
Boyd L. O'Dell
Roger A. Sunde

HANDBOOK OF NUTRITIONALLY ESSENTIAL MINERAL ELEMENTS

CLINICAL NUTRITION IN HEALTH AND DISEASE

Series Editor

LAWRENCE J. MACHLIN
Nutrition Research and Information, Inc.
Livingston, New Jersey

1. Folate in Health and Disease, *edited by Lynn B. Bailey*
2. Handbook of Nutritionally Essential Mineral Elements, *edited by Boyd L. O'Dell and Roger A. Sunde*

Additional Volumes in Preparation

HANDBOOK OF NUTRITIONALLY ESSENTIAL MINERAL ELEMENTS

edited by

Boyd L. O'Dell

Roger A. Sunde

University of Missouri
Columbia, Missouri

MARCEL DEKKER, INC. NEW YORK • BASEL

Library of Congress Cataloging-in-Publication Data

Handbook of nutritionally essential minerals / edited by Boyd L.
O'Dell, Roger A. Sunde.
p. cm. — (Clinical nutrition in health and disease ; 2)
Includes bibliographical references and index.
ISBN 0-8247-9312-9 (hc : alk.)
1. Minerals in human nutrition. I. O'Dell, Boyd L. II. Sunde,
Roger Allan. II. Series.
QP533.H36 1997
612.3'92—dc21

97-55
CIP

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the address below.

This book is printed on acid-free paper.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Current printing (last digit):
10 9 8 7 6 5 4

Printed in the United States of America

Preface

Essential mineral elements play a major role in the nutrition of humans and all animals. In fact, the number of essential mineral elements exceeds the number of vitamins or amino acids that are essential to the diet; however, their quantitative requirements are far less than those for protein, fat, and carbohydrate. Because life evolved in a milieu composed primarily of inorganic molecules, primitive organisms took advantage of the available inorganic elements to serve as catalysts, even as signaling agents, in the support of metabolism. Later, many of the elements were incorporated into protein constructs (typically metalloezymes) that improved their efficiency.

Modern approaches to the determination of mineral nutrient requirements began in the 1920s with the development and use of semipurified diets, but elucidation of the biochemical roles of these nutrients, particularly of the micro (trace) elements, did not emerge until the 1970s. This occurred with the unraveling of now well-established metabolic pathways and protein structures, detailed membrane and subcellular composition, and biochemical mechanisms by which genes are expressed. Over the past 20 years mineral elements have been shown to play key roles at nearly every step of the many life processes. These range from the role of calcium as a second messenger, to the role of zinc and "zinc fingers" in gene transcription, to the selenium-specific codons and insertion elements in mRNA translation. The integration of "mineral nutrition" and molecular biology creates the need for a handbook that encompasses molecular mineral nutrition—a book that provides a single source of advanced and integrated knowledge about all the essential mineral elements.

For historical reasons, the essential mineral elements are commonly divided into macro and micro categories. There is some excuse for this separation based on function, but in general such classification is unjustified; for example, calcium is a major component of bone but micro quantities serve as a second messenger. The number of micro elements recognized as nutritionally essential has increased dramatically since 1948, the end of the era of vitamin discovery. Because of the extraordinary interest in trace elements during the past four decades, numerous reviews and books related to this subject have appeared.

In contrast to the coverage of micro elements, there are few books that cover the

nutritional properties of the macro elements (calcium, phosphorus, potassium, sodium, chloride, and magnesium). In fact, this may be the first single volume that encompasses the roles of both macro and micro elements in nutrition, physiology, and biochemistry. Obviously such broad coverage demands exclusion of some information, but this book distills the essence of knowledge related to the physiological and biochemical functions of all essential mineral elements. To achieve this distillation, the expertise of internationally recognized scholars in their respective areas of mineral nutrition has been assembled.

While the handbook is designed primarily for students and professionals in nutrition (clinical, applied, and basic), it is a valuable source of information for students, teachers, and researchers in all areas of biology, including biochemistry, physiology, pharmacology, and medicine. In addition to the roles of mineral elements in animal nutrition, their analogous roles in plants and microorganisms are discussed where appropriate to provide coverage of the full range of biological functions. Recent work with prokaryotes and plants has provided valuable insights into mineral element function. Consequently, modern molecular nutrition is not restricted to higher animals.

The contributors have prepared and freely incorporated models and figures that can be used in the classroom to present basic concepts. Not only have the authors presented much detailed and specific information, but they have also provided comprehensive references for those who wish to pursue the subject. The editors wish to express their sincere appreciation to the authors for their cooperation and patience as well as their excellent contributions.

Boyd L. O'Dell
Roger A. Sunde

BIBLIOGRAPHY

Burk, R. F. (Ed.). *Selenium in Biology and Human Health.* Springer-Verlag, New York, 1994.

Ciba Foundation Symposium. *Biological Roles of Copper.* Excerpta Medica, New York, 1980.

Mertz, W. (Ed.). *Trace Elements in Human and Animal Nutrition, Fifth Edition.* Vols. 1 & 2. Academic Press, Inc. New York, 1986.

Mills, C. F. (Ed.). *Zinc in Human Biology.* Springer-Verlag, New York, 1989.

Nordin, B. E. C. (Ed.). *Calcium in Human Biology.* Springer-Verlag, New York, 1988.

Prasad, A. S., Oberleas, D. (Eds.). *Trace Elements in Human Health and Disease.* Vols. 1 & 2. Academic Press, New York, 1976.

Contents

Contributors

Manfred Anke Department of Nutrition and Environment, Friedrich Schiller University, Jena, Germany

W. Arnhold Friedrich Schiller University, Jena, Germany

John L. Beard Nutrition Department, Pennsylvania State University, University Park, Pennsylvania

Yitshal N. Berner Harzfeld Geriatric Hospital, Kaplan Medical Center, Gedera, Israel

Felix Bronner Department of BioStructure and Function, University of Connecticut Health Center, Farmington, Connecticut

Edith M. Carlisle† School of Public Health, University of California—Los Angeles, Los Angeles, California

Florian L. Cerklewski Department of Nutrition and Food Management, Oregon State University, Corvallis, Oregon

John K. Chesters Division of Biochemical Sciences, Rowett Research Institute, Bucksburn, Aberdeen, Scotland

Harry D. Dawson Nutrition Department, Pennsylvania State University, University Park, Pennsylvania

C. Drobner Friedrich Schiller University, Jena, Germany

K. Eder Institut für Ernährungphysiologie, Technische Universität München, Freising-Weihenstephan, Germany

M. Glei Friedrich Schiller University, Jena, Germany

Mary-Ellen Harper Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada

†Deceased.

Edward D. Harris Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas

Basil S. Hetzel International Council for Control of Iodine Deficiency Disorders, Adelaide, Australia

H. Illing Friedrich Schiller University, Jena, Germany

M. Jaritz Friedrich Schiller University, Jena, Germany

Jean L. Johnson Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

M. Kirchgessner Institut für Ernährungphysiologie, Technische Universität München, Freising-Weihenstephan, Germany

Roland M. Leach, Jr. Department of Poultry Science, Pennsylvania State University, University Park, Pennsylvania

M. Müller Friedrich Schiller University, Jena, Germany

Forrest H. Nielsen Grand Forks Human Nutrition Research Center, Agricultural Research Service, United States Department of Agriculture, Grand Forks, North Dakota

Boyd L. O'Dell Department of Biochemistry, University of Missouri, Columbia, Missouri

Esther G. Offenbacher St. Luke's–Roosevelt Hospital Center, New York, New York

John Patrick Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada

Linda N. Peterson Departments of Physiology and Paediatrics, University of Ottawa, Ottawa, Ontario, Canada

F. Xavier Pi-Sunyer Division of Endocrinology, Diabetes, and Nutrition, St. Luke's–Roosevelt Hospital Center, New York, New York

A. M. Reichlmayer-Lais Institut für Ernährungphysiologie, Technische Universität München, Freising-Weihenstephan, Germany

U. Schäfer Friedrich Schiller University, Jena, Germany

M. Seifert Friedrich Schiller University, Jena, Germany

Maurice Edward Shils Department of Public Health Sciences, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina

Richard M. Smith Division of Human Nutrition, CSIRO, Adelaide, Australia

Barbara J. Stoecker Department of Nutritional Sciences, Oklahoma State University, Stillwater, Oklahoma

Roger A. Sunde Department of Nutritional Sciences and Biochemistry, University of Missouri, Columbia, Missouri

Maurice L. Wellby Department of Clinical Chemistry, The Queen Elizabeth Hospital, Adelaide, Australia

John S. Willis Department of Cellular Biology, University of Georgia, Athens, Georgia

Kurt R. Zinn Department of Radiology, University of Alabama at Birmingham, Alabama

About the Editors

BOYD L. O'DELL is a Professor Emeritus at the University of Missouri, Columbia. A Fellow of the American Institute of Nutrition and a member of the American Society of Biochemistry and Molecular Biology, the American Chemical Society, and the Society for Experimental Biology and Medicine, he is the author or coauthor of over 200 professional papers. Dr. O'Dell received the A.B. degree (1940) in chemistry and the Ph.D. degree (1943) in biochemistry from the University of Missouri, Columbia.

ROGER A. SUNDE is Food for the 21st Century Nutrition Cluster Leader, Chair of the Graduate Nutritional Sciences Program, and a member of the Department of Biochemistry and the Department of Food Sciences and Human Nutrition at the University of Missouri, Columbia. The author or coauthor of more than 100 professional papers and abstracts that reflect his research interests in the nutritional biochemistry and molecular biology of trace elements, he is a member of the American Society for Nutritional Sciences and the American Society for Biochemistry and Molecular Biology. Dr. Sunde received the B.S. (1972) and Ph.D. (1980) degrees in biochemistry from the University of Wisconsin—Madison.

1
Introduction

Boyd L. O'Dell and Roger A. Sunde
University of Missouri, Columbia, Missouri

The term *mineral element* refers to elements that are not normally volatilized when their organic matrix is ashed to remove the carbonaceous material. *Elements* are substances that cannot be decomposed to simpler substances nor formed by chemical union. The definition of essentiality is more elusive, but in the broad sense, *a nutritionally essential element* is required in the diet of animals and humans in order to allow completion of the life cycle.

I. DISCOVERY OF MINERAL ELEMENT ESSENTIALITY

In 1874 Forster [referenced in (1)] observed that the minerals in the ash of tissues are required to support animal life. This observation established the dietary essentiality of mineral elements. This requirement was distinct and beyond that recognized for carbon, hydrogen, oxygen and sulfur, the elements supplied by water, carbohydrate, fat, and protein. Except for vitamin B_{12}, the dietary requirement for mineral elements is also distinct from that of the vitamins. This Handbook is concerned primarily with the metabolism and metabolic function of these elements in humans and other animals.

The discovery of mineral element essentiality and function has proceeded along multiple routes, employing techniques and observations such as (a) use of purified diets composed of known ingredients, (b) the result of parenteral nutrition, i.e., intravenous infusion of highly purified nutrients, (c) study of animals and people living in ecological niches deficient in specific elements, and (d) determination of the basis of certain genetic diseases. Perhaps the most important tool in the discovery of all essential nutrients, including the mineral elements, has been the purified diet. Total parenteral nutrition is an extension of the purified diet concept, but the discoveries made in the course of its use were

accidental in that the elements were not deliberately omitted, but were found to be deficient in the original formulations. Deficiencies of some elements, such as those of iodine, fluorine, cobalt, and copper, were observed first because the soil, water, or plants in a particular area were deficient in the specific element. Other deficiencies have occurred because the essential element was made biologically unavailable by other dietary constituents. Some genetic diseases result from deficiency of an essential element caused by gene mutations. Such mutations may prevent the production of the proteins required to absorb or metabolize the element in the normal manner. Examples of such genetic diseases in humans include Menkes' disease, which leads to copper deficiency, and acrodermatitis enteropathica, which gives rise to zinc deficiency. Lack of these elements commonly results in defective enzymes whose activities are dependent on the element; e.g., molybdenum deficiency induced by a gene mutation impairs sulfite oxidase activity.

Recognition of the physiologic significance of the mineral elements has developed over a period of some 300 years, but the importance of most of the essential elements has been recognized only during the twentieth century. Table 1 lists chronologically some major observations that have led to the recognition of the essential mineral elements. Many of these observations have stood the test of time, having been confirmed repeatedly by other investigators. The conclusions suggested by some observations may be modified with time, and other elements may be added to the list. More detailed history of each element will be found in the respective chapters.

II. DEFINITION OF ESSENTIALITY

An *essential element* is one that is required to support adequate growth, reproduction and health throughout the life cycle, when all other nutrients are optimal. The difficulty with this definition arises in the interpretation of the word *adequate*. Under normal circumstances, an element is clearly essential if a distinct pathology results when it is omitted from the diet. Essentiality is less clearly defined when there is only a small change in the rate of growth, when the environment is suboptimal, or when there is a microbial infection. Observed improvements in performance upon supplementation with an element may be due to changes in the intestinal microflora, to a pharmacologic effect, or to interaction with another element. Conceivably, a nonessential element might provide temporary improvement in a critical physiologic parameter by releasing a similar but essential element from a less critical site and thus making it available. However, if an element exerts a catalytic or regulatory role in a critical biochemical pathway, there is little or no doubt about its essentiality.

A list of the essential mineral elements is presented in Table 2. For convenience and historical reasons, the elements are divided into two groups, the *macro elements* and the *micro elements*. The requirement for macro elements is in the range of grams per kilogram of diet, while that for the micro elements is in the range of milligrams, or even micrograms, per kilogram. In the case of adult humans, the same distinguishing units apply when expressed as intake per day. The micro elements are also referred to as *trace elements* because the early analytical methods did not allow their quantitation, and they were reported simply as a "trace." For this reason and the lack of sufficiently pure dietary ingredients, the essentiality of most of the micro elements was discovered considerably later than that of the macro elements. The list of essential mineral elements in Table 2 is divided into two categories: those whose essentiality has been confirmed by evidence for an essential biochemical mechanism, involving the element in a catalytic or regulatory role; and those whose essentiality is suggested by impairment of physiological function only.

TABLE 1 Chronological Observations Providing Evidence of Mineral Element Essentiality[a]

Year	Element	Laboratory and reference	Observation
1664	Iron	Sydenham (1)	Iron salts restored skin color in anemic patients.
1747	Iron	Menghini (1)	Blood contains iron.
1842	Calcium	Chossat (2)	$CaCO_3$ prevented fragile bones in pigeons.
1847	Sodium and potassium	Liebig (2)	Tissues contain primarily K, and blood and lymph contain primarily Na.
1849	Sodium	Boussingalt (1)	Oxen fed low Na became depressed, unthrifty, and showed loss of hair.
1881	Sodium and potassium	Ringer (2)	Na and K are essential for maintenance of tissues and organs in vitro.
1908	Iodine	Marine (1)	Goiter in pups can be prevented by giving iodine to dams.
1909	Phosphorus	Huebner (2)	Low-P diet produced rickets in dogs.
1918	Phosphorus	Osborne and Mendel (1)	P restriction in rats retarded growth.
1921	Calcium and phosphorus	Sherman (1)	Ca:P ratio is important for bone formation in rats.
1928	Copper	Hart (1)	Cu as well as Fe is required for prevention of anemia in rats fed milk-based diet.
1931	Magnesium	McCollum (1)	Rats fed low Mg developed dilatation of vessels, extreme hyperirratibility.
1931	Manganese	Hart (1); McCollum (1)	Mice failed to grow and ovulate; rats failed to suckle and survive.
1934	Zinc	Hart (1)	Rats fed low Zn showed growth retardation and hair loss.
1935	Cobalt	Underwood (1); Marston (1)	Co prevented loss of appetite, anemia, and lethargy in sheep.
1937	Chloride	Orent-Keiles (1)	Low Cl caused growth retardation and hypersensitivity; low Na caused "fatigue" and dehydration in rats.
1938	Fluoride	Dean (1)	Dental caries is lower in children who drink water with 1.9 than with 0.2 ppm F.
1953	Molybdenum	Richert and Westerfeld (3)	Mo is a component of xanthine oxidase.
1957	Selenium	Schwarz (4)	Se (Factor3) prevents liver necrosis in rats.
1959	Chromium	Mertz (5)	Cr(III) is the factor involved in maintenance of glucose tolerance (GTF).
1972	Silicon	Carlisle (7); Schwarz (8)	Retarded growth in chicks, rats.
1975	Nickel	Nielsen (9)	Ni deprivation increased perinatal mortality, depressed growth and hematocrit.
1976	Arsenic	Anke (10)	Low (50 ppb) As decreased fertility, birth weight, and survival of goats and minipigs.
1981	Lithium	Anke (11)	Low (1.9 ppm) Li decreased fertility and birth weights of goats.
1981	Lead	Kirchgessner (12)	Low (20 ppb) Pb produced anemia and decreased growth in second-generation rats.
1981	Boron	Nielsen (13)	B added to low (0.3 ppm)-B diet stimulated growth and prevented leg abnormalities in chicks fed diets low in cholecalciferol.

[a]This table lists pertinent observations that led to the discovery of the essential elements, but it is not complete and is not meant to establish priority of discovery. The history of the early literature came from Refs. 1 and 2.

TABLE 2 Essential Mineral Elements

Essentiality confirmed by biochemical mechanism(s)		Essentiality suggested by physiologic impairment	
Macro elements			
Calcium	Chloride		
Phosphorus	Potassium		
Sodium	Magnesium		
Micro elements			
Iron	Cobalt	Fluoride	Arsenic
Iodine	Molybdenum	Chromium	Lithium
Copper	Selenium	Vanadium	Lead
Manganese	Zinc	Silicon	Boron
		Nickel	

While these categories are somewhat arbitrary, they are presented to provide a measure of the validity of their assessment as being essential. It is anticipated that future research will move several elements from the latter list into the confirmed list.

While the macro and micro classes of essential elements tend to differ in function, there is considerable overlap. There are three general physiologic roles for the elements in biology: *structural*, *catalytic*, and *signal transduction*. Of the six macro elements, calcium and phosphorus play important roles in the *skeletal structure* of vertebrates. Phosphorus is also an important component of phospholipids, phosphoproteins, and nucleic acids. Sodium, potassium, and chloride perform major roles in the maintenance of osmotic pressure, water balance, and membrane potentials. Magnesium lies on the border between the macro and micro elements. It is primarily an intracellular element, and it exerts regulatory and catalytic roles in numerous biochemical systems. While the macro elements perform largely structural functions, some also perform regulatory functions involving low or catalytic concentrations. For example, calcium plays a key regulatory role as a messenger in *signal transduction*, notably in nerve and muscle cells. Phosphorus, by way of the phosphorylation–dephosphorylation cycle, exerts a highly important function in the regulation of enzyme activity by changing protein conformation or tertiary structure. Sodium, potassium, and chloride ions are of importance in the function of all cells in the maintenance of water balance. Micro quantities of these elements are important in the function of the nervous system through development of the action potential.

As expected by the low concentrations required, the micro elements serve primarily *catalytic* functions in cells and organisms. Iron performs both major and minor functions. Its deficiency causes anemia, providing clear evidence of iron essentiality, but it is also a component of numerous proteins that exert critical roles in energy metabolism, notably the cytochromes and the enzymes that participate in the electron transport system. Iodine deficiency causes goiter and cretinism, and it is a known component of the important thyroid hormones. Copper deficiency gives rise to many distinct aspects of pathology that can be identified with specific cuproenzymes, e.g., depigmentation and low tyrosinase activity, or aortic rupture and low lysyl oxidase activity. Manganese deficiency results in distinct pathology, including reproductive failure, skeletal defects, and ataxia; the manganese ion is a component of several enzymes, including mitochondrial superoxide dismutase, pyruvate carboxylase, and arginase, although these enzymes may not be related

directly to the observed pathology of manganese deficiency. A similar situation exists in the case if zinc, whose deficiency pathology includes stunted growth, skin lesions, and reproductive difficulties. While zinc is a component of enzymes that catalyze more than 50 different biochemical reactions as well as a component of proteins involved in gene expression, none of these proteins or enzymes has been identified with specific zinc deficiency pathology. Besides its catalytic role in some enzymes, zinc exerts a *structural* role in proteins, particularly in the zinc finger proteins involved in gene transcription. Cobalt is a component of vitamin B_{12} and is thus classed as an essential element, but there is no evidence that the cobalt ion has any other biochemical function. The fact that molybdenum is a component of enzymes, such as xanthine oxidase and sulfite oxidase, provides stronger evidence of its essentiality than does the physiologic response of animals to dietary molybdenum deprivation. Not only does selenium deficiency result in severe pathology, including cardiomyopathy and skeletal muscle defects, it is also a component of several proteins, including the glutathione peroxidases and the iodothyronine-5′-deiodinases. The fact that a biochemical as well as a physiologic function has been established for some elements adds materially to the validity of their classification as essential.

III. MECHANISMS BY WHICH MINERAL ELEMENTS EXERT FUNCTION

Biochemical functions have been established for the macro elements and at least eight of the micro elements listed in Table 2. An obvious challenge for future research is to establish biochemical mechanisms that depend on the other elements listed. Fluoride is probably unique among the essential micro elements in the sense that its function appears to be more protective than catalytic. Low fluoride intake often results in dental caries, but it is not essential to prevent caries under all conditions. Sugar consumption plays a major role in the incidence of caries, because of its effect on the microflora associated with teeth. Fluoride is a structural component of bones and teeth, but there is no evidence that it exerts a specific biochemical function.

Of the eight micro elements whose biochemical functions have been identified, at least in part, six are metals and two, iodine and selenium, are nonmetals. The essential metal ions, which belong to the first two transition series of the Periodic Table, generally function as cations complexed with organic ligands or chelators. Proteins are the most common naturally occurring chelators, but there are many other biochemically important chelators, such as the porphyrins and corrins. Molybdenum differs from most of the other metals in that it commonly exists as an anion containing oxygen (MoO_4^{2-}), or analogous anions in which part or all of the oxygen is replaced with sulfur. In biological cofactors the oxygen and sulfur may be provided by organic molecules. The divalent cations in the first transition series have unfilled *d* orbitals and may be designated by the number of electrons present, e.g., Mn^{d5}, Fe^{d6}, Co^{d7}, and Cu^{d9}. While Zn^{2+} (Zn^{d10}) is not strictly a transition element, because its *d* orbitals are filled, its atomic structure is similar, and it readily forms complexes analogous to those formed by the transition metal ions. All of the transition metal ions form coordinate covalent bonds with ligands that contain the electron-donor atoms N, O, and S, which are found extensively in proteins. There is a degree of selectivity of metal ions for electron-donor atoms—zinc tends to prefer sulfur, and copper prefers nitrogen—but several different donor atoms will complex with each metal ion. The amino acid residues in proteins serve as rich sources of electron-donor atoms; for example, the imidazole group of histidine supplies nitrogen, the carboxyl groups of aspartic and glutamic

acids supply oxygen, and the sulfhydryl group of cysteine supplies sulfur for complexation. Besides proteins that form chelates with the micro elements, porphyrins chelate iron and the corrins chelate cobalt to form specialized enzyme cofactors. The essential micro elements prefer coordination numbers of 4 or 6; i.e., they complex with four or six ligands. Copper and zinc have coordination numbers of 4 and generally form square planar and tetrahedral complexes, respectively. The other cations have coordination numbers of 6 and prefer octahedral complexes. The metal ions complex with chelators with greatly different degrees of affinity. The relative affinities are characterized by thermodynamic dissociation constants that are specific for the ion and the chelator. In general, the stability of a complex is greater if all ligands are present in a single organic molecule, depending on how well their arrangement fits the preferred configuration of the metal ion.

The biologically active molecules that contain the essential nonmetal elements differ distinctly from the metallocompounds. Compounds containing nonmetal elements, such as the iodinated thyroid hormones and the selenoenzymes, bond the respective elements covalently. Thus, the elements in these compounds do not dissociate from the organic moiety and are not in equilibrium with a free ion as in the case of metal ions. The metal ions can associate with preformed apo-proteins, while selenium, for example, is incorporated into proteins as a selenoamino acid at the time of synthesis. Iodine is incorporated into protein posttranslationally, being covalently linked to thyroglobulin after protein synthesis.

