METABOLIC ADAPTATION AND NUTRITION

PAN AMERICAN HEALTH ORGANIZATION
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NOTE

At each meeting of the Pan American Health Organization Advisory Committee on Medical Research, a special session is held on a topic chosen by the Committee as being of particular interest. At the Ninth Meeting, which convened in June 1970 in Washington, D. C., the session focused on the problem of nutrition and metabolic adaptation with an emphasis on new concepts and experimental approaches for future research in the field of human nutrition. In an effort to improve communication among biomedical scientists, and extend the usefulness of the meeting beyond those who attended, the Department of Research Development and Coordination has published this volume which contains the papers presented and the ensuing discussions.

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Special Session on

METABOLIC ADAPTATION AND NUTRITION

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OPENING STATEMENT-Morning

Philip P. Cohen, Chairman

In a presentation at the Eighth Meeting of the Advisory Committee on Medical Research, Pan American Health Organization, last year, a position supporting the need for a more molecular approach was outlined as a basis for advancing our understanding of human biology in health and disease (See Appendix). Particular reference was made to the need for new concepts and experimental approaches in the area of nutrition. The position presented was essentially as follows: The current status of nutritional research indicates that we have today all the basic knowledge needed regarding essential nutrients but what we lack is the effective means for application of this knowledge in the prevention or treatment of primary nutritional disorders. In addition we lack a meaningful definition of health and what constitutes a departure from good health. While there is uncertainty as to the quantitative requirement for one or more nutrients in one situation or other, this search for a magic number seems not likely to yield anything more than a series of quantitative limits which are at best guidelines for the requirement of a given individual, but are of limited value as an index of nutritional adequacy—or of good health.

The area of nutritional research which remains to be explored in any depth has to do with that of regulation of cell function. It is obvious that in any overall process there will be a rate-limiting requirement for any essential ingredient of that process, and any level below that rate-limiting requirement will affect, perhaps in an all-or-none manner, the operation of that process. What we need to know are the consequences of regulatory failure or inadequacy of any of the multiplicity of regulatory processes which result from dietary restrictions. At the same time, we have to know what degree of adaptability exists and the limits of this adaptability in the regulatory process affected by dietary restriction. The means of regulation of cell function are complex and finely controlled; the limits of adaptability in any given dietary situation are yet to be defined and are not well understood. The kind of information needed will result not from the perpetuation of traditional experiments in human nutrition, but rather from experiments designed on the basis of the newer concepts of molecular biology and

cell regulation. It is thus likely that well designed animal model systems will be needed to exploit these newer concepts before suitable experiments can be developed for use with the human.

It is the purpose of the present session on Metabolic Adaptation and Nutrition to explore this area with the hope that new approaches in the diagnosis and treatment of nutritional diseases will emerge.

PHYSIOLOGICAL ADAPTATION: THE TEST OF NUTRITION[1]

Van R. Potter and David F. Scott[2]

Introduction

Metabolic adaptation is a subject that should be of major interest to nutritionists. It represents a common ground where nutritionists, endocrinologists, physiologists, pharmacologists, and now molecular biologists can meet and discuss problems of mutual interest. Metabolic adaptation may also begin to involve the sociologists since cultural adaptation to new dietary practices is part of the total picture. In an earlier paper (Potter 5), Geertz's interesting concepts defining a *culture* as a set of societal mechanisms for controlling or programming individual human behavior were discussed. In that paper, the possibility of defining an optimum environment was presented, and the concept that optimum health or normality could be attained by providing individuals with an environment that required no adaptive responses was questioned. On the contrary, in proposing a list of seven points to consider in defining such an environment, it was emphasized that: "The culture should prepare us for and expect of each individual adaptive responses, continually from birth to death. . . ." In this connection it was stressed that we are, in fact, quite ignorant of how to assist in the evolution of our culture toward better concepts of positive health. The contention was that individuals show a wide variation in their tendency to seek physiological challenges, and the question was raised as to how individuals could be motivated by society to seek their own optimum degree of positive health while maintaining their identity as free individuals. In a later paper (Potter 6), tracing the concept of homeostasis from the era of Claude Bernard and Walter Cannon to the present, it was emphasized that homeostasis in the *milieu intérieur* is achieved by rather heroic variations in certain enzymatic components that contribute small molecules to the common aqueous environment. The two publications already referred to provide background for the present report. Many of the ideas expressed are in harmony with papers presented at this conference by Harper, Waterlow, Mayer, Waters, and others.

In the above-cited papers (*5, 6*), the concept of physiological cost is coupled with the concept of physiological adaptation. The issue is especially applicable in the case of the drug metabolizing enzymes that increase adaptively in response to pesticides or other foreign chemicals in the environment.

Controlled feeding schedules in the study of metabolic adaptation

As a result of our studies, it is believed that much of the literature on enzyme levels in the tissues