IV. QUANTITATIVE INTAKE AND THE PHYSIOLOGIC RESPONSE

As in the case of other essential nutrients, the physiologic effects of the essential mineral elements depend on the level of intake. This concept, based on that of Schulz as proposed in 1888 [referenced in (14)], is depicted in Figure 1. There is a range of intake, the so-called safe and adequate range, which provides optimal function. At intakes progressively below this range there is graded decrease in function until overt signs of deficiency appear. At the same low dietary intake of an element, some organs and functions are affected before others, depending on species and other conditions. For example, copper deficiency adversely affects the cardiovascular system in the pig and chicken before it affects the central nervous system, while in the sheep the nervous system is affected while little or no damage

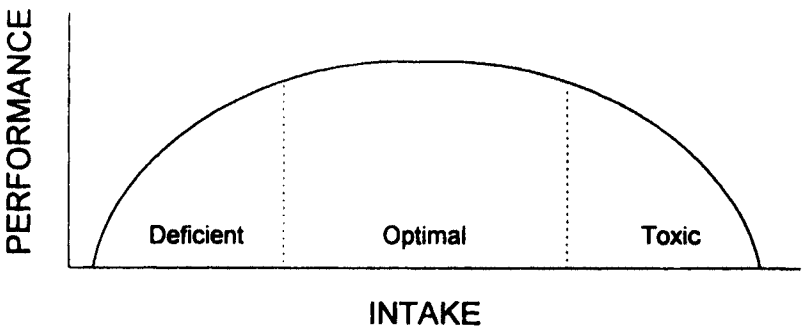

FIGURE 1 Theoretical dose–response curve. The physiologic effect of an essential mineral element is dependent on diet concentration, ranging from deficiency at low intake to toxicity at extremely high intake. The aim of nutritional science is to achieve intakes at safe and adequate levels.

occurs in the vascular system. Zinc deficiency has a more dramatic effect on the nervous system in the guinea pig than it does in the rat. Similar examples can be cited for selenium and other elements. Presumably, these differences occur due to a summation of several factors, including the relative affinities of biologically active proteins for specific metal ions and the relative concentrations and activities of the protein in the different species. The relative rates of expression of critical proteins, the prevalence of alternative protective pathways, as well as the relative rates of generation of substrates could also contribute to the differences.

Signs of toxicity begin to appear when intake exceeds the safe and adequate range. For example, fluoride, selenium, arsenic, and lead have long been considered to be "toxic" elements. As a matter of fact, all of the essential elements are toxic if consumed or administered in excess, although the concentration at which toxicity occurs varies widely. Although it is not valid to arbitrarily classify elements as toxic or nontoxic, the ratio of the minimal toxic level to the minimal adequate level may be a useful index of relative toxicity. For example, although the physiologic significance of selenium was first recognized because of its toxicity in livestock, the ratio of selenium's toxic to its required level is not substantially less than that of many other essential elements, e.g., iron (see Table 3). At concentrations between the safe and toxic levels, there may be a pharmacologic effect of the element. A question that needs to be addressed is, at what levels, if any, do the essential elements act as pharmacologic rather than essential or toxic agents.

Other dietary factors can affect both the requirement and toxicity levels of an element, although the effects on requirement have received the most research attention. Other dietary constituents can increase or decrease the bioavailability of an essential element. By the same token, other dietary components can affect the level of toxicity of both essential and nonessential elements. Relative bioavailability is defined as the proportion of an element in a food that can be absorbed and utilized, compared to a pure standard. Bioavailability of an element is most commonly decreased by its chelation in the gut to form a strong or insoluble complex, or by the presence of a competitive antagonist. For example, phytate, found in seeds, chelates and decreases the bioavailability of zinc; an excess of zinc antagonizes the absorption and utilization of copper. One would expect chelators and antagonists of toxic elements to have an analogous effect on bioavailability of toxic elements. The physiologic effects of the essential elements are concentration-

TABLE 3 Estimated Toxicity: Requirement Ratios of Selected Mineral Nutrients

Element	Highest recommended daily intake (mg)	Estimated minimal toxic daily dose (mg)	Ratio
Calcium	1,200	12,000	10
Phosphorus	1,200	12,000	10
Magnesium	400	6,000	15
Iron	18	100	6
Zinc	15	500	33
Copper	3	100	33
Iodine	0.15	2	13
Selenium	0.2	1	5

Source: Adapted from Ref. 15.

dependent. While the major thrust of this Handbook relates to biological functions of the various elements, generally each chapter gives a brief treatment of the quantitative aspects of requirements and toxicity.

V. MOLECULAR BIOLOGY IN ESSENTIAL MINERAL ELEMENT NUTRITION

A. A New Paradigm for Research

Listed above are four experimental approaches or routes that have been employed successfully to identify and to understand the essential mineral elements. A new paradigm—molecular biology methods of analysis—has emerged in the last decade, and the current and future techniques employing this intellectual base will undoubtedly revolutionize our ability to understand nutrition of the essential elements.

The use of the purified diet in nutrition research was a clever method of analysis that allowed meaningful study of nutrition with little or no knowledge about the underlying processes. Early criteria such as animal survival were subsequently replaced by growth, reproduction, and protection against a specific disease or pathology (16). Later, analytical methods progressed to permit determination of maintenance (balance) of tissue mineral levels. Later still, measurement of biochemical markers, such as circulating alkaline phosphatase or glutamic-oxaloacetic transaminase, or element-specific biochemical markers, such as glutathione peroxidase, angiotensin-converting enzyme, or serum ferritin, gave increasing sophistication to our assessment of mineral requirements. At each increased level of sophistication, additional important knowledge was obtained to help us understand the nutritional biochemistry of the essential elements. These procedures, however, were all dependent on appropriate choice of the parameter on which to make the measurement, and the conclusions were perhaps more dependent on the observer than on the subject of the study. The number of publications in the two decades from 1970 to 1990, reporting discovery of a function for an identified metalloprotein, or of looking for a biochemical mechanisms for an impaired physiologic function (see Table 2), is tremendous. In the late 1980s, the new molecular biology paradigm began to offer exciting routine potential for bridging the gap between a protein and a function. The following examples illustrate this potential.

The ability to sequence proteins, cDNAs, and genes, rapidly and accurately, in combination with the technological revolution that allows rapid searching of the resulting databases, now allows rapid interconversion of information among these three tiers of gene expression. What previously was a seeming miracle of serendipity that married a function and a protein, such as the marriage of hemocuprein and copper, zinc-superoxide dismutase (17), can now be done routinely: for example, the convergent identification of cytosolic aconitase as the iron-regulatory element (IRE)-binding protein (18). In contrast to the hemocuprein story, this regulatory protein is found in trace amounts and would likely not have been detected without the advent of molecular biology.

A second important area is the ability to discern changes in the regulation of protein expression, not only by use of antibodies, but also by use of Northern blotting techniques to monitor changes in mRNA levels, and using footprinting techniques or gel retardation/mobility shift assays to determine interaction of regulatory proteins with nucleic acids. This permits a refinement of our ability to determine the impact of small changes in nutritional status on gene expression and to determine the mechanism of this process at a molecular

level. An exciting recent development, employing variations on the polymerase chain reaction techniques, uses an approach called differential display; without any knowledge of what one is looking for or of the processes involved, one can compare gene expression under two conditions, such as nutrient deficiency versus adequacy, or disease versus health. And the outcome is to identify a series of cDNAs of genes that are potentially regulated differentially under these two conditions.

A third powerful approach is the use of heterologous expression systems, such as the xenopus oocyte (frog), baculovirus (insect), yeast or baby kidney hamster cell systems, to clone, characterize, and identify an animal or human gene from a library. The host system is typically devoid or low in native expression, so modest restoration of activity can be detected when the cells are transfected with a sample containing a gene for the activity of interest. With these heterologous expression techniques, we have powerful ability to identify the proteins and the genes for critical regulatory steps in metabolic pathways which may only be present at the level of a few copies per cell. Because mineral elements were likely critical components of metabolic pathways at the earliest stages of evolution as well as being fundamentally important for maintenance of a constant intracellular environment, these nutrients are emerging as important effectors in gene regulation. The recent identification of a molecular role for copper in iron uptake in yeast, for instance (19), shows the power of this approach in answering long-standing nutritional questions. The identified mechanisms are also likely to play important roles in how nutrition modulates health.

The last general molecular biology technique that we would like to highlight briefly is the powerful technique of producing transgenic and knockout animals. Spontaneous inborn errors of metabolism, such as sulfite oxidase deficiency, have provided tremendous insight into the identification of essential elements, such as molybdenum, that were not readily identified using the purified-diet approach. Today, researchers not only can delete or add a specific gene in a single-celled experimental model, they can also readily create mice with three or four copies of a specific gene, or strains of mice lacking one or more genes. For instance, production of mice lacking both metallothionein I and metallothionein II is helping to specifically identify the essential biochemical function of this regulated copper/zinc/cadmium protein (20).

B. Molecular Biology and Determination of Requirements

The reader will find that most if not all of the nutrient requirements described in the subsequent chapters were established under rather specific conditions, including age, diet, and choice of indicating parameter. Often times, the results were less than clear-cut, and required corollary support from experiments with other species and other methods of requirement determination. Several aspects lie at the heart of this problem. The first is that the easiest, least invasive parameter to be measured may not be a meaningful endpoint, or it may be adversely affected by a number of quite common situations, such as disease, stress, pregnancy, etc. The second is that it is increasingly clear that homeostasis protects organisms from deficiency or excess of a nutrient; this tends to obscure modest or even relatively large changes in nutrient status. Third, attempts to correct for genetic variation, or to provide a margin of safety, further confuse this situation. The emergence of molecular biology techniques and knowledge offers the first true promise for an alternative approach to the determination of biological dietary requirements.

This premise is based on the idea that the status of each mineral element is regulated both intracellularly and at the level of the whole organism. Molecular biology offers the

potential of identifying the specific biochemical mechanism, and it offers the potential to monitor these changes in a meaningful way. A sensor or thermostat, which senses and reacts to changes in the nutrient status, is at the heart of both mechanical and biological feedback systems. Today, work identifying the integrated process for regulating intercellular iron storage (ferritin) and iron uptake (transferrin receptor) via the iron regulatory protein (formally called IRE-binding protein and cytosolic aconitase) suggests that we are close to understanding the specific feedback regulatory mechanism used to control iron status within an individual cell. Similar mechanisms are likely to be obtained in the next decade for zinc, selenium, and many other trace elements.

Equally exciting will be the characterization of these molecular feedback systems. Evolution has undoubtedly refined these sensors to place them at an optimal range for survival; the sensor is neither set so low that it makes the organism prone to deficiency nor so high as to make the organism prone to toxicity. The molecular basis of a specific biological setpoint remains obscure for virtually all nutrient feedback systems, but one can envision an altered three-dimensional protein structure or replacement of an amino acid ligand as mechanisms to modulate the binding constant of a sensor protein for its nutrient target. For instance, replacement of a cysteine with a histidine undoubtedly would have changed the affinity of a regulatory factor for zinc. In just this way, evolution may have adjusted the regulatory factor that maintains intercellular concentrations of elements and or circulating levels of elements at their optimal levels. The use of molecular biology techniques should permit us to determine what nutritional levels correspond to the saturation point of these sensors. For instance, comparison of the minimum dietary selenium requirement for maximum expression of enzyme activity or mRNA level for two distinct intracellular glutathione peroxidase yields dramatic differences (21). The exciting aspect of this fact is not that we can use molecular biology to determine nutrient requirements under the usual conditions that have been used in the past, such as in the rapidly growing animal, but because it will give us the ability to determine optimal nutrient requirements under less than ideal conditions such as during infection, old age, or chronic disease. If the homeostatic mechanisms is still operating, these new techniques will let us determine potentially where the thermostat is set. Equally exciting, we may well be able to determine individual genetic differences for a nutrient. For example, a recent report by Morrison et al. (22) found that the wrong two alleles of the vitamin-D receptor have dramatic impact on bone density, suggesting that a genetic approach has the potential to become, first, an important experimental tool, and second, an important component in the determination of individual nutrient requirements.

The implications of this concept are even greater. It is likely that there will be a gap between saturation of the regulatory thermostat protein and the effector protein that responds to the regulatory protein. Here, too, evolution may have changed the relative affinities of these two proteins to provide optimal survival advantage under typical conditions. The dietary difference between nutrient intakes that trigger the sensor protein and the nutrient intakes that maximize responding protein could be called an evolutionary-derived margin of safety; the gap between the level of dietary selenium necessary for maximal glutathione peroxidase mRNA expression and for maximal glutathione peroxidase activity may be such a margin of safety (23). The point is that careful determination of the molecular mechanism may, for the first time, give us true insight into where evolution has set the nutrient requirements; with this information the nutrient requirement could be based on the intercellular and/or the organism setpoint. Second, we could determine how this setpoint is changed depending on genetic variation, and third, we might no longer have to

determine arbitrarily what margin of safety is necessary. For the mineral nutritionist willing to use molecular biology techniques, the opportunity to unravel these processes is unlimited.

C. Pharmacologic or Conditional Levels of Essential Elements

We are likely, however, to need to contain our excitement about the potential for molecular biology to solve all of the nutrition questions related to nutrient requirements. Numerous epidemiologic studies and prospective studies are identifying conditions that require nutrient intakes outside the usual nutritional realm, and thus outside the formative evolutionary pressures that may have shaped and refined the biochemical requirement setpoints. Examples outside the essential element area include supernutritional levels of vitamin E for prevention of cardiovascular disease; and potential benefit of a synthetic compound, aspirin, to prevent the development of overt cardiovascular disease in middle-aged men and perhaps women. One long-standing nutritional example is fluoride, which may not have a prescribed biochemical role, but which appears to have a defined level in the diet that protects teeth against decay and bones against premature calcium loss. Another example might well be the antitumorigenic effect of selenium at levels well above nutritional levels required to maximize glutathione peroxidase expression. In both of these cases, maximum protective levels may occur just at the onset of apparent toxicity. Second, these effects may be antagonized by normal mechanisms that protect animals against toxicity.

In summary, sophisticated molecular nutrition techniques will be called upon in exciting new ways to set safe and adequate levels of these conditionally essential nutrients. Importantly, the protective regulatory and homeostatic mechanisms on which we have relied to provide protection against day-to-day variation and against drift in requirements due to disease state, health, stress, and age, are likely not to be in effect for these conditional nutrients. Identification of proper reporter markers for setting these requirements is likely to be an important area of nutrition research in the future.

REFERENCES

1. McCollum EV. A History of Nutrition. Boston: Houghton Mifflin Company, 1957.
2. McCay CM. Notes on the History of Nutrition Research, Verzar F, ed. Berne: Hans Huber, 1973.
3. Richert DA, Westerfeld WW. Isolation and identification of the xanthine oxidase factor as molybdenum. J. Biol Chem 1953; 203:915–923.
4. Schwarz K, Foltz CM. Selenium as an integral part of Factor 3 against dietary necrotic liver degeneration. J Am. Chem Soc 1957; 79:3292–3293.
5. Schwarz K, Mertz W. Chromium(III) and the glucose tolerance factor. Arch Biochem Biophys 1959; 85:292–295.
6. Schwarz K, Milne DB. Growth effects of vanadium in the rat. Science 1971; 174:426–428.
7. Carlisle EM. Silicon: an essential element for the chick. Science 1972; 178:619–621.
8. Schwarz K, Milne DB. Growth-promoting effects of silicon in rats. Nature 1972; 239:333–334.
9. Nielsen FH, Myron DR, Givand SH, Ollerich DA. Nickel deficiency in rats. J Nutr 1975; 105:1620–1630.
10. Anke M, Gruen M, Partschefeld M. The essentiality of arsenic for animals. In: Hemphill DD, ed. Trace Elements in Environmental Health—10. Columbia, MO: University of Missouri, 1976:403–409.
11. Anke M, Gruen M, Groppel B, Kronemann H. The biological importance of lithium. Mengen- und Spurenelemente 1981; 1:217– 239.

12. Reichlmayr-Lais AM, Kirchgessner M. Haematologische veraenerungen bei alimentaerem Bleimangel. Ann Nutr Metabol 1981; 25:281–288.
13. Hunt CD, Nielsen FH. Interaction between boron and cholecalciferol in the chick. In: Howell JMcC, Gawthorne JM, White CL, eds. Trace Element Metabolism in Man and Animals (TEMA-4). Canberra: Australian Academy of Science, 1981:597–600.
14. Underwood EJ, Mertz W. Introduction. In: Mertz W, ed. Trace Elements in Human and Animal Nutrition. Vol. 1. New York: Academic Press, 1986:1–19.
15. Committee on Diet and Health, Food and Nutrition Board. Diet and Health. Washington, DC: National Academy Press, 1989:518.
16. Schneider HA. Rats, fats, and history. In: Perspectives in Biology and Medicine. Chicago: University of Chicago Press, 1986; 29:392–406.
17. McCord JM, Fridovich I. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244:6049–6055.
18. Kaptain S, Downey WE, Tang CC, et al. A regulated RNA binding protein also possesses aconitase activity. Proc Natl Acad Sci (USA) 1991; 88:10109–10113.
19. Dancis A, Yuan DS, Haile D, et al. Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. Cell 1994; 76:393–402.
20. Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD. Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. Proc Natl Acad Sci (USA) 1994; 91: 584–588.
21. Lei XG, Evenson JK, Thompson KM, Sunde RA. Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated by dietary selenium. J Nutr 1995; 125:1438–1446.
22. Morrison NA, Qi JC, Tokita A, et al. Prediction of bone density from vitamin D receptor alleles. Nature 1994; 367:284–287.
23. Sunde RA. Intracellular glutathione peroxidases—structure, regulation and function. In: Burke RF, ed. Selenium in Biology and Human Health. Vol. 5 45–47, New York: Springer-Verlag, 1994:45–57.

2
Calcium

Felix Bronner
University of Connecticut Health Center, Farmington, Connecticut

I. INTRODUCTION

A. Distribution of Calcium in Nature

Calcium is the fifth most abundant element of the globe. It is found in a variety of rocks, such as aragonite or dolomite, and throughout most waters. The concentration of calcium in seawater varies from 1 to 10 mM (1); concentrations of calcium ion in fresh waters tend to be one to two orders of magnitude lower than in the oceans, but may reach the higher concentrations found in the ocean if the ground waters come from reservoirs in limestone. The latter are typically known as "hard" waters and are usually also high in magnesium. The average calcium concentration of the crust of the earth is almost 1 mol/kg, with more than 80% of the calcium found there occurring in the form of limestone ($CaCO_3$) deposits, which therefore constitute the principal forms of calcium in the earth. Kretsinger (1), in discussing these relationships, has called attention to the close link between carbon and calcium in living organisms. Moreover, since calcium phosphate is fairly insoluble and since phosphates and phosphorylation serve cells in a variety of ways (1)—to assure hydrophilicity for metabolites, as leaving groups in nucleophilic displacement reactions, as significant forms of energy storage (ATP), to link RNA and DNA—cells have to find ways to keep calcium from tying up phosphate and preventing its utilization.

As cells evolved, they learned to get rid of excess calcium, adapting calcium extrusion to a variety of cellular processes—protein secretion, intracellular signaling, and the many calcium-dependent processes that have come to light in recent years. Thus the conquest of the ocean by living systems, uni- and multicellular, was paralleled by their ability to deal with the calcium in their surroundings. When organisms began to leave the ocean to conquer the land, they required mechanisms of support and developed external and

ultimately internal skeletons, calcium deposits which also served as storage depots, i.e., for the accumulation and release of calcium, thereby maintaining the plasma calcium concentration constant, typically 2.5 mM.

B. Distribution of Calcium in the Body

The adult human body contains easily 1 kg of Ca, with less than 1% of that amount found outside the skeleton. The body's calcium traffic, which will be described in detail later, involves multiple gradients of calcium concentration and changes in state from solid to liquid and back. Thus an individual may ingest 1 g of Ca daily, of which perhaps two-thirds is in solution (as in milk) or readily solubilized, as from cheese. The remainder may be ingested in solid form and require both digestion and solubilization before it moves across the intestinal epithelium. The calcium concentration in the luminal fluid of the intestine can readily reach 10 mM or higher, whereas the free calcium concentration of lymph and blood is maintained at 0.5 and ~1 mM, respectively. Calcium enters bone from blood and is precipitated in the form of various calcium phosphate phases, e.g., whitlockite, carbonate, phosphate, hydroxyapatite.

When calcium is mobilized from bone, it changes its state from solid to solution and returns to the circulation. Excretion in the urine involves a change in concentration, from 0.5 to 3 mM. Excretion in sweat and in the stool as endogenous fecal calcium (see below) probably involves no change in concentration as the calcium passes through the skin or into the intestinal lumen, but evaporation of sweat and water reabsorption in the intestine markedly change the calcium concentration of the final product.

Total cellular calcium varies between 1 and 5 mmol/kg, but the free intracellular calcium is ~100 nmol/L in most cells. Thus there is a very large gradient between free and bound intracellular calcium, the latter accounting for nearly the entire cellular calcium. It is undoubtedly this huge gradient that has made it possible for calcium to be such an effective intracellular signal. The requirement for separation of calcium and phosphate signifies that entry into the cell must be severely restricted, while cellular calcium extrusion must be at a maximum. How these characteristics affect transcellular calcium transport and bone calcium deposition and removal will be discussed in the relevant sections on transport and bone calcium.

C. Calcium Movement in the Body

Figure 1 summarizes in schematic form the pathways that calcium follows as it is ingested, absorbed, transported to and from the skeleton, and excreted from the body. Details of the processes involved will be presented in the appropriate sections.

II. CALCIUM IN FOOD AND DIET

If one classifies foods on the basis of their calcium content, then foods that contain more than 2.0 mmol per portion include (2) milk, cheese, ice cream, broccoli, cheese pizza, custard, Cream of Wheat, a whole orange (but not orange juice). Block et al. (3) have done a detailed study of the dietary sources of calcium in the second U.S. National Health and Nutrition Examination Survey (1976–1980) and found that dairy products contributed about 55% of dietary calcium. However, if one considers that most calcium in white bread, in many flours, and in other foods is contributed by the addition of milk powder, one can estimate that calcium derived from dairy products constitutes about 70% of the calcium

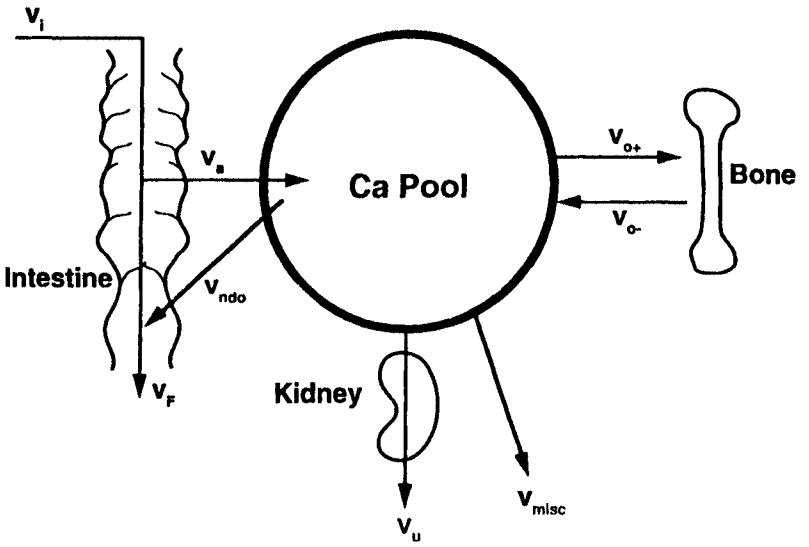

FIGURE 1 Schematic diagram of the flow of calcium through the body. The Ca pool includes calcium in solution in blood plasma, in the extracellular fluid, and in, or associated with, bone, described in units of mass, e.g., mmol. v_i = Ca ingested in food; v_a = Ca absorbed from food; v_{ndo} = endogenous Ca lost in stool; v_u = Ca excreted in urine; v_F = Ca excreted in stool; v_{misc} = Ca lost from the body via sweat, semen, menstrual fluid, milk; v_{o+} = Ca deposited in bone; v_{o-} = Ca resorbed from bone, described in units of mass per unit time, e.g., mmol/day. v_T = rate at which Ca enters or leaves the pool, i.e., = $v_a + v_{o-} = v_u + v_{ndo} + v_{o+} + v_{misc}$; In a nonlactating organism, v_{misc} is generally negligibly small. For illustrative numbers of the various parameters, see Table 4.

intake, not very different from an earlier estimate (4). In 1984, U.S. women 35–50 years old consumed on average 13.3 mmol of calcium, a mean value not much lower than what was reported in 1959 (5). Both values are significantly lower, however, than the daily recommended dietary allowance (RDA) for women of 20 mmol (6).

Men aged 19–50 years, on the other hand, consumed 125% of their RDA for calcium when surveyed for one summer day in 1985 (7).

Increasingly, the diet of people in industrialized countries is made up of foods that have undergone substantial processing. While processing of foods may alter their vitamin content, the nature of their proteins, or cause the loss of trace minerals (8), the calcium content of these foods is less likely to be affected. Moreover, processing may render a given food more digestible, and fortification with nonfat dry milk powder enriches the food's calcium content.

III. CALCIUM ABSORPTION

A. Digestion and Solubilization

Calcium becomes absorbed when it moves across the intestinal epithelium. Obviously, only calcium that is in solution in the intestinal lumen can undergo absorption. Calcium may already be in solution in a food before it is ingested, as in milk, or it may become solubilized

as the result of the combined actions of gastric acid and intestinal enzymes and of intestinal contractions and peristalsis, all of which act to bring about digestion and solubilization.

One aspect of the bioavailability of calcium is the degree to which it can be solubilized from a given food. Clearly, the more completely a given food is digested, the larger the fraction of its calcium that becomes available for absorption.

Consider the situation when $CaCO_3$ is fed as a salt and is the only calcium source in the diet. In an individual with normal acid production in the stomach, the chyme as it leaves the stomach will have a fairly low pH and—unless the quantity of $CaCO_3$ that has been ingested is very large—all the calcium will be in ionic form. Some will then undergo absorption in the duodenum. As the chyme travels caudad, its pH will go up and, depending on the amount of calcium still in solution, some of the calcium may in fact precipitate. At the neutral or slightly alkaline pH found in the lower intestine, no more than about 4 mmol of $CaCO_3$ will dissolve per liter (9), and that is the maximum available for absorption in that portion of the intestine. To be sure, as calcium is absorbed from the lumen, precipitated calcium goes into solution. Hence the rate of absorption and the length of the time that the chyme remains in a given intestinal segment will determine the amount absorbed.

If more than one calcium salt is present—e.g., carbonate and phosphate—the ratio of the respective solubility products for each salt will determine how much calcium, derived from each salt, will be in solution. In the case of monobasic calcium phosphate and calcium carbonate, virtually all of the calcium in solution will be due to the phosphate, because the phosphate is 18 times more soluble than $CaCO_3$. In other words, the availability of calcium from $CaCO_3$ is markedly reduced if other sources of calcium are present in addition to the carbonate. In terms of overall availability of calcium, the fact that only a tiny faction of the absorbed calcium is derived from carbonate, with most coming from other sources, is of course of no practical significance, provided enough calcium is present in the lumen for calcium absorption to proceed throughout the period the chyme remains in the intestinal lumen. In the rat this is typically 3–4 h (10,11).

B. Transcellular Movement

Calcium moves across the intestinal epithelium by two routes (12): through the cell, i.e., transcellularly; and between cells, i.e., paracellularly. The transcellular movement is saturable, regulated (largely by vitamin D; see Sec. VIII.A), and takes place in the proximal intestine, mostly the duodenum (Fig. 2). Paracellular transport occurs throughout the intestine and does not appear to be subject to acute regulation.

Transcellular movement of calcium is a metabolically active process, requiring oxygen, and is against a chemical gradient. It is experimentally demonstrated by means of everted intestinal sacs (13,14), filled with and immersed in the same calcium-containing buffer (15). When the outside solution of such a preparation is oxygenated, the tissue accumulates calcium on the inside of the everted sac. Only sacs from the proximal intestine transport calcium actively (12,15).

Intestinal loops can be prepared from all three segments of the intestine. As shown in Figure 3, calcium absorption in the duodenum and upper jejunum, when evaluated by the in-situ intestinal loop procedure (16), involves the sum of two processes, saturable and nonsaturable, whereas no saturable step is observed in the ileum. Figure 4 shows the time course of duodenal calcium transport, as evaluated by in-situ loops. As can be seen, completion of absorption is concentration-dependent; i.e., it is completed by 30 min when the instilled solution contains 10 mM Ca, whereas it is only half complete at 2.5 h when the concentration of the instilled solution is 150 mM. If absorption were due to simple diffusion

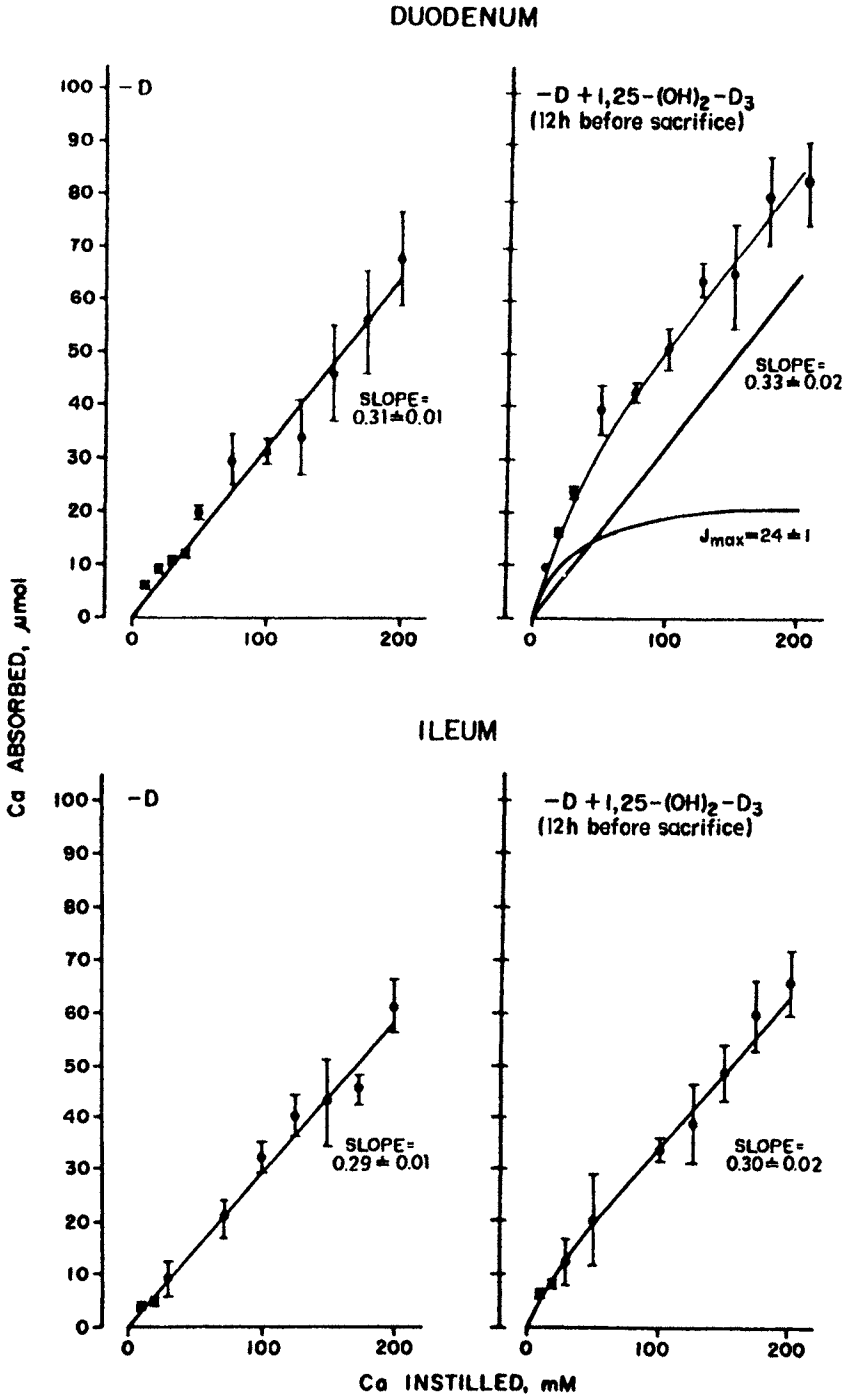

FIGURE 2 Calcium absorption in duodenum and ileum of vitamin D-deficient rats before and after treatment with 1,25-dihydroxyvitamin D_3. Male weanling Sprague-Dawley rats were fed a vitamin D-deficient diet (1.5% calcium, 1.5% phosphorus) and about 4 weeks later, when they weighed 116 ± 4 g and their mean plasma calcium level was 5.6 ± 0.1 mg/dL, calcium absorption studies by the in-situ loop procedure were initiated. The left panels show calcium absorption of the duodenum and ileum of the vitamin D-depleted animals (n = 72); the right panels show calcium absorption in the duodenum and ileum of vitamin D-deficient animals that had received 3 ng of 1,25-$(OH)_2$-D_3 by i.p. injection 12 h before sacrifice. (Reproduced by permission from Ref. 16.)

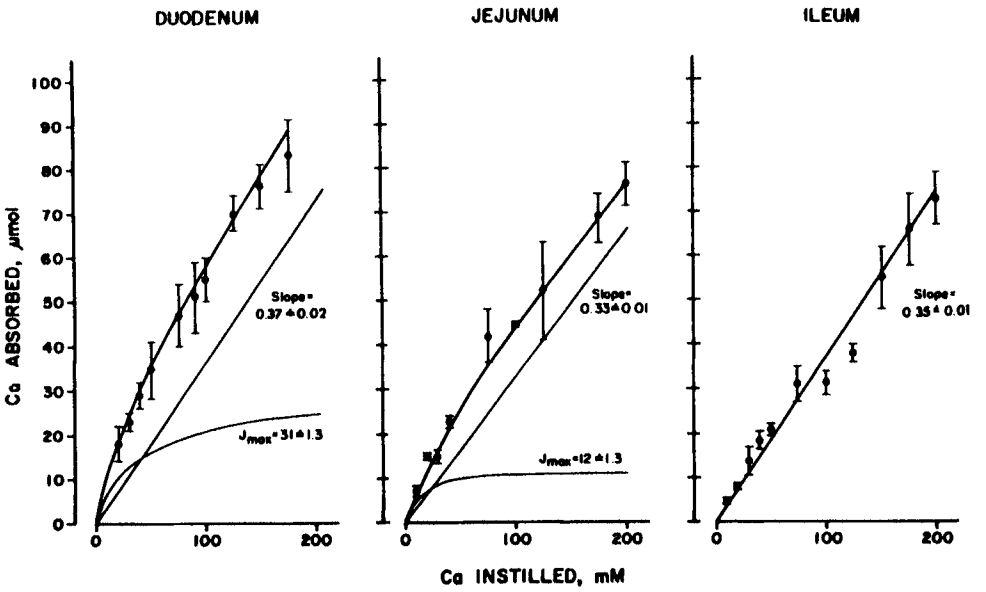

FIGURE 3 Calcium absorption in three intestinal segments of rats. Seventy male weanling rats were fed a high-Ca diet (1.5% Ca, 1.5% P) for 10 days. When their body weight averaged 77 ± 1.2 (SE) g, calcium absorption was analyzed by an in-situ loop procedure. J_{max} (equivalent to V_m) (μmol), and slope P are given as mean values ± SE. The experiment lasted 150 min, so the slope averaged 0.14 h^{-1}. (Reproduced with permission from Ref. 16.)

alone, the extraction rate would be identical at all concentrations. Hence a saturable process is involved.

The simplest description of a saturable process is the Michaelis-Menten relationship:

$$a = \frac{-d[\text{Ca}]_L}{dt} = \frac{V_m[\text{Ca}]_L}{K_m + [\text{Ca}]_L} \tag{1}$$

where v_a = initial rate and V_m = maximum rate of luminal Ca ($[\text{Ca}]_L$) efflux or rate of uptake, and K_m = that concentration of calcium in the lumen, $[\text{Ca}]_L$, when $v_i = V_m/2$. Integration of Eq. 1 between the limits $[\text{Ca}]_0$ at time = 0 and $[\text{Ca}]_t$ at time = t yields

$$\ln\frac{[\text{Ca}]_t}{[\text{Ca}]_0} + \frac{[\text{Ca}]_t - [\text{Ca}]_0}{K_m} = \frac{-V_m t}{K_m} \tag{2}$$

When K_m is large compared to $[\text{Ca}]_t - [\text{Ca}]_0$, the second term of Eq. 2 becomes negligible. Figure 5 is a plot of $\ln [\text{Ca}]_t/[\text{Ca}]_0$ against t. At low calcium concentrations (5 and 10 mM), a straight line, with slope V_m/K_m, describes the data well. At a calcium concentration of 25 mM, the function is clearly curvilinear and the value of K_m must be markedly smaller than the prevailing calcium concentrations. It has been shown (12) that the function that fits the loop data at 25 mM has a V_m of 23 μmol/g/h and a K_m of 3.9 mM; with the aid of these values, theoretical time courses were calculated for calcium efflux at concentrations from 50 to 150 mM (Fig. 5). At the highest concentration, the theoretical time course no longer fitted the data, which diverged widely from the theoretical curve. This can only mean that

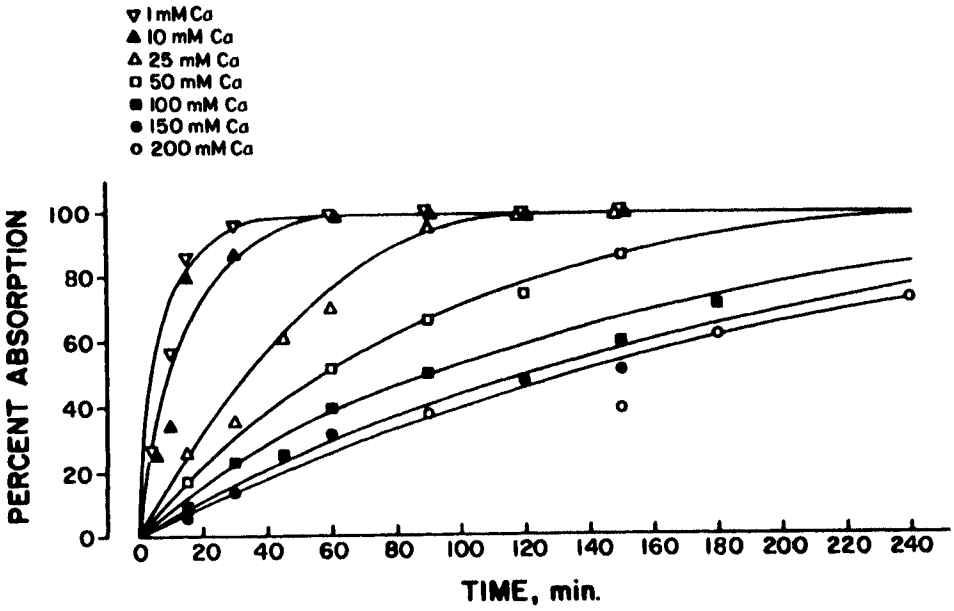

FIGURE 4 Time course of calcium efflux from in-situ duodenal loops. Male Sprague-Dawley rats, 40–50 days of age and 120–160 g body weight, were fed a semisynthetic diet containing 1.5% calcium and 1.5% phosphate for approximately 10 days. Two days before experiment, animals were fed a low-Ca diet (0.05% Ca, 0.2% P). Calcium at various concentrations, shown in the figure, was instilled in the intestinal lumen. Efflux, estimated from the amount of ^{45}Ca lost in the indicated time period, is shown as percent absorbed. Each point represents a mean estimate of 2–6 loops; average SE is 7%. (Reproduced by permission from Ref. 12.)

at high calcium concentrations, efflux from the loop was substantially augmented by a process other than that which saturates at low calcium concentrations (12).

Transcellular movement of calcium involves three sequential steps: entry across the brush border membrane; intracellular movement from one to the other pole of the cell; and extrusion at the basolateral cell membrane. For each of these steps one can ask at what rate and by what mechanism each takes place.

1. Calcium Entry

Calcium enters intestinal cells through the brush border membrane. It travels down an electrochemical gradient, inasmuch as the luminal calcium concentration is in the millimolar range, whereas the free intracellular calcium concentration is approximately 100 nM. Also, the intracellular content is electronegative with respect to the intestinal lumen. In principle, one can envision one of two possible modes of calcium entry into the cell: via a channel or via facilitated diffusion, utilizing a transport molecule. Calcium uptake by isolated brush border vesicles that have been sheared off the intestinal cells is appropriately described by a curvilinear function. The latter has been interpreted as being due to a transmembrane flux that is linear, followed by binding to the inside of the vesicle membrane (17,18). Others (19–21) have analyzed the curvilinear function in terms of a linear and a saturable component, interpreting the existence of the latter as evidence for mediated

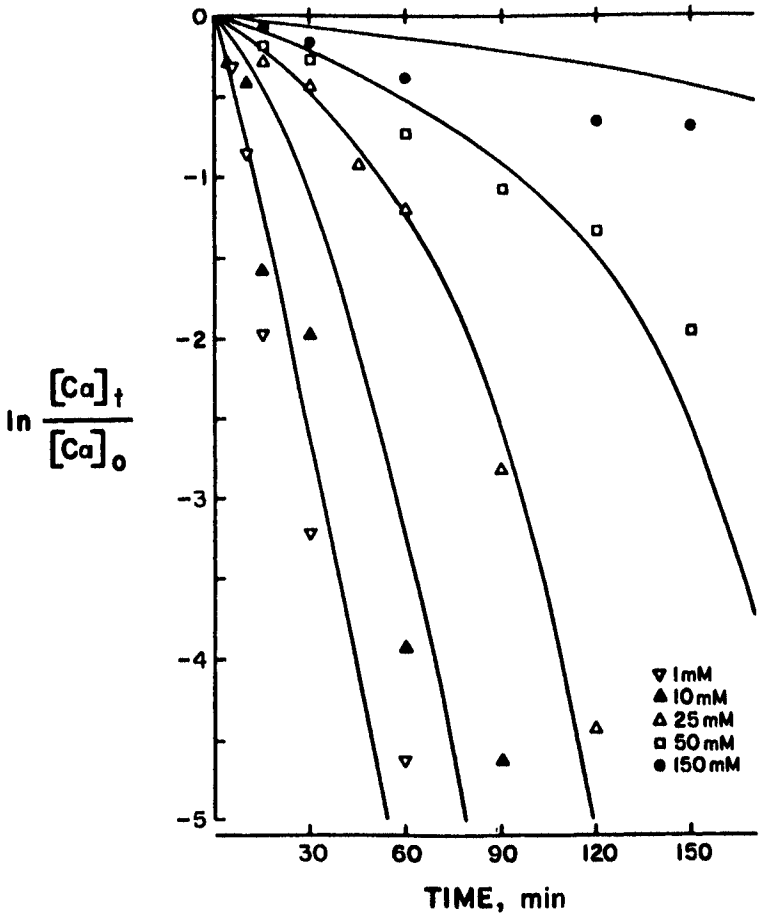

FIGURE 5 A plot of $\ln[Ca_t/[Ca]_0$ against time, using the data of Figure 4. Lines shown are calculated with use of Eq. 2, with V_m = 22.25 μmol/h/loop and K_m = 3.85 mM, with $[Ca]_0$ = 1, 10, 25, 50, and 150 mM (left to right). (Reproduced by permission from Ref. 12.)

transport, i.e., a transporter. While current evidence is insufficient to reject the possibility of a brush border transporter—an idea first advanced by Holdsworth (22)—calcium movement is down an electrochemical gradient and calcium would flood the cell if there existed no barrier to its entry (23). Consequently, the existence of a transporter seems unlikely. Additional arguments against the existence of a transporter include the only modest effect of vitamin D on calcium entry (17), even though vitamin D is the major regulator of active, transcellular calcium transport (Fig. 2).

The ubiquitous presence of calcium channels makes it likely that entry into the cell is in fact mediated by such channels. Both the nature and the number of channels in the intestinal cell are as yet unknown. No report has appeared detailing the location of calcium channels on the brush borders of intestinal cells; the existence of calcium channels on the basolateral pole of ileal cells has been reported (24). In the colon, tension development depends on extracellular calcium influx, mediated in turn by potential-dependent calcium channels (25). Such channels have also been observed in the rabbit nephron (26). Based on

analogy with calcium channels in guinea pig ventricular cells (27), a rat duodenal cell has been estimated to contain 873 channels (12), a reasonable number. Functional evidence for the existence of calcium channels in clonal rat osteosarcoma cells has been reported (28,29). These cells have osteoblastlike characteristics and respond within seconds to 1,25-dihydroxyvitamin D_3 with a 50–100% increase in intracellular free calcium (29,30). Conceivably, the enhanced calcium uptake by stimulated intestinal cells (31) and by brush border membranes isolated from vitamin D-repleted rats (17) utilizes a similar mechanism, i.e., a calcium channel that contains a vitamin D-sensitive element. A large, brush border membrane-related particulate complex, a part of which is vitamin D-dependent, has been identified (32) and assigned a transport role (33). Miller and colleagues (34) have identified a membrane-bound, vitamin D-dependent calcium-binding protein from the brush border which differs importantly from the cytosolic calbindin (CaBP, see below) and which may represent the monomer of the larger protein of Kowarski and Schachter (32).

In summary, it is probable that calcium enters the duodenal cell through calcium channels which may contain a vitamin D-dependent calcium-binding component. Entry is down an electrochemical gradient at a rate that is probably not limiting for transcellular calcium transport.

2. Intracellular Diffusion

Once calcium has entered the cell, it must diffuse through the cell interior to the basolateral membrane where extrusion takes place. The rate at which a calcium ion diffuses through the interior of an intestinal cell is given by Fick's law, which may be written as

$$F = \frac{AD_{Ca}}{L}([Ca]_1 - [Ca]_2) \tag{3}$$

where

F = transcellular flux rate of the calcium ion
A = cross-sectional area of the intestinal cell
D_{Ca} = diffusion coefficient for calcium ion in the cell sap at 37°C
$[Ca]_1$ = calcium concentration at the brush border pole of the cell
$[Ca]_2$ = calcium concentration at the basolateral pole of the cell
L = Length of the diffusion path, i.e., length of the intestinal cell

For a rat intestinal cell, $A = 80\ \mu m^2$, $L = 10\ \mu m$, and the difference in calcium concentration between the two poles can be reasonably estimated at 200 nM, since the average free intracellular calcium ion concentration is typically 100 nM. With $D = 3 \times 10^{-3}$ cm²/min (35), F becomes 96×10^{-18} mol/min/intestinal cell. When the experimental value for the V_m of transcellular calcium flux is expressed on a cell basis, however, its value is some 70 times greater than the calculated self-diffusion rate of calcium. This means that, in the duodenal cell, calcium moves transcellularly at a rate some 70 times greater than the rate at which the ion alone can diffuse. This implies that in the calcium-transporting duodenal cell there must exist a mechanism that significantly and markedly enhances the self-diffusion of the calcium ion. This is accomplished by the presence in the cell of a calcium-binding protein, calbindin D-9K ($M_r \approx 9$ kDa).

This molecule, discovered by Wasserman and colleagues (36–38), whose biosynthesis is totally dependent on vitamin D (39–41), contains two calcium-binding sites, has a quaternary structure resembling an E–F hand (42), a pI of 4.3, and K_d for calcium of about 0.3 μM (43,44). Under conditions of maximum V_m (Fig. 6), the calbindin (CaBP)

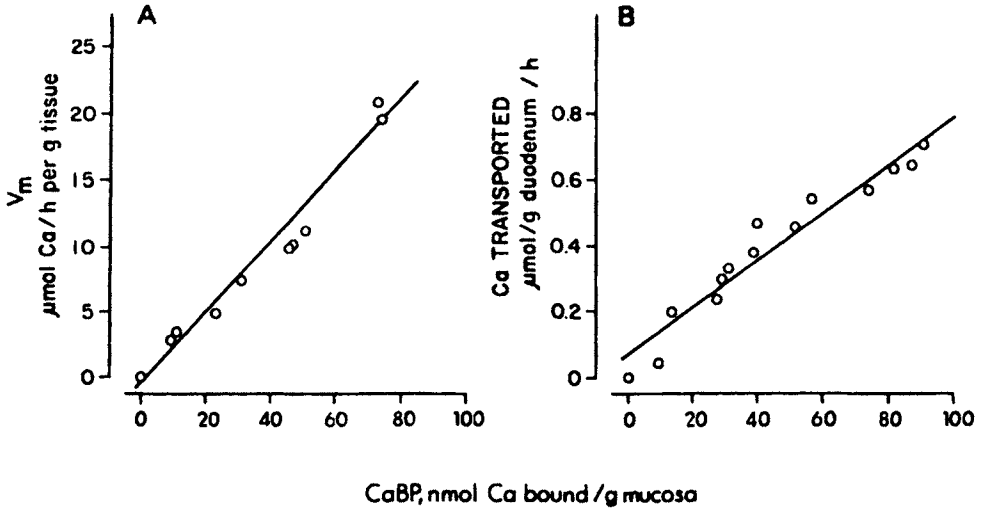

FIGURE 6 Relation between intestinal calcium transport and calbindin (CaBP) content. A. V_m, calculated from in-situ duodenal, jejunal, and ileal loop experiments, shown as a function of CaBP content. Equation describing the relationship is $V_m = 0.59 + 0.26$ CaBP, units as shown, $n = 10$, $r = 0.98$. B. Calcium transport, as evaluated from everted duodenal sac experiments (90 min incubation, 0.25 mM calcium), shown as a function of CaBP content. Equation describing relationship is Ca transport = 0.070 + 0.00714CaBP, units as shown, $n = 14$, $r = 0.97$. Data from male Sprague-Dawley or Wistar rats, 70 to 150 g body weight, fed a semisynthetic, high-calcium regimen (1.5% calcium and 1.5% phosphate), with or without vitamin D. (Reproduced by permission from Ref. 12.)

concentration in the cell is 0.2–0.4 mM (45), a concentration sufficient to augment the diffusion rate of calcium to the required value.

Stein (23) has shown how Eq. 3 can be transformed to yield an expression that predicts the augmentation factor for calcium diffusion in the cell:

$$\text{AUG} = \frac{D_{\text{CaBP}} \times \text{Tot CaBP} \times K_{\text{CaBP}}}{D_{\text{Ca}} \times (K_{\text{CaBP}} + [\text{Ca}]_1)\,(K_{\text{CaBP}} + [\text{Ca}]_2)} \tag{4}$$

where

AUG = augmentation of calcium flux, i.e., the ratio of calcium flow due to calbindin compared with calcium flow in the absence of calbindin
D_{CaBP} = diffusion coefficient of calbindin
Tot CaBP = total calbindin concentration
K_{CaBP} = dissociation constant of calbindin with calcium
$[\text{Ca}]_1$, $[\text{Ca}]_2$ = Ca^{2+} concentrations at the two poles of the cell

As predicted (12,23) and found experimentally (Fig. 6), Feher et al. (46) report in their in-vitro studies that "the enhancement of transcellular [calcium] transport was nearly linearly dependent on calbindin-D9K concentration."

Stein (23) has shown that the augmentation factor predicted from Eq. 4 equals the amplification needed, i.e., some 70-fold, and called attention to the three components of Eq. 4 that contribute most to the efficiency of the transcellular transport systems: (1) a high

ratio of calbindin to the free calcium ion concentration [$(2 \times 10^{-4}$ M)/10^{-7} M]; (2) a high ratio of the diffusion coefficient of the bound to that of the free substrate (It should be borne in mind, however, that the bound calcium, being attached to a large carrier molecule, will always diffuse more slowly than the free calcium ion.); (3) an optimal value for the dissociation constant of the substrate-carrier complex. Feher et al. (46) have shown that the experimental value of the K_d of calbindin obtained in the presence of 0.1 M KCl, a situation similar to what prevails in the cell sap, is optimum for enhancing transport. The value they utilized, 4.3×10^{-7} M (46,47), was also used by Stein (23).

We thus see that intracellular calcium diffusion, the second of the three steps making up transcellular calcium transport, is assured by and is in direct proportion to the quantity of the intracellular calcium-binding potein, calbindin-D9K, whose action may be compared to that of an intracellular ferry. It should be pointed out that this process is "exactly equivalent to the process postulated to occur within the muscle cell (48), where myoglobin is thought to ferry oxygen across the cell, thus speeding up the loading and unloading of oxygen from and to the tissue" (23).

3. Calcium Extrusion

The intracellular free calcium concentration is in the 100-nM range, whereas the concentration of calcium in the extracellular fluids is in the millimolar range; calcium extrusion therefore is necessarily against a chemical gradient. Since, moreover, the cell interior is electronegative with respect to the outside of the cell, calcium extrusion is also against an electrical gradient. It is effected by Ca-ATPase, a large, membrane-spanning molecule ($M_r \approx 123$ kDa) that "couples the hydrolysis of ATP to the transport of Ca^{2+}" (49). Available information, based on the primary structure of the pump molecule from a cDNA isolated from a human teratoma library (50), indicates that on the cytoplasmic side of the membrane there is a calmodulin-binding domain and a cAMP-dependent phosphorylation site. The latter is located near the carboxyl terminus. Also on the cytoplasmic side is a putative calcium-binding domain somewhat nearer the N terminus, and a "hinge region," thought to bring about the conformational change in the transmembrane elements. This change would then propel the calcium through the channel-like transmembrane elements to the outside of the cell. The conformational change is thought to be the sequel to a phosphorylation step, and the site on the molecule that is thought to undergo phosphorylation has been identified (51). While the general aspects of the molecular structure and pump action are likely to apply to all Ca-ATPases, it should be emphasized that no detailed structure of the intestinal Ca-ATPase has been published. Until specific information is available, the picture of how the enzyme works must be regarded as speculative.

An important question with respect to the enzyme is whether pump activity is sufficient to cope with transcellular calcium traffic. All cells are probably equipped with a calcium extrusion mechanism, but is its capacity sufficient to meet the needs of the duodenal cell? Ghijsen et al. (52) have estimated the V_m of isolated basolateral membrane vesicles from rat intestine to be about 8.5 nM Ca/min/mg protein, a number subsequently confirmed by Wasserman et al. (53). Inasmuch as only inside-out vesicles can dephosphorylate ATP that is added to a vesicle preparation and only about half of the vesicles are likely to be inside-out, the actual V_m of Ca transport by these vesicles and therefore by the basolateral membrane is more likely to be near 20 nM Ca/min/mg protein. Carafoli (54) has estimated the V_m of the general plasma membrane Ca-ATPase to be about 30 nM Ca/min/mg protein. The enzyme therefore seems capable of handling maximum loads. Nevertheless, membrane preparations from vitamin D-replete animals seem to have maximum

transport capacities that exceed those of preparations from vitamin D-deficient animals by a factor of two to three (12,53,55), with no apparent change in the K_m (53). This suggests that vitamin D acts to increase the number of pumps per cell, but the mechanism by which this occurs is not known.

In addition to a Ca-ATPase, many cells contain a Na/Ca exchanger, but this enzyme is less widely distributed than is the Ca-ATPase (56). Na^+/Ca^{2+} exchange is an antiport system, i.e., the translocation of calcium is coupled to sodium movement in the opposite direction. Sodium enters the cell via the Na/K-ATPase, and the inward concentration gradient thus established for sodium provides the energy for calcium extrusion (56). In intestinal cells, Na/Ca exchange does not appear to play a significant role in calcium extrusion (57), in contrast with Ca-ATPase which is the major mechanism for Ca extrusion from duodenal cells.

C. Paracellular Movement

As pointed out above (Fig. 2), the nonsaturable component of transepithelial calcium transport is found throughout the small intestine, with the rate of calcium movement independent of the calcium concentration. This is evident from the fact that the slope of calcium absorption, when plotted as a function of the calcium concentration of the instilled solution (Fig. 2), is relatively constant throughout the intestine.

A compilation of in-situ loop experiments (58) showed that the average rate of nonsaturable transepithelial movement of calcium was 0.16 h^{-1}. In other words, approximately 16% of the luminal calcium concentration was being moved per hour spent in intestinal sojourn. This was verified in subsequent experiments (59), where the rate of nonsaturable calcium movement was compared to the rate with which phenol red moved transepithelially. Phenol red does not enter cells (60). The overall rate of transmucosal movement of the ion and the dye were the same, 16% h^{-1}.

Scattered through the literature are reports on changes in calcium absorption that in all likelihood have resulted from a modification of the paracellular flow rate. Thus, Pansu et al. (61) showed that the addition of either glucose or xylose to a solution instilled in a jejunal loop—where active calcium transport is quite limited (Fig. 3)—doubled the rate. Presumably this resulted from the fact that the solution was hyperosmolar so that body fluid entered the lumen and distended the tissue, thereby increasing fluid transport out of the lumen (62) and raising the net absorption of calcium. The same explanation is likely to apply to the enhancement of calcium absorption in the ileum by various carbohydrates (63,64).

Lactose has long been known to enhance calcium absorption, but the mechanism has been uncertain (63–65). That lactose must act, as other sugars do, by enhancing paracellular transport of calcium, presumably due to hyperosmolar distention of the extracellular passage, was shown (66) in experiments where rats were fed 30% lactose in their diet. Their total calcium absorption increased, their fractional absorption decreased, and the active CaBP-related transport was downregulated. Thus adding lactose in sufficient concentrations to the diet caused the net level of calcium absorption to increase in a manner equivalent to increasing the calcium content of the regimen from 0.4% to 0.7% (67). As pointed out above, CaBP and active calcium transport vary inversely with calcium intake, whereas net absorption varies linearly with intake (66,67).

Other substances whose effect on calcium absorption may be mediated by their effect on paracellular transport are amino acids such as L-lysine (68) or medium-chain triglycerides (69). In neither of these cases does it seem probable that the active transport process is modified; rather, modification of the intercellular pathway seems more probable. Some detergents and bile salts may similarly enhance calcium absorption (69) by acting on the paracellular pathway.

D. In Vivo Applications

As is evident from the above discussion and as was formulated many years ago by Wasserman and Taylor (64), calcium absorption is the sum of a saturable and a nonsaturable process which may be formulated as follows:

$$v_a = \frac{V_m \times [Ca]_L}{K_m + [Ca]_L} + b[Ca]_L \tag{5}$$

where

v_a = amount of calcium absorbed per unit time e.g., mmol/h of mg/d
V_m = maximum amount transported by the saturable component
$[Ca]_L$ = calcium concentration of the luminal fluid
K_m = concentration of $[Ca]_L$ when $V_m/2$ has been attained
b = an apparent permeability constant, e.g., 0.16 h^{-1} in the rat (58)

Under normal conditions, $[Ca]_L$ is proportional to the calcium intake, v_i, so that one can substitute v_i for $[Ca]_L$ in Eq. 5. If, however, the ingested calcium is highly insoluble, as when $CaCO_3$ constitutes the only source, then raising intake beyond the solubility of calcium in the intestinal fluid will lead to no further absorption (70).

To apply Eq. 5 to what happens in vivo necessitates taking into account the functional history of the organism under study, inasmuch as the value of V_m at any time is the result of prior calcium intake, vitamin D status, sex, age, and reproductive status. Figure 7 shows how, in a group of male rats fed a high-calcium diet from weaning, V_m and CaBP (= calbindin-D9K) varied in the course of development, whereas the passive component of transepithelial calcium transport remained invariant after about 28 days of age. Absolute values of V_m and CaBP would be higher in rats raised on a low-calcium intake (67), and active calcium transport seems to be downregulated with age (71,72).

Active calcium transport being totally vitamin D-dependent, it proved possible (12) to estimate the value of V_m from experiments (73) with vitamin D-replete rats whose calcium intake (v_i) ranged from 45 to 115 mg/day, and vitamin D-deficient rats, whose calcium intake ranged from 18 to 85 mg/day.

The values arrived at (12) were $V_m = 12$ mg Ca/day and $K_m = 9.5$ mg Ca/day. Inserting these in Eq. 5 and using the experimentally derived mean value of 0.53 for the fraction of calcium absorbed by these rats by the nonsaturable route in a 24-h period yields

$$v_a = \frac{12 \times v_i}{9.5 + v_i} + 0.53v_i \tag{6}$$

The values predicted for v_a on the basis of Eq. 6 have been shown (70) to agree well with experimental ones, if one takes into account that the numerical values for V_m and K_m

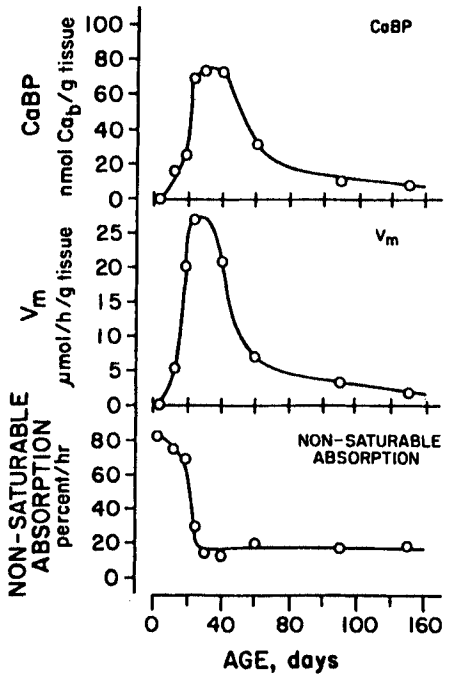

FIGURE 7 The developmental course in rats of calbindin (CaBP), active calcium transport (V_m), and nonsaturable absorption, i.e., of the paracellular route. Data obtained from experiments with male rats fed a high-Ca diet (1.5% Ca, 1.5% P) from weaning. (Adapted from Ref. 206.)

are only approximate. An even better fit is likely if the value for V_m is derived from the relationship between V_m and calbindin, following experimental determination of the calbindin content in a given study. This relationship, derived from Figure 6, is

$$V_m = -0.59 + 0.52 \text{ CaBP} \tag{7}$$

where

V_m = μmol Ca transported/h/g
CaBP = nmol calbindin/g

(Note: 1 nmol calbindin = 2 nmol Ca_{bound}.)

On the basis of the above analysis, it is evident that the most efficient way of increasing the amount of calcium that is absorbed is to increase intake of calcium in a form that is readily digested and solubilized. Increased intake will, with time, lead to diminished absorption by the active route. The amount transported by the passive route, on the other hand, increases with increased intake. Because of the downregulation of the active transport route with increased calcium intake, the relative role played by the active transport route also decreases as calcium intake increases. This is readily appreciated by inspecting Eq. 5, whose first term approaches V_m as the luminal calcium concentration, $[Ca]_L$, which normally varies with intake, v_i, becomes large in relation to K_m. As a result, the proportion of v_a contributed by the second term of Eq. 5, i.e., by the paracellular route, becomes dominant, particularly as V_m goes down with increased calcium intake (67). In the vitamin D-replete rat on a high-calcium intake, CaBP and presumably V_m respond very rapidly to exogenous

1,25-dihydroxyvitamin D_3, increasing within 1 h of the injection of the metabolite (74). Vitamin D-deficient rats take several hours to increase CaBP levels in response to exogenous 1,25-$(OH)_2$-D_3 (40,74). Since dietary manipulation leads to relatively rapid changes in the serum levels of 1,25-$(OH)_2$-D_3, up- and downregulation of the active transport component will happen relatively quickly—within 24 h in the rat—so that dietary manipulation of calcium intake is an effective and safe procedure. Administration of vitamin D will, to be sure, enhance the active component of calcium transport, but it needs medical supervision and may lead to other complications, such as hypercalcemia, hypercalciuria, and depressed bone turnover (75).

IV. CALCIUM IN BLOOD

A. Biological Description

Virtually all blood calcium is found in the plasma, so the plasma calcium content, 2.5 mM, is the parameter dealt with in biology and medicine. The plasma calcium concentration is closely regulated in vertebrates. It has about the same value in all species and varies little with age and physiological status. A notable exception is the laying hen, whose total plasma calcium level may reach 5 mM, but whose dialyzable free plasma calcium concentration, like that of all other vertebrates, lies between 1 and 1.5 mM. The major calcium-binding protein that is responsible for the high calcium concentration of chicken plasma during the egg-laying cycle is vitellin.

Table 1 lists the compounds and forms of calcium in human blood, typical of mammalian blood. As shown in Table 1, somewhat less than half of the total plasma calcium is bound to protein, albumin being the major component. Somewhat more than half of the plasma calcium is not bound to protein, mostly in the form of the free ion, and a small portion, ~ 6%, is bound to citrate, phosphate, and other complexes. Although dissociation constants between calcium and its ligands in the plasma vary, the rate at which equilibrium is established with albumin is so rapid (<5 s; 77) that total and ionized calcium concentrations will give equivalent functional information under nearly all circumstances, even though it is the ionized calcium concentration that is the relevant functional variable. Automated methods for measuring ionized calcium in plasma now exist, but in general still seem to be less reliable than automated methods for measuring total plasma calcium. In any event, current data support the older literature that the ionized calcium concentration in human plasma is between 1.1 and 1.2 mM (for further discussion, see Ref. 78).

TABLE 1 Distribution of Calcium in Normal Human Plasma

Form	mM	Percent of total
Ionized	1.18	47.5
Protein-bound	1.14	46.0
Phosphate	0.04	1.6
Citrate	0.04	1.7
Other complexes	0.08	3.2
Total	2.48	100.0

Reproduced from Ref. 76.

Plasma calcium levels tend to be higher in men than in women (79) and to decrease with age (79–81). As will be shown later, the age-dependent decrease in plasma calcium is related to the increasing proportion of hydroxyapatite as bone mineral matures. As a result the average calcium-binding sites in the skeleton have a higher affinity for calcium, causing the plasma calcium to drop.

The plasma calcium is in extremely rapid, dynamic equilibrium with calcium in the extracellular fluid, plasma constituting about 17% of the extracellular fluid volume (82,83). Evidence for this comes from experiments in which calcium was injected intravenously or intraperitoneally, and the plasma concentration dropped with a $t_{1/2}$ of <1 min to about one-sixth of what it would have been if none of the injected calcium had left the plasma compartment (84,85). Following this initial expansion, the calcium load is disposed of in a strictly exponential manner; in other words, the plasma calcium concentration, elevated due to a positive load or depressed due to a "negative" load—e.g. intravenous injection of a calcium chelator such as EDTA (86)—returns to the pretreatment (baseline) value with a $t_{1/2}$ that approximates the time it would take for the plasma to be cleared of half of its positive or negative load in about 27 circulations (87). This value was arrived at because, in the dog, approximately 5% of the cardiac output goes to the skeleton (88). Moreover, 50% of the ^{45}Ca that enters the circulation of a dog's femur is extracted from the blood (89). If one assumes that this figure also applies to the reduction of calcium load, then 2.5% of the plasma calcium would be cleared by bone in one circulation and it would take 27 circulations (ln 0.5/ln 0.975) to clear the blood of half of its circulating (positive or negative) load. Circulation times multiplied by 27 should then represent the $t_{1/2}$ of load reduction in various species.

In addition to bone, the other major gateways for calcium entry to and exit from the circulation are the gut and the kidney. The latter in particular has often been implicated as a significant regulator of blood calcium (90). If, however, one calculates the time required for a load to be cleared by the kidney or the gut, it is apparent (87) that neither of these organs can compete effectively with bone.

The initial pool of virtual distribution of calcium, i.e., what is likely to represent the extracellular water, seems to be largely independent of the functional state of the organism. In other words, a calcium load will be diluted approximately sixfold, whatever the state of the organism. On the other hand, as evident from Table 2, the rate at which the blood is cleared by the skeleton of its calcium load is very much dependent on the endocrine status and the age of the individual. Thus, as Table 2 shows, the rate of clearance—i.e., the rate at which the error in the plasma calcium is being corrected—is much faster in young than in old dogs and much faster in euparathyroid than parathyroidectomized rats.

B. Regulatory Model

To explain how bone can overcome a calcium load, it is first necessary to analyze the nature of the interaction of plasma calcium with bone. As shown by Table 2, the time of return of the plasma calcium to the preload level is the same whether the load is positive or negative. It therefore appears that each time the blood plasma courses over bone, half of the positive or negative difference between the concentration of the plasma calcium as it enters and leaves the bone is abolished. Consequently, the plasma calcium seems to be in rapid, dynamic equilibrium with calcium-binding sites in bone.

The concept of a dynamic equilibrium derives support from the analysis of plasma disappearance curves following the intravenous administration of tracer calcium. When

TABLE 2 Response Times to Calcium Loads

Species	Hormonal status[a]	Ca load and route[b]	$t_{1/2}$ (min)
Rat	N	3.8 mg/animal, i.v.	14
	N	2–18 mg/animal, i.p.	22.5 ± 2.0
	−D	2–15 mg/animal, i.p.	51.0 ± 2.5
	PTX	8 mg/animal, i.p.	53
	CTX	8 mg/animal, i.p.	41
Dog	N	10 mg/kg, i.v. infusion	58
	N	−10 mg/kg, i.v. infusion	80
	N (young)	not given	23.0 ± 0.8
	N (mature)	−4.3 to −8.3 mg/kg, i.v. infusion	78.0 ± 12

[a]N = normal; −D = vitamin D-deficient; PTX = parathyroidectomized; CTX = endogenous thyrocalcitonin supply excised.

[b]A negative sign before the load indicates that a calcium chelator was given to depress the plasma calcium to the same extent to which a positive load would have raised it; i.v. = intravenous route; i.p. = intraperitoneal route.

Reproduced from Ref. 87 with permission.

analyzed in terms of a four-termed exponential expression (91,92), the first term, with a half-time of less than 1 min, represents dilution into the extracellular fluid, as described above, whereas the second term, with a half-time in terms of minutes, is the equivalent of the clearance of calcium by bone (93–95). The later terms of the disappearance equation have been taken to represent losses from the calcium pool due to excretion and long-term bone calcium deposition (91,92,96).

The qualitative and quantitative similarity between results obtained with the use of calcium loads and isotopic calcium gives special significance to the observation (89), already referred to, that about half of the tracer calcium is extracted in a single passage of the circulation that enters bone, yet the total calcium concentration of blood that enters and leaves bone is unchanged. Bone must therefore contain calcium-binding sites that are in rapid and dynamic equilibrium with the plasma calcium.

Such a dynamic equilibrium is generally considered in terms of the solubility product of ionic calcium and phosphate in the blood. However, the equilibrium for bone calcium-binding sites can be equally well described in terms of an apparent half-concentration, K_m. When there is no net gain or loss of calcium from the plasma, the free energy of calcium binding must be zero. Consequently, the plasma calcium level [Ca_s] must equal the mean K_m of the bone calcium-binding sites. In other words, $K_m = [Ca_s]$.

The rapidity with which the equilibrium between blood and bone is established can be assessed by calculating the apparent permeability constant P for a dog of 10 kg whose trabecular bone surface area, A, is 1.6×10^4 cm^2 (97). $P = (0.693V)/At_{1/2}$, where V is the volume of plasma cleared. With a circulation time of 40 s (99), the value of P is 0.5×10^{-5} cm^{-1} (87). This value is comparable to what can be conservatively calculated for the vitamin D-deficient rat duodenum (12), where calcium moves paracellularly, but not transcellularly. As pointed out earlier (Sec. III.B), it is the presence of the intracellular, vitamin D-dependent calbindin-D9K that assures transcellular calcium transport at a rate compatible with the experimental V_m (Fig. 6). It is as yet uncertain whether bone cells contain any calbindin, but its concentration in bone cells is at best 1% of that in the vitamin D-replete duodenal cell (45). The low calbindin content of bone cells, combined with the

relatively high apparent permeability of bone for calcium, makes it unlikely that calcium enters bone by fluxing through bone cells. Rather, calcium must move between bone cells, with the calcium-binding sites therefore located on (or in) the bone mineral itself.

The nature and type of calcium-binding sites in bone mineral have not been described as such. However, at least five phases of calcium phosphate are likely to occur in bone (Table 3). Inasmuch as calcium-binding affinity varies inversely with solubility, then the K_m must also. Because the calcium phosphate phases with the highest calcium-to-phosphorus ratio are least soluble, they must have the lowest K_m. Moreover, the average K_m of a given section of bone will depend on the relative content of the various bone salts. Mature bone, having a high content of hydroxyapatite, would therefore be associated with a low K_m and a relatively depressed plasma calcium level, whereas in newly forming bone the bone salt would be less mature and its average K_m and the associated plasma calcium level would be relatively high. Shifts in the mean K_m and associated plasma calcium level would then be brought about by shifts in the proportions of calcium-binding sites in bone.

The question then arises how those rapid shifts in the proportion of calcium-binding sites, leading to a change in the K_m, can be brought about. To illustrate the speed with which plasma calcium changes, it is only necessary to recall that, following parathyroidectomy, the plasma calcium drops with a $t_{1/2} < 2$ h (86) from the normal or reference level of 2.5 mM to approximately 1.5 mM. This means that the average K_m of calcium-binding sites in bone has changed. Conversely, if parathyroid hormone (PTH) is administered, the well-documented rise in plasma calcium that follows must parallel a comparable rise in the average K_m. Calcitonin injection leads to a rapid ($t_{1/2} < 15$ min) transient drop in plasma calcium (100), which would result from the postulated drop in the average K_m of the binding sites.

The model of regulation that has been proposed (87) is based on the rapid changes in shape that bone cells have been observed to undergo (101,102). Osteoblasts and osteoclasts, together with the bone-lining cells and osteocytes, constitute the cell population of the mammalian skeleton, but only osteoblasts and osteoclasts are metabolically active (103) and are the cells that primarily regulate bone growth and turnover.

Osteoblasts possess receptors (102) for PTH and 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2$-D_3, whereas only osteoclasts possess receptors for calcitonin (102,104,105), the third of the three major calcitropic hormones (see Sec. VIII). A very early response of osteoblasts to PTH (101) or 1,25-$(OH)_2$-D_3 (102) is to round up, followed by a cascade of

TABLE 3 Solid Phases of Calcium Phosphate Linked to Biological Calcification[a]

Name	Formula	Molar ratio of Ca to P
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	1.66
Whitlockite	$(Ca,Mg)_3(PO_4)_2$	1.50
Amorphous $CaXPO_4$	$Ca_9(PO_4)_6$(variable)	1.30–1.50
Octacalcium phosphate	$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	1.33
Brushite	$CaHPO_4{\cdot}(2H_2O)$	1.00

[a]Listed in order of increasing acidity, solubility, and, by inference, increasing K_m, i.e., decreasing calcium-binding affinity.

Note: At least 90% of the cystalline solid is hydroxyapatite, based on X-ray diffraction analysis (98).

Reproduced by permission from Ref. 98.

events such as inhibition of collagen synthesis (106,107), characteristic either of an arrest or a marked slowing of metabolic activity. Calcitonin, on the other hand, induces a rounding up of osteoclasts and inhibition of their metabolic activity (102,104).

The rounding up of osteoblasts is thought to lead to an opposite shape change in osteoclasts, i.e., their spreading out and extending apposition to the bone surface (108). Contrariwise, the rounding up of osteoclasts, as under the influence of calcitonin, allows osteoblasts to extend and to enhance their metabolic activity. For example, calcium repletion of calcium-deficient rats, whose PTH levels are high (109), has led to lowering of PTH levels and to rapid positional changes in osteoclasts, these cells clearly lifting off the bone surfaces (110).

Shape changes of bone cells may be thought to be associated with changes in the average calcium-binding affinity of bones by causing a change in the relative proportion of high- and low-affinity calcium-binding sites. It is therefore hypothesized (87) that osteoblasts, when extended and apposed to bone, block the low-affinity extremes of the population of calcium-binding sites (Fig. 8A). But when osteoblasts retract under the action of PTH or 1,25-$(OH)_2$-D_3, they expose these low-affinity sites (Fig. 8B).

Conversely, osteoclasts may be thought to be associated with binding sites that have high affinity for calcium. Thus, when osteoclasts are caused to contract, as under the action of calcitonin (Fig. 8C), high-affinity calcium-binding sites are exposed.

When osteoblasts contract, the proportion of low-affinity binding sites goes up for two reasons: More are exposed as a result of the contraction of osteoblasts; and osteoclasts, which are associated with binding sites that have a higher calcium-binding affinity, tend to spread out over the bone surface, covering up high-affinity binding sites. The result is a rise in K_m and in the plasma calcium.

When osteoclasts contract, the proportion of high-affinity calcium-binding sites goes up because high-affinity binding sites are exposed and because osteoblasts, in extending over the bone surface, now cover more low-affinity binding sites. Consequently, the proportion of high-affinity sites increases, the K_m goes down, and the plasma calcium falls.

That osteoclasts and osteoblasts are associated with bone surfaces that differ in calcium-binding capacity can be inferred from studies using biphosphonates (111) and gallium (112,113). Both of these substances inhibit osteoclastic action specifically, presumably by becoming associated with bone salt sites that would normally be attacked by osteoclasts.

Changes in the rate of ^{45}Ca movement into and out of bone have been shown to be quite rapid after hormone addition. In chicks and rats, calcium uptake by the femur was reduced to nearly half within 3 min of PTH injection (94). In chicks, following the injection of 16,16-dimethyl prostaglandin E2, calcium uptake by bone was markedly reduced, whereas cAMP levels in the bone cells increased in parallel (114). These responses are too rapid to be accounted for by changes in the synthesis or degradation of proteins that regulate calcium deposition or removal. They are of the right time scale, however, to have resulted from changes in cell shape, presumably involving such hormonally induced biochemical signals as changes in ion channel permeability (115) and in cAMP (116).

Shape changes of osteoblasts and osteoclasts need not constitute the sole mechanism for changes in binding-site affinity. Cell-mediated changes in the pH of the immediate surroundings of a calcium-binding site or liberation of protein factors may contribute to affinity changes. Moreover, shape changes are only a first step in the cascade of events that lead to long-term bone deposition and remodeling.

Four factors play major roles in determining the rate at which the skeleton responds to a calcium load: (a) circulation time, which increases with age (117); (b) the fraction

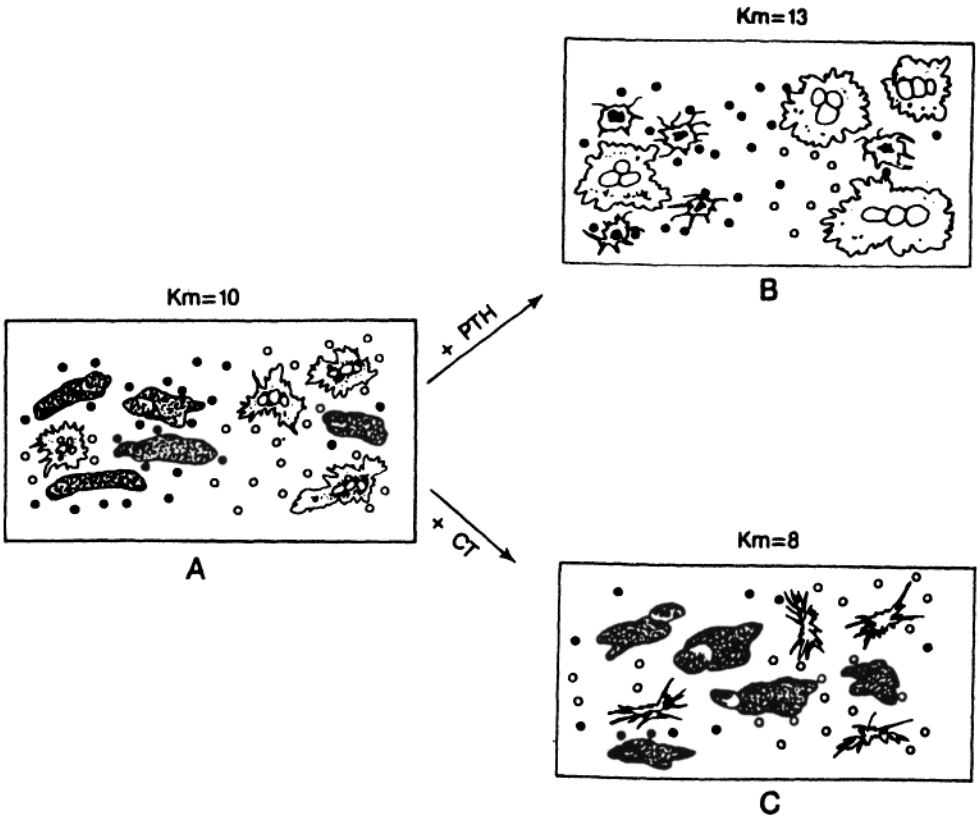

FIGURE 8 Diagram representing the effect of the acute administration of parathyroid hormone (PTH) or calcitonin (CT) on the spatial relationships of osteoblasts and osteoclasts on a bone surface. The open and closed circles represent, respectively, the K_m values of high-affinity and low-affinity bone calcium-binding sites. Diagram A represents a normocalcemic situation, with an equal number of high- and low-affinity sites. Diagram B represents the result of PTH administration, where the shrinkage of osteoblasts has exposed low-affinity sites and the associated expansion of osteoclasts has blocked high-affinity sites, leading to an average K_m of 13 and hypercalcemia. Diagram C represents the result of CT administration, where the shrinkage of osteoclasts has exposed high-affinity sites and the consequent expansion of osteoblasts has blocked low-affinity sites, leading to an average K_m of 8 and hypocalcemia. *Note*: High-affinity sites are considered to have an apparent K_m of 5 mg Ca/dL, and low-affinity sites an apparent K_m of 15 mg Ca/dL. For the sake of convenience, the K_m's refer to total plasma calcium, rather than the theoretically correct ionic calcium concentration, approximately half of the total. Bone mineral with a high Ca/P ratio is considered to have a relatively higher affinity for calcium binding than bone mineral with a low Ca/P ratio (see text and Table 3). (Reproduced with permission from Ref. 87.)

of cardiac output that goes to bone, which decreases with age (118); (c) the nature of the bone mineral, which affects the extraction rate; and (d) the driving force, i.e., the differential between the plasma calcium level as it enters the skeleton and the mean K_m of the bone calcium-binding sites.

Factors (a), (b), and (c) may be considered responsible for the slowing of the rate of return (or reduction of the error correction rate) that differentiates young and old dogs

(Table 2). The absolute plasma calcium level of young and old dogs is similar, hence their mean K_m—i.e., the proportion of high- to low-affinity bone calcium-binding sites—must be similar. The difference in the error correction rate would arise therefore from a slower circulation rate, a smaller fraction of the cardiac output going to bone, and perhaps a smaller extraction rate.

Rats deprived of their endogenous calcitonin have a slower error correction rate than normal animals (Table 2), yet their skeletons are neither smaller nor less well mineralized than those of controls (100), their plasma calcium is the same as that of controls (100), and there is no reason to believe that cardiac output or circulation time is altered. Hence factors (a), (b), and (c) would not come into play. Instead, a difference in driving force can be postulated, as follows. In normal mammals, calcitonin is released when plasma calcium rises acutely (119), acting directly on osteoclasts (104). This leads to osteoclast retraction and the exposure of high-affinity binding sites. In turn, this results in a greater driving force for calcium deposition and an increase in the rate at which normocalcemia is restored. In animals without calcitonin, this response cannot occur and the half-time of error correction is correspondingly lengthened.

A difference in driving force can also explain why parathyroidectomized (PTX) animals require more time to overcome a positive calcium load (see Ref. 87 for details).

The explanation involving a driving force cannot apply to vitamin D deficiency, which in some ways resembles parathyroidectomy, yet vitamin D-deficient rats have maximum levels of circulating PTH (109). It is the low absolute amounts of bone in vitamin D deficiency (73) that are likely to cause calcium extraction to be reduced [factor (c)], hence leading to a lengthening of $t_{1/2}$ of the error reduction rate.

The preceding discussion makes it obvious that ablation of hormonal control, due to PTH, calcitonin, or vitamin D, reduces the rate at which an error in plasma calcium is overcome by bone. It is bone, however, whether or not under hormonal control, that is the principal regulator of the plasma calcium and that is responsible for causing the plasma calcium to return to its preload level and to maintain it at that level.

V. INTRACELLULAR CALCIUM

Three mechanisms contribute to maintaining a low intracellular calcium concentration: (a) a limited entry rate, governed by a limited number of plasma calcium channels, probably less than 1000/cell (12); (b) a significant intracellular store of calcium-binding sites, found in the various cell membranes, the endoplasmic reticulum, in and on the various organelles, with mitochondria playing a major role; and (c) efficient extrusion mechanisms, i.e., Ca-ATPase and Na/Ca exchanger.

Entry and extrusion mechanisms have already been described in the relevant sections on transcellular calcium transport (Sec. III.B). Different cells and tissues may have specific entry and extrusion mechanisms that differ in structural and/or functional details from the general model. For example, the vitamin D-dependent protein (32,33) or peptide (34) that occurs in intestinal cells, and that may play a role in modulating calcium entry into the intestinal cell, may be specific to that cell, as there is no logical requirement that calcium entry into cells which do not transport calcium be enhanced by vitamin D. Similarly, while vanadate is an effective inhibitor of the plasma membrane Ca-ATPase, it has little effect on the renal Ca-ATPase (120). Whereas calmodulin modulates the activity of the Ca-ATPase of plasma membrane or cardiac sarcoplasmic reticulum, it does not seem to do so in the case of the liver plasma membrane enzyme (121).

In resting liver cells, as in most cells, the free intracellular Ca^{2+} concentration is in the 100-nM range. In stimulated hepatocytes, the intracellular free calcium concentration reaches 600 nM and, when the cells are under oxidative stress, intracellular calcium may reach micromolar concentrations. Moreover, total cytosolic calcium of liver cells is 3–4 mmol/kg dry weight. It is obvious, therefore, that liver cells, like other cells, have significant cellular calcium stores. These include the rough and smooth endoplasmic reticulum, the Golgi complex, mitochondria, inner and outer leaflets of the plasma membrane, lysosomes, and possibly secretory granules. (For documentation and further discussion of the liver cell, see Ref. 122.) The endoplasmic reticulum probably contains the largest fraction of intracellular calcium, perhaps a quarter of the total (123)—easily four times that found in the mitochondria. This quantitative disparity would not necessarily make it unlikely for mitochondria to play a major role in intracellular calcium regulation, as proposed by Lehninger et al. (124), since mitochondrial calcium might turnover more rapidly than that of other organelles, but more recent analyses have made it less likely that mitochondria play a significant regulatory role (125).

External signals to cells, such as hormones, neurotransmitters, or growth hormones, are detected by receptors located on the outer aspect of the plasma membrane and transmitted to the cell interior by second messengers. 1,2-Diacyl glycerol and myo-inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) are two major messenger molecules (122). Ins 1,4,5-P_3 causes calcium to be released reversibly from intracellular calcium stores, typically the endoplasmic reticulum; the released calcium is the "calcium signal" or "second messenger." The released calcium travels to various intracellular locations and, depending on the cell in question, induces a calcium-dependent reaction. Since the discovery of these events in 1983 (126; for more details see 127) a very large number of cellular events have been shown to involve and/or be mediated by calcium signaling. Typical examples include cell volume regulation (128), egg activation (129) and fertilization (130), growth factor-induced changes in cell proliferation (131), secretion, as in adrenal chromaffin cells (132), transmitter secretion from presynaptic nerve terminals (133), and platelet activation (134), to name just some of the many processes that seem to involve calcium signaling.

While Ins 1,4,5-P_3 is involved in all instances in the release of the calcium signal from an internal source, it is less certain whether this inositol molecule is also involved in calcium gating and/or the uptake of calcium from the extracellular milieu (127). The quantity of calcium released for signaling is generally so low that this alone would not necessarily lead to increased calcium influx. In other words, whether calcium channels, the entry ports for calcium, are closely linked to or regulated by phosphatidyl-inositol turnover is not established. Moreover, while Ins 1,4,5-P_3 plays a key role in mediating intracellular release of calcium, it has not yet been demonstrated that this molecule is formed fast enough to account for the very rapid release of Ca^{2+} (250 ms, Ref. 134). Protein kinase C and elevation of cAMP inhibit calcium mobilization (134) and may therefore inhibit processes stimulated by the calcium signal, e.g., secretion, contraction, or changes in conductivity.

An interesting but as yet incompletely understood phenomenon of calcium signaling—or intracellular calcium movement—involves discrete transients or oscillations (135). Oscillations may result from fluxes across the plasma membrane or from fluxes across intracellular membranes. In hepatocytes (136) agonists that act via Ins 1,4,5-P_3 provoke dose-dependent increases in intracellular free calcium, with oscillation frequency increasing, the latency period decreasing, but the amplitude of the oscillations remaining constant.

In the case of the hepatocytes, the changes in intracellular calcium concentrations are initiated at a specific subcellular domain, adjacent to the plasma membrane, and are then propagated through the cell in the form of a wave. The most reasonable explanation of this phenomenon at present is a calcium-induced calcium release. Something like this is also thought to occur in the muscle cell of the heart (137; see also below). Thomas and Renard (136) propose that the oscillating calcium signal may function to allow all parts of the cell to respond to the stimulus.

The muscle cell is probably where intracellular calcium traffic is most elaborate and tied most closely to its function. Langer (137) has reviewed the sequence of calcium movements in the muscle cell in the course of a contraction cycle. When the cell becomes depolarized, calcium enters via L-type calcium channels, the best characterized voltage-sensitive calcium channels (for further description, see Ref. 138). Entry occurs during the plateau phase of the action potential and induces calcium release via the "feet," i.e., the connection between the sarcoplasmic reticulum and the sarcolemma. Calcium that is released is the major source of the calcium-activating contraction. As calcium enters the narrow subsarcolemmal space, calcium concentration of the space goes up transiently, thereby activating the Na/Ca exchanger, which then extrudes calcium even as the myofilaments are being activated. The rate of calcium efflux is such as to maintain the cellular calcium at a steady state.

As the myocardial cell repolarizes, calcium is pumped into the longitudinal sarcoplasmic reticulum, at the same time diffusing into the cistern. Calcium extrusion via the exchanger continues and calcium is pumped out through the diastole by the calcium pump of the sarcolemma, the pump being a Ca-ATPase (139).

In summary, it appears that intracellular calcium is very closely controlled, with rises in intracellular calcium, when due to external signals such as hormones or neurotransmitters, serving as intracellular signals which in turn elicit calcium-dependent processes such as secretion, contraction, etc. Transient rises in intracellular free calcium are readily overcome by calcium becoming bound to a large number of organelles. Calcium entry is via channels, and calcium extrusion is via the Na/Ca exchanger and the Ca-ATPase. In specialized cells such as muscle cells, intracellular calcium is shuttled back and forth via intracellular pumps.

VI. CALCIUM IN BONE

Calcium, along with phosphate, constitutes the principal structural substance of the skeleton that gives vertebrates their internal support and, in the case of people, allows them to be erect. The skeleton constitutes about 16% of an adult's body weight. Forty-seven percent of the skeletal weight is dry, fat-free bone, and 26% of the latter is calcium (140). Table 3 lists the various phases in which calcium phosphate occurs in bone. The precise order in which calcium is precipitated and the path it follows as calcium salts mature and become increasingly less soluble is not yet known. The final calcium salt is a carbanato-hydroxyapatite, i.e., an apatitic type of calcium phosphate that also contains, at least on the surface, calcium carbonate. The mechanism of calcification, many years of study notwithstanding, is not fully known. The initial calcium phosphate is deposited on or inside collagen fibrils that have formed extracellularly from collagen molecules that had been extruded by osteoblasts, the bone-forming cells. Once the calcium phosphate molecule is in place, additional calcium phosphate is deposited by accretion so that the calcium salt is

arranged in a very orderly fashion. The crystals are platelike, with a long axis that is 30–70 nm, and a short axis that is 10–25 nm (98). The surface layers of the bone crystals constitute 50% of the newly formed crystals, but ultimately, in the fully mature crystal, they constitute only about 25% of the fully mature crystal volume (98).

The principal function of the metabolically active osteoblast is to produce the collagen which represents the major component of the extracellular matrix on which calcium phosphate precipitates. Depending on species, age, and type of bone, osteoblasts cover some 5–10% of the bone surface. They are more numerous in trabecular than in cortical bone, more numerous in young than in mature bone, and their capacity to respond to fracture—i.e., the ability of connective tissue cells to respond to the osteoinductive stimulus that results from fracture—is diminished with age. Osteoblastic activity also diminishes with age (98). From Eriksen's data, as tabulated by Simmons and Grynpas (98), bone remodeling by osteoblasts and osteoclasts takes around 10 days in humans, but mineralization of sites laid down by osteoblasts takes an order of magnitude longer, i.e., some 100+ days. The extracellular matrix in which mineralization takes place also contains numerous molecules; some bind calcium, all in some way seem to aid in bone formation and mineralization, even though in many instances specific functions are not known. For a more detailed review of these molecules, see Refs. 141–143.

Calcium exists in bone in two forms: in solution, as part of the fluid that surrounds and exists in bone, and in a solid state, as the phases of calcium phosphate described above and listed in Table 3. The bone calcium pool contains five to six times more calcium than is found in the extracellular fluid, but constitutes only a small fraction of the total body calcium. For example, in a 220-g male rat, the extracellular calcium amounts to 0.67 mmol of Ca, the bone calcium pool contains about 3.45 mmol of Ca, whereas the total skeletal calcium amounts to about 120 mmol of Ca (144).

The bone calcium pool serves two functions: as the source of calcium that becomes deposited as bone salt, and as the source of exchangeable calcium, i.e., the calcium that leaves and reenters blood, with the concentration of blood plasma calcium determined by the average calcium-binding capacity of the bone salt.

In the example of the male rat quoted above, the bone calcium pool turns over about 1.3 times per day, with bone calcium deposition constituting about 43% of the total calcium flow into the bone calcium pool. The fraction of calcium entering the bone calcium pool that then undergoes a phase transformation to become bone salt diminishes with age more than does the total flow of calcium into the bone calcium pool (Table 4).

Bone calcium resorption, i.e., the phase transformation of solid to solution and the return of that calcium into the circulation, also decreases with age, but somewhat less rapidly than does the bone calcium deposition rate. As a result, the individual goes into negative bone calcium balance; i.e., the bone mass decreases. This is illustrated in Table 4. Note in particular in Table 4 how the bone calcium deposition rate decreases with age and sex in humans and rats, with corresponding decreases in the calcium balance.

Modeling and remodeling of bone also involve structural changes, i.e., osteoclast-mediated dissolution of calcium and partial or total degradation of the extracellular matrix from which the bone salt has been removed. These structural changes proceed necessarily more slowly than the initial steps which involve calcium binding and release by the bone salt.

Thus bone formation and bone resorption are terms, each of which designates a cascade of events, with calcium constituting the principal cation of bone salt, the major product of the osteoblasts.

TABLE 4 Parameters of Calcium Metabolism in People and Rats

	Young male human	Postmenopausal female human	Young male rat	Young female rat	Old female rat
Body wt (kg)	76	55	0.23	0.17	0.30
Age (yr)	17	62	0.19	0.33	0.83
Plasma calcium (mg/dL)	10.3	9.9	11.0	9.5	n.d.
Calcium pool (mg)	6100	3500	165	148	42
Pool turnover (mg/day)	1500	650	84	71	31
v_i = calcium intake (mg/day)	1160	625	70	51	n.d.
v_u = urinary calcium output (mg/day)	250	100	1	1	n.d.
v_{ndo} = fecal endogenous output (mg/day)	150	75	5	9	n.d.
v_F = total fecal Ca (mg/day)	860	550	34	20	n.d.
Δ = calcium balance (mg/day)	+50	−25	35	30	n.d.
v_a = calcium absorbed (mg/day)	450	150	41	30	n.d.
Percent calcium absorption	39	24	59	59	n.d.
v_{o+} = bone calcium deposition (mg/day)	1100	475	78	61	15
v_{o-} = bone calcium removal (mg/day)	1050	500	43	31	n.d.
Fraction to bone, v_{o+}/v_T	0.73	0.73	0.93	0.86	0.50
Fraction excreted, $v_u + v_{ndo}/v_T$	0.27	0.27	0.07	0.14	0.50

The plasma calcium is part of the calcium pool (Fig. 1).
Pool turnover, $v_T = v_a + v_{o-} = v_{o+} + v_u + v_{ndo}$
n.d. = not determined.
40 mg Ca = 1 mmol.
Data based on reports in Refs. 75, 109, 144–146.

VII. CALCIUM EXCRETION

A. Urine

Of the calcium circulating in the blood, less than 0.1% is typically excreted in the urine. Thus, in an adult man of 70 kg, the plasma volume is about 2.5 L and contains about 6 mmol calcium. Cardiac output would be 5.1 L/min (99), with 20–25% of the cardiac output being presented to the kidney and half of that amount filtered at the glomerulus. Thus the nephron handles approximately 1.5 mmol of Ca per minute or some 2100 mmol of Ca/day. The urinary calcium output of the typical 70-kg man might be 7 mmol/day, or 0.3% of the filtered load. Actual calcium excretion in the urine is a complicated function of an individual's prior calcium intake, vitamin D and nutritional status, sex, reproductive status (if female), as well as age and therefore skeletal maturity.

Calcium in the lumen of the nephron is subject to two transport processes (147): A paracellular, concentration-dependent transepithelial movement, and a transcellular movement that constitutes active transport against an electrochemical gradient. Calcium is reabsorbed, i.e., moves transepithelially, in the proximal tubule (straight and convoluted), mostly by a passive mechanism, although some movement may be transcellular and

involve entry via calcium channels and extrusion via Ca-ATPase (148). Very little calcium is reabsorbed as the luminal fluid passes through the thin limbs of Henle. As the fluid enters the thick ascending limb of the nephron, calcium reabsorption is resumed, largely by a passive process. Active transepithelial calcium transport occurs principally in the distal convoluted tubule (DCT; 147,148). Once the luminal fluid has passed the region of the DCT, it enters those regions of the nephron designated as connecting and collecting ducts, where transport is essentially by a paracellular route.

Active calcium transport in the DCT, as across the duodenal cell, involves three steps: entry across the brush border region of the cells lining the lumen of the DCT, intracellular diffusion, and extrusion across the basolateral pole of the renal cell. Entry is likely to be via calcium channels, the existence of which in the kidney has been documented (26).

Intracellular self-diffusion of the calcium ion through the DCT cell has been estimated as nearly two orders of magnitude too slow (149), compared with the experimentally determined V_m (150). In other words, transcellular calcium transport in the kidney, i.e., in the DCT, requires the same kind of amplification that is provided in the duodenal cell by the intestinal calbindin. Indeed, there exists a vitamin D-dependent, intracellular calcium-binding protein, termed calbindin D28K, found almost exclusively in the kidney (151,152). It is a separate gene product from calbindin D9K. Both calbindin D9K and calbindin D28K are found in the embryonic kidney, but as development proceeds, expression of calbindin D9K is repressed and calbindin D28K remains as the only calcium-binding protein with an E-F hand. Calbindin D28K has a molecular weight of 28,000 Da, has four calcium-binding sites, and has $K_D = 2 \times 10^{-6}$ M. It occurs principally in the DCT (153) and therefore may serve as a calcium ferry. It has been calculated (149) that the amplification provided by the renal calbindin is similar to that due to the intestinal calbindin, i.e., about 76-fold, if its concentration in the DCT is similar to that in the duodenal cell, a reasonable possibility. All available evidence thus indicates that the renal calbindin fulfills the same kind of ferrying role in the DCT as does the intestinal calbindin, thus providing the molecular basis for calcium reabsorption in the DCT.

Calcium extrusion from the renal cell is against an electrochemical gradient and is effected by the Ca-ATPase in the basolateral membrane and perhaps also by the Na/Ca exchanger. The nature and characteristics of the renal Ca-ATPase are likely to be similar to those of the intestinal Ca-ATPase, but no detailed report on the renal Ca-ATPase has come to this reviewer's attention.

Whereas in the intestine the Na/Ca exchanger appears to play no role in calcium extrusion (57), this may not be true in the kidney. However, it is not clear whether the exchanger's principal role is calcium extrusion or whether it is even involved in calcium traffic. There exists in the renal proximal tubule a reciprocal relationship between sodium reabsorption and intracellular calcium concentration, a rise in calcium depressing sodium reabsorption (154). The Na/Ca exchanger may regulate sodium channels indirectly, inasmuch as channel activity is diminished as intracellular calcium rises (155). Whether these observations also apply to the DCT and how sodium metabolism modulates active calcium transport is not clear. It is probable, however, that a disturbance of sodium metabolism of the renal cell—or, for that matter, of the duodenal cell—will play havoc with calcium transport (14).

Paracellular calcium transport accounts for most of the calcium reabsorption in the kidney. The mechanisms by which this occurs have not been fully clarified.

In addition to vitamin D, the major regulator of urinary calcium excretion is parathyroid hormone (PTH), causing increased reabsorption of calcium from the renal tubule,

while phosphate excretion is simultaneously increased. The mechanism by which PTH enhances calcium reabsorption is unclear. PTH causes a rise in 1,25-dihydroxyvitamin D_3 ($1,25\text{-}OH_2\text{-}D_3$), so that increased calcium reabsorption due to elevated PTH levels may have resulted from an increased level of the renal calbindin. However, the initial response to PTH seems faster (156) than if only a biosynthetic pathway were involved. Whether PTH acts on the entry or extrusion steps of transcellular transport or causes changes in the configuration of the tight junctions via a cAMP-mediated intracellular event has not been clarified.

In summary, urinary calcium excretion represents significantly less than 1% of the calcium that is circulated to the kidney. Most of the calcium filtered by the kidney is reclaimed by the body by passive transport, with active transport, mediated by the renal calbindin ($M_r \sim 28{,}000$ Da), occurring largely in the distal convoluted tubule.

B. Stool

Calcium in the stool has two sources: unabsorbed food calcium and unreabsorbed "body" calcium, i.e., calcium that entered the intestinal lumen with the various body fluids such as bile, succus entericus, etc., but that was not reabsorbed. One can therefore write

$$v_F = v_i - v_a + v_{ndo} \tag{8}$$

where

v_F = fecal calcium excretion rate
v_i = calcium ingestion rate
v_a = rate of calcium absorption
v_{ndo} = endogenous fecal calcium excretion rate
units = mg Ca/day

To determine the rates in Eq. 8, it is necessary to differentiate between $(v_i - v_a)$, on the one hand, and v_{ndo}, on the other hand. Otherwise all that can be measured is S_i, net absorption, that is, $v_i - v_F$. In either case, whether measuring S_i or v_a and v_{ndo}, it is necessary to use fecal markers to take into account the time delay between ingestion and excretion. The simplest way to resolve Eq. 8 is to administer a calcium tracer intravenously at about the time the intestinal balance measurement is initiated. The amount of tracer collected in the stool during the intestinal balance period can be converted to v_{ndo} by simultaneously estimating the area under the plasma tracer curve during the same period and dividing that area into the amount of fecal tracer collected between fecal markers. Thus, combining an intestinal mass balance with a tracer study will yield numerical values for v_F, v_i, and v_{ndo} in Eq. 8, so that all four values are determined. See Refs. (58) and (91) for equations and practical details.

Concerning the endogenous fecal calcium, v_{ndo}, one can ask whether it plays a regulatory role in calcium metabolism. The origin of endogenous fecal calcium is calcium secreted into the intestine in bile juices, pancreatic juices, the *succus entericus*, and calcium from cellular debris.

It seems unlikely that bile juices are secreted in response to the body's calcium needs. Moreover, over 90% of bile fluids are reabsorbed as hepatic circulation (157). Pancreatic juices and the various secretions that are poured into the intestinal lumen appear to function mostly in facilitating digestion and absorption, with the bulk of the fluid presented to the entire intestine being reabsorbed (158). The calcium content of these fluids is not well known, but they form largely by osmotic equilibration in response to the contents of the

upper intestine. Therefore their calcium content, like their monovalent ion content, probably simply reflects the body fluid composition (158). On the basis of these considerations, a direct regulatory role of v_{ndo} in calcium metabolism seems unlikely.

In summary, fecal calcium excretion consists of unabsorbed food calcium and endogenous calcium that has not been reabsorbed. In human subjects, the latter is approximately equal in amount to the urinary calcium. In rats, it is 10 times greater than the calcium lost from the body in urine. There is no indication, however, that endogenous fecal calcium is subject to regulation or plays a regulatory role in calcium metabolism.

VIII. REGULATORS OF CALCIUM METABOLISM

A. Calcitropic Hormones*

Three systemic hormones are involved in the regulation of calcium metabolism: parathyroid hormone (PTH), vitamin D, and calcitonin (CT). PTH and CT are both peptides; vitamin D is a secosterol whose hormonelike action was recognized less than 30 years ago. PTH and CT interact with plasma membrane receptors on their target cells, whereas vitamin D, after a two-step transformation in liver and kidney to 1,25-dihydroxyvitamin D, acts on target cells via a gene-mediated mechanism. As a result, PTH and CT have quite rapid effects, whereas the vitamin D-mediated responses tend to be somewhat slower (102). One target of vitamin D is the parathyroid cell (159); consequently, there exists some interaction between vitamin D and PTH, but most responses associated with one hormone occur independently of the action of the other two.

1. Parathyroid Hormone

Chemistry, Biosynthesis, and Metabolism

PTH is a protein of 84 amino acids (Fig. 9). It is synthesized in the form of a precursor, termed for historical reasons the "prepro PTH," a protein of 115 amino acids. The "pre" sequence of 25 amino acids is thought to be a signal sequence that permits establishing a polyribosomal/membrane junction in the rough endoplasmic reticulum. The second cleavage, i.e., the removal at the amino terminal of the six-amino acid sequence that constitutes the "pro" segment, occurs in the Golgi complex about 15 min after the signal sequence has been cleaved off. The hormone molecule, i.e., the 84-amino acid polypeptide, is then extruded, probably by means of exocytosis. It is thought that the hormone is located inside a vesicle or granule whose limiting membrane fuses with the plasma membrane. This step is followed by lysis of the membrane and eventual extrusion or release of the hormone molecule into the circulation (161).

The circulating PTH is transported to its target tissues, bone and kidney, as well as to the liver. In the liver, the molecule undergoes further cleavage, either between residues 33 and 34 or at several other sites that are near the 33/34 site; it is also cleaved near the middle of the molecule. Whether this cleavage has functional significance is not known, but it may yield a fragment that still can exert PTH action. Other fragments also result from this and

*The literature on the endocrine aspects of calcium metabolism is very extensive, and appropriate treatment would far exceed the confines of this chapter. For this reason, the text of Section VIII has been adapted from Ref. 159. Where references to a particular phenomenon are multiple, the reader is referred to appropriate reviews. As a result, cited references are not exhaustive and may not reflect all publications that document a point or conclusion.

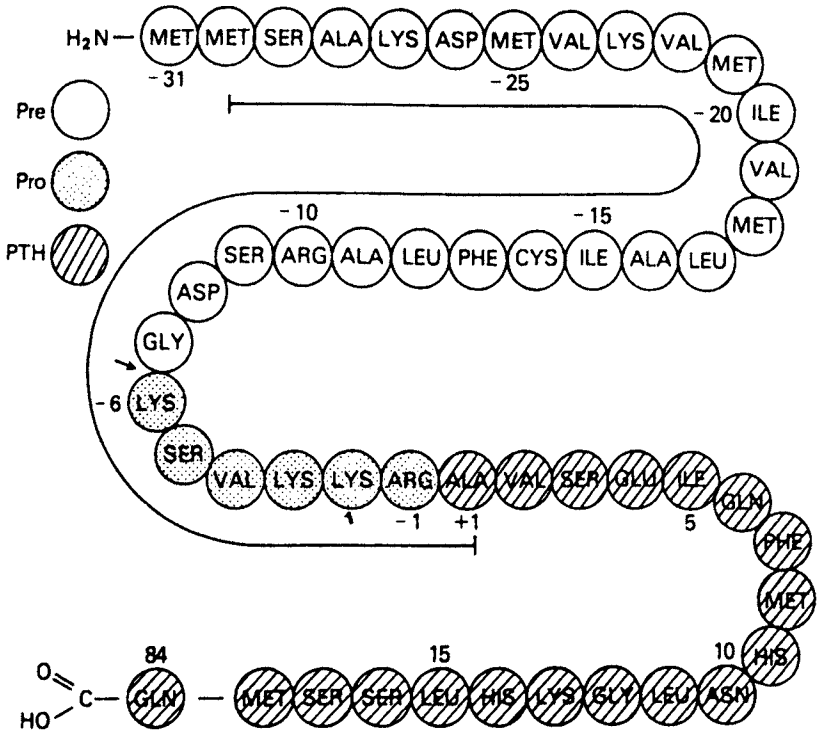

FIGURE 9 Amino acid sequence of parathyroid hormone, the prepro- and pro-hormone. (Adapted from Ref. 160.)

other cleavages. Most fragments, except the 1–34 amino-terminal sequence, have no or only limited biological activity. The 1–34 fragment has biological activity that appears to be identical, both qualitatively and quantitatively, to that of the intact hormone. The functional significance of the 35–84 sequence of the intact hormone is not known.

The genes of murine, bovine, and human PTH have been cloned (162). The human and bovine gene, like their hormone products, are more alike, while the rat gene (and hormone) exhibits fewer homologies with the human gene (and hormone). The human gene is located near the end of the short arm of chromosome 11, close to the genes encoding the hormones calcitonin and insulin. Transfection studies in which the PTH gene has been inserted in lines of cells that do not normally express PTH are under way and should yield information on gene activation, transcription, and translation.

When PTH interacts with the receptor site on the plasma membrane of kidney or bone cells, there results a stimulation of the adenyl cyclase system leading to increased production of 3′,5′-cAMP (162,163). This is a specific test for PTH, inasmuch as only target cells respond to PTH by an increase in their adenyl cyclase activity and their cAMP content.

One of the earliest responses of the osteoblast, the bone cell that is equipped with PTH receptors, is a shape change, i.e., a retraction of peripheral lamellipodia (101,102), probably modulated via cAMP. The change in shape would then be the first step in the cascade of steps that ultimately leads to increased bone resorption, mediated by the osteoclast.

Function

The principal function of PTH is to regulate the calcium concentration of extracellular fluid. This is accomplished by altering the transfer of calcium from and into bone (144), by changes in renal calcium reabsorption (147), and by indirectly inducing changes in intestinal calcium absorption (164).

In the normal mammalian organism, major regulation of the bone calcium balance is effected by modulation of bone calcium resorption. Figure 10 shows that in the parathyroidectomized animal, the relationship between bone calcium deposition and resorption rates is altered both quantitatively and qualitatively. In the normal organism, the rate of bone calcium deposition is virtually constant, regardless of how much calcium enters the body via the gut. Bone resorption drops in direct proportion to the amount of calcium absorbed. In the organism without functioning parathyroid glands, on the other hand, the intensity of both parameters is reduced to nearly one-half. Moreover, as calcium comes in from the gut, bone resorption goes down, but at a lesser rate than in the normal animal (165). At the same time, the rate of bone calcium deposition goes up, at a rate that is almost twice as fast as in the normal animal (144). In other words, in the presence of PTH, regulation of calcium flow to the skeleton is exerted almost exclusively by means of the resorptive processes, whereas in the absence of PTH, the regulatory action of bone calcium resorption, although still dominant, is lessened.

These effects, observed some years ago (144), may now be explained as having resulted from the complementary behavior of osteoblasts and osteoclasts. When PTH is released from the parathyroid glands, in response to a drop in the plasma calcium, osteoblasts assume their contracted state and their metabolic activities such as collagen synthesis and secretion are largely arrested. Osteoclasts in turn spread out, occupying some of the space freed up by osteoblastic retraction, and their metabolic action is intensified,

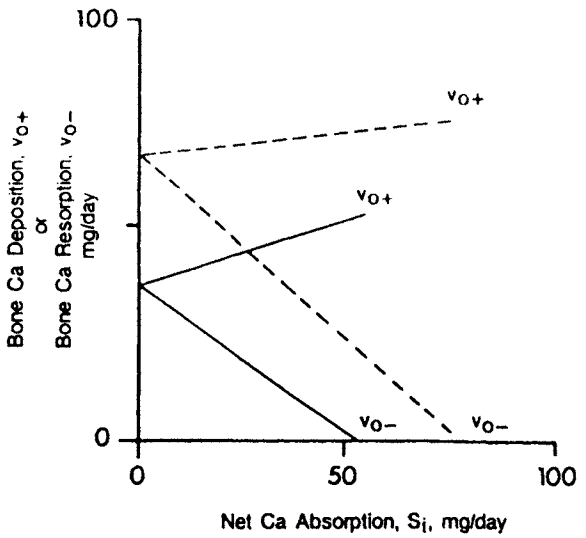

FIGURE 10 Effect of parathyroidectomy on bone calcium deposition and resorption in rats. Dashed line, euparathyroid; solid line, parathyroidectomized animals. The experimental points have been omitted for the sake of clarity. (Adapted from Ref. 144.)

leading to resorption of bone mineral and degradation of the extracellular matrix and collagen fibers (Sec. VI).

The second organ that responds to PTH is the kidney. One response involves the release into the urine of cAMP (166), about half of which is of renal origin. It is the latter that is significantly increased as a result of an infusion of PTH, and the increase in nephrogenous cAMP may be used to assess the amount of circulating PTH (163). The hormone also causes a marked phosphaturia; this is the best-known clinical effect. Finally, PTH leads to an increase in the glomerular clearance of calcium, i.e., the amount of calcium that is cleared from the luminal fluid and returned to the plasma (167).

The mechanisms by which PTH causes increased phosphate excretion and diminished calcium excretion have not been fully clarified. The phosphaturic effect of PTH may be due to a direct effect of PTH on phosphate transport and/or to its effect on sodium or bicarbonate reabsorption (168). The effect of PTH on calcium reabsorption may involve a modulation by the hormone of the intercellular space and a resultant increase in paracellular calcium flux (147).

The indirect effect of PTH on calcium absorption is mediated by 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2$-D_3, the active metabolite of vitamin D, the renal hydroxylation of which is stimulated by PTH (168). The mechanism by which PTH acts to enhance either the rate of hydroxylation of 25-(OH)D_3 or the amount that becomes converted to the dihydroxylated form is not known. The increase in 1,25-$(OH)_2$-D_3 leads to increased synthesis of intestinal calbindin (CaBP) (164). Shifting animals to a low-calcium diet leads to a rise in intestinal CaBP, whether or not the animals are parathyroidectomized; however, the response in the parathyroidectomized animal seems smaller (164).

The normal plasma calcium level is quite invariant at 2.5 mM (Sec. IV). Following parathyroidectomy, plasma calcium drops to about 1.5 mM and remains much less constant. Diurnal variations, due partly to variations in intake, which are small in the normal state, are greatly magnified, as is the increase in plasma calcium in response to higher intakes (144). This increase in the euparathyroid state is about 2%, but is about 30% in the parathyroidectomized state. Thus, the ability to maintain the plasma calcium steady is markedly impaired in the parathyroidectomized or parathyroprivic state. In the euparathyroid animal, the presence of the hormone permits inflow of calcium from bone during periods when inflow from the gut is nil or low, as in nonfeeding periods (165). This compensatory mechanism is absent in the parathyroidectomized animal and partly accounts for the greater diurnal variability of the plasma calcium. In addition, as calcium turnover in bone is much slower in animals without parathyroids (73), the calcium pool expands more when calcium enters it from the gut, even if most of the incoming calcium is absorbed by the paracellular route only (Sec. III).

PTH is released from the gland when the extracellular calcium level drops, and its release is inhibited when extracellular calcium rises. The extracellular changes in calcium concentration are paralleled by changes in intracellular calcium, but the mechanism by which intracellular calcium alters hormone release and directly or indirectly affects hormone synthesis is not known.

2. Calcitonin

Chemistry and Biosynthesis

Calcitonin is a 32-amino acid polypeptide (Fig. 11) that is synthesized in the parafollicular cells, also known as "C," or "clear" cells of the thyroid gland. These cells arise embry-

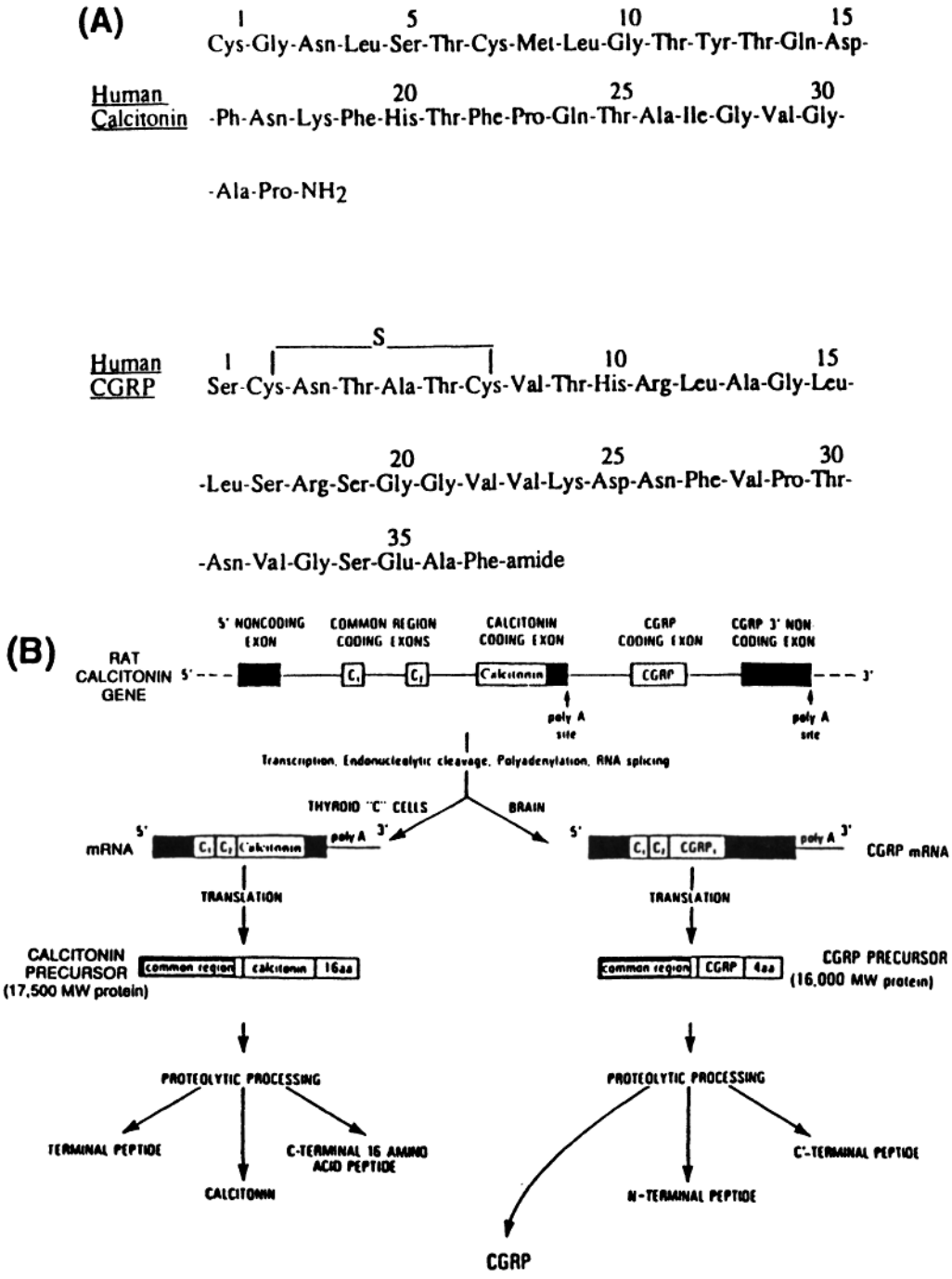

FIGURE 11 A. Amino acid sequence of calcitonin and CGRP. B. Transcription of calcitonin gene, translation of calcitonin and CGRP. (Reproduced by permission from Ref. 159.)

ologically from the fifth branchial pouch and migrate into the thyroid gland in mammals, but in fish they form the ultimobranchial body, a distinct entity (for review, see Ref. 169). Remarkably, there is considerable similarity between calcitonin from fish and mammals, and the bovine and human molecules are more distinct than human and salmon calcitonin. The latter exists in three forms, one of which is the most potent, biologically speaking, of all calcitonins that have been sequenced (163).

Calcitonin was one of the first hormones to be sequenced, but its function has remained a mystery. It has a pronounced, transient hypocalcemic effect; it is this effect that was utilized to purify and determine its structure. However, its role in calcium metabolism is unclear.

Studies of the calcitonin gene have shown that it gives rise by differential splicing (Fig. 11B) to two products, calcitonin and another protein, called the calcitonin gene-related peptide (CGRP; Fig. 11B). Whereas calcitonin is expressed in the C cells of the thyroid, different controls lead to the expression of CGRP, largely in the central nervous system. As is true for many gene products, the messenger RNA for calcitonin is translated into a precursor molecule of 136 amino acids (in the rat), which is then cleaved into three fragments, one of which is the hormone (163). In the case of the messenger RNA for CGRP, the precursor is again split into three peptides, one of which is CGRP.

The function of CGRP is unknown. Systemic and local administration of CGRP increase the rate and force of contraction of the isolated atrium of the heart (170). Administration of CGRP to normal human subjects leads to vasodilatation and to flushing of the face. Some of these effects can also be induced by calcitonin, but require much greater doses. This can be explained by cross-reaction of receptor binding, plus the existing homology of these two products of a single gene. CGRP is a 37-amino acid polypeptide.

Function

Calcitonin interacts with a receptor located on the plasma membrane of osteoclasts (104,105). This interaction leads to a stimulation of the adenyl cyclase system, with increased accumulation of cAMP. The interaction of calcitonin with the osteoclast causes the latter to stop its searching action, expressed by the movement of pseudopodlike extensions that move over the extracellular bone surface and presumably precede the release of lysosomes and dissolution of bone (171,172). Osteoblasts do not have receptors for calcitonin.

At the level of the whole organism, the function of calcitonin is not understood. When calcitonin was first discovered, it was thought to function in a push–pull fashion with PTH, PTH causing bone resorption to be enhanced, while calcitonin inhibited bone resorption. However, extensive studies of animals deprived of endogenous calcitonin have failed to reveal major changes in bone metabolism (100,169). Animals that have been deprived of their C cells exhibit no change in their steady-state plasma calcium level and the rates of bone calcium deposition and resorption are unchanged, as are their urinary calcium and endogenous fecal calcium excretions. Calcium absorption is also unchanged. Patients with medullary thyroid carcinoma and excess amounts of circulating calcitonin also seem to have no obvious disturbance of calcium metabolism (173).

The major effect of a deficiency in calcitonin is the inability to overcome a hypercalcemic challenge as readily as when calcitonin can be released normally. When a large amount of calcium is given so as to expand the calcium pool, the rate at which the expanded pool returns to the preinjection value in a calcitonin-deficient organism is about half what it would be normally (Table 2; Ref. 84).

3. Vitamin D

In contrast with the peptides, PTH, and calcitonin, vitamin D is a secosteroid, a cyclopentanoperhydrophenanthrene-related structure. The amount of the vitamin that the body can produce from precursors in the absence of sunlight is insufficient to meet the need for this compound, especially in growing infants and children living in northern climates. When it was identified in the early 1920s, the compound was classified as a vitamin, i.e., as an essential micronutrient. About 40 years after its identification, as the metabolism of vitamin D became known, it became clear that the mode of action of the biologically active metabolite derived from vitamin D was close to that of steroid hormones, that is, the genomic induction of specific proteins.

Chemistry and Metabolism*

Cholesterol is the precursor compound that, in a series of steps, becomes converted to 7-dehydrocholesterol, the provitamin D. The latter accumulates in the skin and, under the action of ultraviolet light, is converted to vitamin D in two steps. In the first step, one of the rings (B) is opened up, yielding the secosteroid structure typical of the vitamin. The second step involves a rearrangement of the "previtamin D" to vitamin D. Neither of these will take place in the absence of sunlight (or ultraviolet light), hence the need for ingesting preformed vitamin D in the absence of sunlight.

Vitamin D undergoes two sequential hydroxylations, one in the liver, the other in the kidney. In the liver cell, vitamin D undergoes hydroxylation at carbon 25. In the kidney cell, 25-hydroxyvitamin D_3 is further hydroxylated to 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$, biologically the most active vitamin D metabolite. Alternatively, 25-hydroxyvitamin D_3 can be hydroxylated at the 24-carbon, to form 24,25-$(OH)_2D_3$.

From the regulatory viewpoint, the relative amount of 1,25-$(OH)_2$-D or 24,25-$(OH)_2$D plays a key role, as the major functions of vitamin D in kidney, intestine, and bone are associated with the effects due to 1,25-$(OH)_2$-D. Thus, under conditions of calcium deficiency, production of 1,25-$(OH)_2$-D is increased leading to an increase in the active step of intestinal calcium absorption (67,74), enhanced renal reabsorption (109,147), and stimulation of bone metabolism (73). Under conditions of calcium excess, the production of 1,25-$(OH)_2$-D is downregulated and larger amounts of 24,25-$(OH)_2$D are produced. How the renal enzyme systems respond to what must be changes in intracellular calcium concentrations so as to favor production of one or the other vitamin D metabolite is not known.

Function

The major functions of vitamin D are to enhance active calcium transport in intestine and kidney and to enable bone cells to function at a suitably high level of intensity. In addition, vitamin D appears to have a series of pleiotropic functions related to development and cell differentiation (174).

Intestine: Table 5 summarizes the quantitative and qualitative effects of vitamin D on the various steps of transcellular calcium transport. Calcium entry is moderately enhanced as a result of the action of the vitamin (17). This may involve a vitamin D-dependent gating molecule that either regulates or amplifies the putative calcium channel (32–34).

*For greater detail and additional references, see Ref. 174.

TABLE 5 Effects of Vitamin D on Transcellular Calcium Transport in the Intestine

Step	Mechanism or structure	Effect of vitamin D	Mechanism
Entry across brush border	Down chemical gradient (via channel?)	Enhances 20–30% (17)	Possibly via integral Ca-binding protein (32–34)
Binding to fixed cellular sites (buffering)	Golgi apparatus, RER,[a] mitochondria	Enhances 100% (175)	Unknown
Intracellular movement	Diffusion	Facilitates in direct proportion (≈ 100-fold) (15)	Biosynthesis of soluble CaBP (M_r 8800 in mammals, 28,000 in birds), which acts as Ca ferry (12,46,205)
Extrusion	Pumping against a gradient, Ca-ATPase, Na/Ca exchange	Increases action Ca-ATPase 200–300% (12,53)	Unknown

[a]Rough endoplasmic reticulum.
Adapted from Ref. 12. Numbers in parentheses refer to reference citations.

Once calcium enters the cell, it encounters a series of calcium-binding sites associated with either mobile or fixed molecules or organelles. Vitamin D seems to increase the number of these calcium-binding sites in the Golgi apparatus and endoplasmic reticulum (175). The synthesis of the intestinal calbindin (CaBP) is totally dependent on vitamin D. As explained in Section III, active calcium transport varies directly and proportionately with the cellular content of CaBP. The latter molecule acts as a ferry, effectively shuttling calcium from the luminal to the serosal pole of the intestinal cell at a rate directly proportional to the amount of CaBP (Fig. 6).

Calcium extrusion is also enhanced by vitamin D. The number of pump molecules is increased (53), although the mechanism may be indirect, as most Ca-ATPases are not—and would not be expected to be—vitamin D-dependent.

Quantitatively speaking, as also shown in Table 5, the major effect of vitamin D is on the intestinal CaBP, which in turn varies linearly and directly with the V_m of active transport (Fig. 6). The enhancing effect on calcium entry is only some 30%. Calcium extrusion and binding to fixed sites are enhanced twofold to threefold. It has been estimated (12) that neither entry, nor extrusion, nor binding to fixed intracellular sites is the limiting rate; only intracellular diffusion in the absence of the cellular CaBP is severely limiting.

Kidney: The action of vitamin D in the kidney is to increase calcium reabsorption (Sec. VII). Vitamin D regulates transcellular calcium movement in the distal convoluted tubule (DCT) in a manner analogous with what it does in the intestine. The biosynthesis of the renal calcium-binding protein ($CaBP_r$, calbindin D28k, M_r 28 kDa) is totally dependent on vitamin D (176). The protein is thought to function in the DCT by amplifying transcellular transport of calcium by a factor of about 70 (149). To what extent vitamin D also plays a role in the entry or extrusion processes in the DCT cell is not known, but it may do so, in analogy with what is known in the intestine.

Bone: The effect of vitamin D on bone is to increase the rates of bone formation and resorption (73). Receptors for 1,25-$(OH)_2$-D have been found only in osteoblasts (102), so the effect of vitamin D on bone resorption is likely to be indirect, i.e., via the osteoblast. The principal molecular effect of 1,25-$(OH)_2$-D on osteoblasts is to increase their production of the extracellular matrix molecule, the Gla protein (also known as osteocalcin; 177). Treatment of osteoblasts in culture leads to a state equivalent to enhanced differentiation—that is, a higher level of alkaline phosphatase and shape changes—but the relationship of these observations to the in vivo effect of vitamin D has not been clarified.

The role of vitamin D in mineralization is problematical from the viewpoint of mechanisms. Soft bones and the clinical signs associated with rickets are well known, but many of these can be attributed to a phosphate deficiency that is aggravated by the lack of vitamin D (178).

Experimental vitamin D deficiency leads to minor bone changes in rats (73,109). Hypocalcemia due to vitamin D deficiency is not generally associated with obvious skeletal changes visible on radiographic examination. Moreover, it has been reported that in some animals the provision of abundant minerals, either in the diet or systemically, can overcome the skeletal defects associated with vitamin D deficiency. While it seems probable that vitamin D plays a hormonelike role in bone cells, as it does in cells of duodenum and the DCT of the kidney, the nature of this role and its relationship to mineralization are not yet clarified. There is little doubt, however, that bone turnover of calcium is diminished in vitamin D deficiency (109).

B. Other Systemic Hormones

Parathyroid hormone, calcitonin, and vitamin D are considered the primary hormones that regulate calcium metabolism, as discussed earlier. Other hormones that act directly or indirectly on calcium fluxes or bone include the gonadal steroids, the adrenal glucocorticoids, the thyroid hormones, growth hormone and somatomedin, and insulin and glucagon. A general discussion of these hormones is beyond this chapter's scope, but their specific roles in calcium metabolism will now be discussed briefly.

1. Gonadal Steroids (168)

Mineral metabolism and bones and cartilage are profoundly affected in conditions of excess or deficiency of the gonadal steroids. Osteoblasts have receptors that respond equally to estrogens (179) or testosterone (180). The existence of gonadal hormone receptors in osteoclasts is less certain. Avian osteoclasts may possess such receptors (181), and in-vitro studies (182) suggest that mammalian osteoclast development may be enhanced by the absence of estrogens; in other words, estrogens may, perhaps by action on precursor cells via cytokines, inhibit osteoclast development and/or differentiation. It has also been shown that the inhibitory effect exerted by parathyroid hormone on osteoblasts (Sec. VI) is diminished when estrogen or androgen is added to an osteoblast culture (183,184).

Estrogen administration leads to diminished urinary calcium output (185). Estrogens probably do not affect intestinal calcium absorption directly (185).

Testosterone seems to have mechanisms of action that are similar to those of estrogen. At one time, testosterone was used to treat women with postmenopausal osteoporosis, partly because testosterone administration leads to significant improvement in the nitrogen balance (186). However, because of the undesirable masculinizing effect, it is rarely used today.

2. Corticosteroids

Cortisol, corticosterone, and deoxycorticosterone are the major products of the zona fasciculata of the human adrenal gland and represent the chemical response of the body to stress. They have a pronounced effect on bone metabolism, inhibiting cartilage growth and development, impairing synthesis of the bone matrix, and inhibiting bone formation. These antianabolic actions of the corticosteroids may be the result of their inhibition of protein synthesis. As a result, bone absorption is stimulated.

Glucocorticoids seem to have no acute effect on urinary calcium excretion (187). Chronic administration causes calcium excretion to rise (185). Long-term administration of glucocorticoids or glucocorticoid excess may induce excess calcium mobilization from the skeleton and thus lead to hypercalciuria. The major metabolic effect of glucocorticoids is on bone. An excess of these hormones, as in Cushing's disease, or when administered exogenously, is associated with decreased bone growth in children, loss of bone volume in adults, and the impairment of fracture healing (185). Glucocorticoids inhibit incorporation of sulfate into cartilage (188) and of amino acids into cartilage collagen (189); thus, they have an antianabolic effect. Glucocorticoid insufficiency, if untreated, is life-threatening. It is associated with hypercalcemia, which may be the result of excessive osteoclastic activity that would normally be modulated by these hormones (185).

3. Thyroid Hormones

The two principal thyroid hormones are triiodothyronine and thyroxine, the latter containing four atoms of iodine. Even though they are peptides and not steroids, the most probable mechanism of action of the thyroid hormones involves nuclear localization and genomic activation by a ligand-receptor complex. This in turn leads to selective synthesis of specific proteins. Consequently, the action of the thyroid hormones can be likened to that of steroid hormones (168).

Both thyroid hormone excess and hypothyroidism have significant effects on bone metabolism. Hyperthyroidism is associated with a marked increase in bone resorption, particularly in cortical bone, caused mainly by increased osteoclastic activity (185). At the same time, bone turnover is increased. However, the stimulation to bone calcium resorption seems greater than that of bone calcium deposition. As a result, the bone calcium balance, especially in cortical bone, is diminished. Biochemically, hyperthyroidism is often associated with an increase in hydroxyproline excretion in urine and sometimes with increased hydroxyproline levels in serum, indications of heightened collagen turnover (190). Alkaline phosphatase levels are increased in hyperthyroidism, the enzyme presumably originating in the skeleton (185). Thyroid hormones appear to have a direct effect on osteoclasts, as enhanced radiocalcium release from labeled bones can be demonstrated in bone organ cultures (191). Hyperthyroidism is associated with hypercalcemia, hyperphosphaturia, and hypercalciuria. Calcium absorption from the intestine tends to be diminished in conditions of excess thyroid hormone (185). This appears to be due to a diminution of the production of intestinal calbindin, mediated by a diminution of $1,25\text{-}(OH)_2\text{-}D_3$ production consequent to the hypercalcemia.

In hypothyroidism, the effects on calcium metabolism are the obverse of what occurs in the case of thyroid hormone excess. Bone turnover is slowed, hydroxyproline excretion is diminished, and there is a tendency to mild hypocalcemia and diminished serum phosphate levels. Hence, urinary calcium and phosphate excretion are also lower than normal. The bone calcium pool is diminished.

Thyroid hormone promotes skeletal growth in prepubertal children by acting on cartilage growth directly and by stimulating the growth hormone–somatomedin pathway. Therefore, hypothyroidism in children is associated with diminished skeletal growth and an increase in density in the zone of provisional calcification and delayed appearance of the centers of epiphyseal ossification (192).

4. Growth Hormone and Somatomedin

The growth hormone molecule is a large protein consisting of 191 amino acids, the active core of which involves the N-terminal two-thirds of the sequence, while the remainder, the C-terminal one-third, is thought to stabilize the molecular structure (168). Growth hormone is the major hormonal growth factor, acting on bone and cartilage. The hormone promotes growth through the somatomedins, low molecular weight (~7000 Da) peptides synthesized in the liver. Somatomedin receptors have been found in adipocytes, hepatocytes, lymphocytes, and bone cells. Of the three human somatomedins—A, B, and C—the latter, C, is identical with the insulin-like growth factor.

Growth hormone is essential for normal growth and development (185). One form of human dwarfism is caused by defective production of growth hormone, an autosomal recessive characteristic. Growth hormone is essential for somatomedin synthesis (168). Patients with acromegaly, i.e., enlarged stature due to excess growth hormone production, exhibit increased rates of calcium uptake and release by the skeleton, with formation stimulated more than resorption (185). Acromegaly is associated with hypercalciuria and, in some patients, with increased calcium absorption from the intestine. The mechanism(s) by which growth hormone and/or somatomedin act on these processes is unknown; direct action on transcellular calcium transport seems unlikely.

5. Insulin and Glucagon (168,185)

It seems probable that bone cells are a direct target of the action of insulin, although other tissues—adipose, muscle, and liver—are generally considered major targets.

Insulin deficiency leads to a diminution of bone turnover, with bone calcium removal markedly enhanced in insulin-deprived animals. Insulin-deprived rats in the growth phase are only slightly hypocalcemic, but have stopped adding calcium to their skeletons. In other words, their bone balance, instead of being positive, is zero or slightly negative (193). Insulin deficiency is associated with a drop in active calcium absorption in the intestine and a rise in urinary calcium output; this suggests a diminution of the calcium-conserving capacity of the kidney (193).

Glucagon administration produces transient hypocalcemia in several mammalian species, including humans, presumably by direct interaction with the skeleton (185). There is evidence that glucagon inhibits parathyroid-stimulated osteoclastic action, but probably has no effect in unstimulated bone cells (194,195).

IX. OSTEOPOROSIS—AN INTEGRATIVE VIEW

Osteoporosis may be defined as a condition in which the mass of bone per unit volume is significantly decreased, to the point where the risk of fracture is high. While the disorder is clinically relevant only when a fracture has occurred—typically a vertebral or hip fracture—it is prevention rather than treatment of the fracture that seems the more effective approach (196).

As shown in Figure 12, bone mass increases from infancy until the late teens, thereafter remains at a plateau until the mid-thirties, and then begins to decline. In women

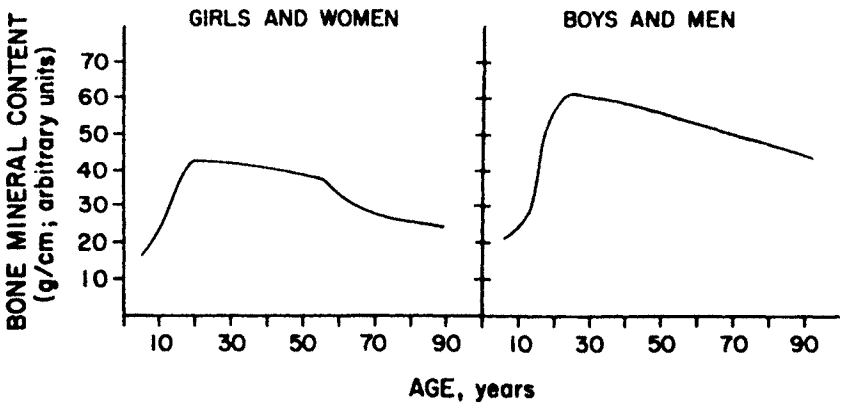

FIGURE 12 Bone mineral content as a function of age, determined by photon absorptiometry. (Unpublished data provided by C. Christiansen, Denmark.)

there occurs a rather drastic decline in the years following the menopause, whereas in men the decline is more gradual. Some 10 years after the menopause, the rate of decline of bone mass in women parallels that in men. Total bone mass is lower in women than in men.

Bone mass in a given individual is genetically programmed, but the final expression is modulated both qualitatively and quantitatively by environmental factors. Thus, in the absence or with reduced availability of the bone minerals, calcium and phosphate, the genetically determined optimum expression of the skeleton is diminished; bone then is thinner and therefore weaker at any age, and the risk of fracture is greater. This is illustrated by the now classical study of Matkovic and colleagues (197), who reported a higher and earlier incidence of certain fractures in a Yugoslav population consuming by custom low quantities of calcium, as compared to a presumably comparable population, genetically speaking, that by custom ingested large quantities of calcium. Similarly, the Japanese, who before World War II were largely vegetarian and consumed only about 10 mmol of Ca per day (91), are now, two generations later, closer to a Western diet in terms of calcium and protein intake and are now also taller.

While the attained bone mass can be modified by calcium intake, the question arises whether the age- and hormone-dependent drop in bone mass can be altered by a change in calcium intake (and absorption). There can be little doubt that a negative calcium balance will increase the rate at which bone mass is lost. The reverse, however, is far from obvious. There is reasonable evidence that the rate of cortical bone loss is similar in individuals of different statures (198), and that this rate is relatively independent of calcium intake. It is therefore not surprising that increased calcium intake in women with postmenopausal osteoporosis had virtually no effect on their rate of bone loss (199), whereas estrogen replacement therapy (200), accompanied by a nutritionally adequate intake of calcium, arrested the loss of trabecular bone characteristic of the menopause (201) or of oophorectomy (202).

The mechanism by which gonadal hormones stimulate bone cells is not well understood. Both male and female gonadal hormones interact with receptors on the osteoblast, causing these cells to increase in activity and number. Part of the responses of osteoblasts seems to involve a reduced release of cytokines, leading in turn to diminished osteoclastic activity and number (203). Osteoclasts, when relieved of the repression exerted by the

gonadal hormones—whether directly or indirectly—become more active, with the result that bone resorption exceeds bone formation.

Of the two general classes of bone, trabecular and cortical, it is the former that undergoes more rapid turnover and is the site of the majority of osteoblasts and osteoclasts (97). Consequently, in the menopause, with gonadal hormone function ceasing abruptly, it is trabecular bone that is more particularly affected. When elements of trabecular bone, the trabeculae, are destroyed, rebuilding seems no longer to be possible. Lost cortical bone can be replaced more readily. In males, gonadal hormone loss, or at least loss of function, is more gradual, as is loss of bone mass in aging men (Fig. 12).

To combat osteoporosis thus involves several approaches: (a) assuring optimum genetic expression of skeletal size and strength by providing the needed nutrients, calcium in particular. This is of especial importance for girls and young women, whose average calcium intake tends to be half of what appears to be needed (204); (b) replacing, under suitable medical supervision, the gonadal hormones whose concentration drops abruptly at the menopause or gradually with aging; (c) assuring an adequate calcium intake throughout life; (d) recognition of the age-dependent drop of active calcium absorption from the intestine with maturity of the skeleton and compensation therefor by increased intake; (e) recognition that slowing the rate of bone loss, preferably by stimulating bone formation, can have long-term benefits in the decrease, and perhaps the severity, of fractures.

X. OUTLOOK

The past two generations have seen significant advances in the understanding of extracellular calcium metabolism and its regulation. The molecular nature of hormones, their receptors, and intracellular events that occur in response to hormonal stimuli are being studied intensively, and many significant factors are bound to be uncovered in coming years. What is less well understood at present is the relationship between intracellular and extracellular calcium homeostasis. The constancy of the free calcium concentration, now recognized to prevail inside essentially all cells, raises the question of why it is necessary for both intra- and extracellular calcium concentrations to be so exquisitively regulated, and whether and how maintenance of the calcium concentration of the two milieus is related or interconnected. The discovery of calcium-responsive proteins in cell membranes will contribute to that understanding, as will analysis of their evolution in relation to the fundamental cellular task of separating calcium from phosphate (1).

Another aspect of calcium metabolism that needs substantial development is the construction of quantitative schemes of extracellular calcium metabolism. The transformation of qualitative into quantitative information, the development of hierarchical relationships between various cellular and tissue events, and the ability to predict quantitatively how changes in, say, calcium intake will affect bone metabolism both acutely and over the long-term are needed if calcium research is to yield information that has both theoretical and practical importance for human and animal health. Ultimately, such information will also permit effective planning of the cultivation and distribution of food supplies, thereby contributing in turn to the betterment of population health.

REFERENCES

1. Kretsinger RH. Why cells must export calcium. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990: 439–457.

2. Latham MC, McGandy RB, McCann M, Stare FJ, eds. Scope Manual on Nutrition. Kalamazoo, MI: Upjohn Co, 1972: Table B, Appendix.
3. Block G, Dresser CM, Hartman AM, Carroll MD. Nutrient sources in the American diet: quantitative data from the NHANES II Survey. I. Vitamins and minerals. Am J Epidemiol 1985; 122: 13–26.
4. Eckelman R, Kulp J, Schubert AR. Strontium-90 in man. II. Science 1958; 127: 266–274.
5. Morgan AF, ed. Nutritional status USA. Calif Agr Bull 769, 1959.
6. Recommended Dietary Allowances, 10th ed. Washington, DC: National Academy Press, 1989: 284.
7. U.S. Department of Agriculture. CSF II. Nationwide food consumption survey. Continuing survey of food intakes by individuals. Men 19–50 years, 1 day, 1985. NFCS, CSFII, 85-3, November 1986. Hyattsville, MD: USDA.
8. American Medical Association. Nutrients in Processed Foods—Proteins; Vitamins, Minerals; Fat, Carbohydrates. Acton, MA: Publishing Sciences Group, 1974.
9. Washburn EW, ed. International Critical Tables of Numerical Data, Physics, Chemistry and Technology. Vol. 3. New York: McGraw-Hill, 1928: 377.
10. Cramer CF, Copp DH. Progress and rate of absorption of radiostrontium through intestinal tracts of rats. Proc Soc Exp Biol Med 1959; 102: 514–517.
11. Marcus CS, Lengemann FW. Absorption of Ca^{45} and Sr^{85} from solid and liquid food at various levels of the alimentary tract of the rat. J Nutr 1962; 77: 155–160.
12. Bronner F, Pansu D, Stein WD. An analysis of intestinal calcium transport across the rat intestine. Am J Physiol 1986; 250 (Gastrointest Liver Physiol 13): G562–G569.
13. Schachter D, Dowdle EB, Schenker H. Active transport of calcium by the small intestine of the rat. Am J Physiol 1960; 198: 263–268.
14. Martin DL, DeLuca HF. Influence of sodium on calcium transport by the rat small intestine. Am J Physiol 1969; 216: 1351–1359.
15. Roche C, Bellaton C, Pansu D, Miller A III, Bronner F. Localization of vitamin D-dependent active Ca^{2+} transport in rat duodenum and relation to CaBP. Am J Physiol 1986; 251 (Gastrointest Liver Physiol 14): G314–G320.
16. Pansu D, Bellaton C, Roche C, Bronner F. Duodenal and ileal calcium absorption in the rat and effects of vitamin D. Am J Physiol 1983; 244 (Gastrointest Liver Physiol 7): G695–G700.
17. Miller A III, Bronner F. Calcium uptake in isolated brush-border vesicles from rat small intestine. Biochem J 1981; 196: 391–401.
18. Miller A III, Li ST, Bronner F. Characterization of calcium binding to brush border membranes from rat duodenum. Biochem J 1982; 208: 773–782.
19. Schedl HP, Wilson HD. Calcium uptake by intestinal brush border membrane vesicles. Comparison with in vivo calcium transport. J Clin Invest 1985; 76: 1871–1878.
20. Wilson HD, Schedl HP, Christensen K. Calcium uptake by brush-border membrane vesicles from the rat intestine. Am J Physiol 1989; 257 (Renal Fluid Electrolyte Physiol 26): F446–F453.
21. Takito J, Shinki T, Sasaki T, Suda T. Calcium uptake by brush-border and basolateral membrane vesicles in chick duodenum. Am J Physiol 1990; 258 (Gastrointest Liver Physiol 21): G16–G23.
22. Holdsworth ES. Vitamin D_3 and calcium absorption in the chick. Biochem J 1965; 96: 475–483.
23. Stein WD. Facilitated diffusion of calcium across the intestinal epithelial cell. J Nutrition 1992; 122: 651–656.
24. Homaidan FR, Donowitz M, Weiland GA, Sharp GWG. Two calcium channels in basolateral membranes of rabbit ileal epithelial cells. Am J Physiol 1989; 257 (Gastrointest Liver Physiol 20): G86–G93.
25. Butler DJ, Hillier K. Calcium and human large-bowel motility. Ann NY Acad Sci 1989; 560: 447–450.
26. Saunders JCJ, Isaacson LC. Patch clamp study of Ca channels in isolated renal tubule

segments. In: Pansu D, Bronner F, eds. Calcium Transport and Intracellular Calcium Homeostasis. Heidelberg: Springer, 1990: 27–34.
27. Hess P, Tsien RW. Mechanism of ion permeation through calcium channels. Nature 1984; 309: 453–458.
28. Guggino SE, Lajeunesse D, Wagner JA, Snyder SH. Bone remodeling signaled by a dihydropyridine-and phenylalkylamine-sensitive calcium channel. Proc Natl Acad Sci USA 1989; 86: 2957–2960.
29. Caffrey JM, Farach-Carson MC. Vitamin D_3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. J Biol Chem 1989; 264: 20265–20274.
30. Lieberherr M. Effects of vitamin D_3 metabolites on cytosolic free calcium in confluent mouse osteoblasts. J Biol Chem 1987; 262: 13168–13173.
31. Bronner F, Pansu D, Bosshard A, Lipton LH. Calcium uptake by isolated rat intestinal cells. J Cell Physiol 1983; 116: 322–328.
32. Kowarski S, Schachter D. Intestinal membrane calcium-binding protein: vitamin D-dependent membrane component of the intestinal calcium transport mechanism. J Biol Chem 1980; 255: 10834–10840.
33. Schachter D, Kowarski S. Isolation of the protein 1M Cal, a vitamin D-dependent membrane component of the intestinal transport mechanism for calcium. Fed Proc 1982; 41: 84–87.
34. Miller A III, Ueng TH, Bronner F. Isolation of a vitamin D-dependent, calcium-binding protein from brush borders of rat duodenal mucosa. FEBS Lett 1979; 103: 319–322.
35. Washburn WE, ed. International Critical Tables of Numerical Data, Physics, Chemistry and Technology. Vol. 5. New York: McGraw-Hill, 1929; 66.
36. Wasserman RH, Corradino RA, Taylor AN. Vitamin D-dependent calcium binding protein: purification and some properties. J Biol Chem 1968; 243: 3978–3986.
37. Wasserman RH, Feher JJ. Vitamin D-dependent calcium-binding proteins. In: Wasserman RH, Corradino RA, Carafoli E, Kretsinger RH, MacLennan DH, Siegel FL, eds. Calcium Binding Proteins and Calcium Function. New York: Elsevier-North Holland, 1977: 293–302.
38. Wasserman RH, Fullmer CS, Taylor AN. The vitamin D-dependent calcium binding proteins. In: Lawson DEM, ed. Vitamin D. London: Academic Press, 1978: 133–166.
39. Thomasset M, Desplan C, Parkes O. Rat vitamin D-dependent calcium-binding proteins. Specificity of mRNAs coding for the 7500-Mr protein from duodenum and the 28000-Mr protein from kidney and cerebellum. Eur J Biochem 1983; 129: 519–524.
40. Perret C, Desplan C, Thomasset M. Cholecalcin (a 9-kDa cholecalciferol-induced calcium-binding protein) messenger RNA. Distribution and induction by calcitriol in the rat digestive tract. Eur J Biochem 1985; 150: 211–217.
41. Perret C, Lomri N, Gouhier N, Auffray C, Thomasset M. The rat vitamin D-dependent calcium-binding protein (9-kDa CaBP) gene. Complete nucleotide sequence and structural organization. Eur J Biochem 1988; 172: 43–51.
42. Kretsinger RH. Calcium-binding proteins. Annu Rev Biochem 1976; 45: 239–266.
43. Ueng TH, Golub EE, Bronner F. The effect of age and 1,25-dihydroxyvitamin D_3 treatment on the intestinal calcium-binding protein of suckling rats. Arch Biochem Biophys 1979; 196: 624–630.
44. Ueng TH, Bronner F. Cellular and luminal forms of rat intestinal calcium binding protein as studied by counter ion electrophoresis. Arch Biochem Biophys 1979; 197: 205–217.
45. Thomasset M, Parkes CO, Cuisinier-Gleizes P. Rat calcium-binding proteins: distribution, development and vitamin D-dependence. Am J Physiol 1982; 243 (Endocrinol Met 6): E483–E488.
46. Feher JJ, Fullmer CS, Wasserman RH. Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. Am J Physiol 1992; 262 (Cell Physiol 31): C517–C526.
47. Martin SR, Linse S, Johnson C, Bayley PM, Forsen S. Protein surface changes and Ca^{2+} binding to individual sites in calbindin D_{9k}: stopped flow studies. Biochemistry 1990; 29: 4188–4193.

48. Wyman J. Facilitated diffusion and the possible role of myoglobin as a transport molecule. J Biol Chem 1966; 241: 115–121.
49. Garrahan PJ, Rega AF. Plasma membrane calcium pump. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990; 271–303.
50. Verma AK, Filoteo AG, Stanford DR, et al. Complete primary structure of a human plasma membrane Ca^{2+} pump. J Biol Chem 1988; 263: 14152–14159.
51. Carafoli E, James P, Strehler EE. Structure-function relationships in the calcium pump of plasma membranes. In: Peterlik M, Bronner F, eds. Molecular and Cellular Regulation of Calcium and Phosphate Metabolism. New York: Wiley-Liss, 1990: 181–193.
52. Ghijsen WEJM, Van Os CH, Heizmann CW, Murer H. Regulation of duodenal Ca^{2+} pump by calmodulin and vitamin D-dependent Ca^{2+}-binding protein. Am J Physiol 1986; 251 (Gastrointest Liver Physiol 14): G223–G229.
53. Wasserman RH, Chandler JS, Meyer SA, et al. Intestinal calcium transport and calcium extrusion processes at the basolateral membrane. J Nutr 1992; 122: 662–671.
54. Carafoli E. Membrane transport of calcium: an overview. Meth Enzymol 1988; 157(Q): 3–11.
55. Van Os CH. Transcellular calcium transport in intestine and renal epithelial cells. Biochim Biophys Acta 1987; 906: 195–222.
56. Reeves JP. Sodium-calcium exchange. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990: 305–347.
57. Nellans HN, Popovitch JR. Role of sodium in intestinal calcium transport. In: Bronner F, Peterlik M, eds. Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects. New York: Alan R. Liss, 1984: 301–306.
58. Bronner F. Calcium absorption. In: Johnson LR, Christensen J, Jacobson ED, Jackson MJ, Walsh JH, eds. Physiology of the Gastrointestinal Tract. 2d ed. Vol. 2. New York: Raven Press, 1987: 1419–1433.
59. Bronner F, Spence K. Non-saturable Ca transport in the rat intestine is via the paracellular pathway. In: Bronner F, Peterlik M, eds. Cellular Calcium and Phosphate Transport in Health and Disease. New York: Alan R. Liss, 1988: 277–285.
60. Cassidy MM, Tidball CS. Cellular mechanisms of intestinal permeability alterations produced by chelation depletion. J Cell Biol 1967; 32: 685–698.
61. Pansu D, Chapuy MC, Milani M, Bellaton C. Transepithelial calcium transport enhanced by xylose and glucose in the rat jejunal ligated loop. Calcif Tiss Res 1976; 21: 45–52.
62. Diamond J, Tormey JM. Role of long extracellular channels in fluid transport across epithelia. Nature 1966; 210: 817–820.
63. Vaughn DW, Filer LJ. The enhancing action of certain carbohydrates on the intestinal absorption of calcium in the rat. J Nutr 1960; 71: 10–14.
64. Wasserman RH, Taylor AN. Some aspects of the intestinal absorption of calcium, with special reference to vitamin D. In: Comar CL, Bronner F, eds. Mineral Metabolism—An Advanced Treatise. Vol. 3. New York and London: Academic Press, 1969: 321.
65. Armbrecht HJ. Effect of age and the milk sugar lactose on calcium absorption by the small intestine. Adv Exp Med Biol 1989; 249: 185–192.
66. Pansu D, Bellaton C, Bronner F. Effect of lactose on duodenal calcium-binding protein and calcium absorption. J Nutr 1979; 109: 509–512.
67. Pansu D, Bellaton C, Bronner F. The effect of calcium intake on the saturable and non-saturable components of duodenal calcium transport. Am J Physiol 1981; 240 (Gastrointest Liver Physiol 8): G32–G37.
68. Wasserman RH, Comar CL, Nold MM. The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium^{45} and strontium^{89} in the rat. J Nutr 1956; 59: 371–383.
69. Kehayoglou CK, Williams HS, Whimster WF, Holdsworth C. Calcium absorption in the normal, bile duct ligated and cirrhotic rat, with observations on the effect of long- and medium-chain triglycerides. Gut 1968; 9: 597–603.

70. Pansu D, Duflos C, Bellaton C, Bronner F. Solubility and intestinal transit time limit calcium absorption in rats. J Nutr 1993; 123: 1396–1404.
71. Armbrecht HJ, Zenser TV, Bruns MEH, Davis BB. Effect of age on intestinal calcium absorption and adaptation to dietary calcium. Am J Physiol 1979; 236 (Endocrinol Metab Gastrointest Physiol 5): E769–E774.
72. Armbrecht HJ, Zenser TV, Davis BB. Effect of vitamin D metabolites on intestinal calcium absorption and calcium-binding protein in young and adult rats. Endocrinology 1980; 106: 469–475.
73. Hurwitz S, Stacey RE, Bronner F. Role of vitamin D in plasma calcium regulation. Am J Physiol 1969; 216: 254–262.
74. Buckley M, Bronner F. Calcium-binding protein biosynthesis in the rat: regulation by calcium and 1,25-dihydroxyvitamin D_3. Arch Biochem Biophys 1980; 202: 235–241.
75. Hall BD, MacMillan DR, Bronner F. Vitamin D-resistant rickets associated with high fecal endogenous calcium output. A report of two cases. Am J Clin Nutr 1969; 22: 448–457.
76. Walser M. Ion association VI. Interaction between calcium, magnesium, inorganic phosphate, citrate and protein in normal human plasma. J Clin Invest 1961; 40: 723–730.
77. Carr CW. Studies on the binding of small ions in protein solutions with the use of membrane electrodes II. The binding of calcium ions in solutions of bovine serum albumin. Arch Biochem Biophys 1953; 43: 147–156.
78. Bronner F. Calcium homeostasis. In: Bronner F, Coburn JW, eds. Disorders of Mineral Metabolism. Vol. 2. New York: Academic Press, 1982: 43–102.
79. Roof BS, Piel CF, Hansen J, Fudenberg HH. Serum parathyroid hormone levels and serum calcium levels from birth to senescence. Mech Ageing Develop 1976; 5: 289–304.
80. Ryckewaert A, Ricket G, Lemaire V, Begue MC, Fenelon JP. Etude de la calcémie dans une population de 6048 hommes. Variation avec l'age. Correlations avec d'autres valeurs biologiques. Rev Rheumat 1974; 41: 473–478.
81. Franke H, Gall L, Gross W, Mull E, Weisshaar D. Klinisch-chemische Befunde bei 41 Hundertjährigen und Vergleich mit jüngeren Alterstufen. Klin Wchschr 1973; 51: 183–190.
82. Edelman IS, Leibman J. Anatomy of body water and electrolytes. Am J Med 1959; 27: 256–277.
83. Robinson RA. Observations regarding compartments for tracer calcium in the body. In: Frost HM, ed. Bone Biodynamics. Boston: Little Brown, 1964: 423–439.
84. Bronner F, Sammon PJ, Nichols C, Stacey RE, Shah BG. Thyrocalcitonin and plasma calcium homeostasis in the rat. In: Talmage RV, Belanger LF, eds. Parathyroid Hormone and Thyrocalcitonin. Amsterdam and New York: Excerpta Medica, 1968: 353–369.
85. Bronner F, Bosco JJ, Stein WD. Acute plasma calcium regulation in rats: effect of vitamin D deficiency. Bone and Mineral 1989; 6: 141–154.
86. Copp DH, Moghadam H, Mensen ED, McPherson GD. The parathyroids and calcium homeostasis. In: Greep RO, Talmage RV, eds. The Parathyroids. Springfield, IL: C C Thomas, 1961: 203–219.
87. Bronner F, Stein WD. Modulation of bone calcium-binding sites regulates plasma calcium: an hypothesis. Calcif Tissue Int 1992; 50: 483–489.
88. Ray RD, Kawabata M, Galante J. Experimental studies of peripheral circulation and bone growth. Clin Orthop & Rel Res 1967; 54: 175–185.
89. Ray RD. Circulation and bone. In: Bourne GH, ed. The Biochemistry and Physiology of Bone. Vol. IV. New York: Academic Press 1976: 385–402.
90. Peacock M. Renal excretion of calcium. In: Nordin BEC, ed. Calcium in Human Biology. London & Berlin: Springer Verlag, 1988: 125–169.
91. Bronner F. Dynamics and function of calcium. In: Comar CL, Bronner F, eds. Mineral Metabolism—An Advanced Treatise. Vol. 2A. New York: Academic Press, 1964: 341–444.
92. Aubert JP, Milhaud G. Méthode de mesure des principales voies du métabolisme calcique chez l'homme. Biochim Biophys Acta 1960; 39: 112–139.
93. Thomas RO, Litovitz TA, Rubin MI, Geschickter CF. Dynamics of calcium metabolism. Time distribution of intravenously administered radiocalcium. Am J Physiol 1952; 169: 568–575.

94. Dacke CG, Shaw AJ. Studies of the rapid effects of parathyroid hormone and prostaglandins on ^{45}Ca uptake into chick and rat bone *in vivo*. J Endocrinol 1987; 115: 369–377.
95. Bronner F. Disposition of intraperitoneally injected calcium-45 in suckling rats. J Gen Physiol 1958; 41: 767–782.
96. Heaney RP. Calcium kinetics in plasma: as they apply to the measurements of bone formation and resorption rates. In: Bourne GH, ed. The Biochemistry and Physiology of Bone. Vol. IV. New York: Academic Press. 1976: 105–133.
97. Polig E, Jee WSS. Bone structural parameters, dosimetry and relative radiation risk in the beagle skeleton. Rad Res 1989; 120: 83–101.
98. Simmons DJ, Grynpas MD. Mechanisms of bone formation *in vivo*. In: Hall BK, ed. Bone: The Osteoblast and Osteocyte. Vol. 1, NJ: Telford Press, 1990: 193–302.
99. Spector WS, ed. Handbook of Biological Data. Philadelphia: Saunders, 1956: 584.
100. Sammon PJ, Stacey RE, Bronner F. Further studies on the role of thyrocalcitonin in calcium homeostasis and metabolism. Biochem Med 1969; 3: 252–270.
101. Miller SS, Wolf AM, Arnaud CD. Bone cells in culture: morphologic transformation by hormones. Science 1976; 192: 1340–1342.
102. Sato M, Rodan GA. Bone cell shape and function. In: Stein WD, Bronner F, eds. Cell Shape Determinants, Regulation and Regulatory Role. New York: Academic Press, 1989: 329–362.
103. Jee WSS. Introduction to skeletal function: structural and metabolic aspects. In: Bronner F, Worrell RV, eds. A Basic Science Primer in Orthopaedics. Baltimore: Williams & Wilkins, 1991: 3–34.
104. Chambers TJ, McSheehy PMJ, Thompson BM, Fuller K. The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 1985; 60: 234–239.
105. Boyde A, Jones SJ. Early scanning electron microscopic studies of hard tissue resorption: their relation to current concepts reviewed. Scanning Microsc 1987; 1: 369–381.
106. Kream BE, Rowe DW, Givorek SC, Raisz LG. Parathyroid hormone alters collagen synthesis and procollagen mRNA levels in fetal rat calvaria. Proc Natl Acad Sci (USA) 1980; 77: 5654–5658.
107. Raisz LG, Kream BE, Smith M, Simmons HA. Comparison of the effects of vitamin D metabolites on collagen synthesis and resorption of fetal rat bone in organ culture. Calcif Tissue Int 1980; 32: 135–138.
108. Rodan GA, Martin TJ. Role of osteoblasts in hormonal control of bone resorption—a hypothesis. Calcif Tissue Int 1981; 33: 349–351.
109. Bronner F, Golub EE, Fischer JA. The effect of vitamin D on renal calcium clearance. In: Fleisch H, Robertson WG, Smith LH, Vahlensieck W, eds. Urolithiasis Research. New York: Plenum, 1976: 383–388.
110. McMillan PJ, Dewri RA, Joseph EE, Schultz RL, Deftos LJ. Rapid changes of light microscopic indices of osteoclast-bone relationships correlated with electron microscopy. Calcif Tissue Int 1989; 44: 399–405.
111. Thompson DD, Seedor JG, Weinreb M, Rosini S, Rodan GA. Aminohydroxybutane biphosphonate inhibits bone loss due to immobilization in rats. J Bone and Mineral Res 1990; 5: 279–286.
112. Bockman RS, Repo MA, Worrell RP Jr, et al. Distribution of trace levels of therapeutic gallium in bone as mapped by synchrotron x-ray microscopy. Proc Natl Acad Sci (USA) 1990; 87: 4149–4153.
113. Hall TJ, Chambers TJ. Gallium inhibits bone resorption by a direct effect on osteoclasts. Bone and Mineral 1990; 8: 211–216.
114. Shaw AJ, Dacke CG. Cyclic nucleotides and the rapid inhibitions of bone ^{45}Ca uptake in response to bovine parathyroid hormone and 16,16-dimethyl prostaglandin E_2 in chicks. Calcif Tissue Int 1989; 44: 209–213.
115. Sachs F. Ion channels as mechanical transducers. In: Stein WD, Bronner F, eds. Cell Shape. Determinants, Regulation and Regulatory Role. San Diego, CA: Academic Press, 1989: 63–92.

116. Rodan GA, Bourret LA, Harvey A, Mensi T. 3′,5′-cyclic AMP and 3′,5′-cyclic GMP: mediators of the mechanical effects on bone remodeling. Science 1975; 189: 467–469.
117. Weinman DT, Kelly PJ, Owen CA, Orvis AL. Skeletal clearance of Ca^{47} and Sr^{85} and skeletal blood flow in dogs. Proc Staff Meetings, Mayo Clin 1963; 38: 559–570.
118. Brandfonbrener M, Landowne M, Shock NW. Changes in cardiac output with age. Circulation 1955; 12: 559–570.
119. Care AD, Cooper CW, Duncan J, Orimo H. A study of thyrocalcitonin secretion by direct measurement of in vivo secretion rates in pigs. Endocrinology 1986; 83: 161–169.
120. Tsukamoto Y, Suki WN, Liang CT, Sacktor B. Ca^{2+}-dependent ATPases in the basolateral membranes of rat kidney cortex. J Biol Chem 1986; 261: 2718–2724.
121. Lin HS, Fain JN. Ca^{2+}-Mg^{2+}-ATPase in rat hepatocyte plasma membranes: inhibition by vasopressin and purification of the enzyme. In: Bronner F, Peterlik M, eds. Epithelial Calcium and Phosphate Transport: Molecular and Cellular Aspects. New York: Liss, 1984: 25–31.
122. Gerok W, Helmann C, Spamer C. Regulation of intracellular calcium by endoplasmic reticulum of hepatocytes. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Liss, 1990: 139.
123. Somylo AP, Bond M, Somlyo AV. Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly in vivo. Nature 1985; 314: 622–625.
124. Lehninger AL, Fiskum G, Vercesi A, Tew W. Ca^{2+} transport by mitochondria: a survey. In: Bronner F, Peterlik M, eds. Calcium and Phosphate Transport Across Biomembranes. New York: Academic Press, 1981: 73–82.
125. Crompton M. Role of mitochondria in intracellular calcium regulation. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990: 181–209.
126. Streb H, Irvine RF, Berridge MJ, Schultz J. Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-triphosphate. Nature 1983; 306: 67–69.
127. Petersen OH. Regulation of calcium entry in cells that do not fire action potentials. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990: 77–96.
128. McCarty NA, O'Neill RG. Calcium signaling in cell volume regulation. Physiol Rev 1992; 72: 1037–1061.
129. Bement WM. Signal transduction by calcium and protein kinase C during egg activation. J Exp Zool 1992; 263: 382–391.
130. Shen SS. Calcium signaling at fertilization. Curr Opin Genet Devel 1992; 2: 642–646.
131. Villereal ML. Calcium signals in growth factor signal transduction. Rev Physiol, Biochem, Pharmacol 1992; 119: 67–121.
132. Cheek TR. Calcium signalling and the triggering of secretion in adrenal chromaffin cells. Pharmacol Therapeut 1991; 52: 173–189.
133. Augustine GJ, Adler EM, Charlton MP. The calcium signal for transmitter secretion from presynaptic nerve terminals. Ann NY Acad Sci 1991; 635: 365–381.
134. Rink TJ, Sage SO. Calcium signaling in human platelets. Annu Rev Physiol 1990; 52: 431–449.
135. Muallem S. Calcium transport by resting and stimulated cells. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1989: 349–380.
136. Thomas AP, Renard DC. Spatial and temporal organization of calcium signalling in hepatocytes. Cell Calcium 1991; 12: 111–126.
137. Langer GA. Calcium and the heart: exchange at the tissue, cell, and organelle levels. FASEB J 1992; 6: 893–902.
138. Miller RJ, Fox AP. Voltage-positive calcium channels. In: Bronner F, ed. Intracellular Calcium Regulation. New York: Wiley-Liss, 1990: 97–138.
139. Carafoli E. Sarcolemmal calcium pump. In: Langer GA, ed. Calcium and the Heart. New York: Raven Press, 1990: 109–126.
140. Bronner F, Lemaire R. Comparison of calcium kinetics in man and the rat. Calcif Tiss Res 1969; 3: 238–248.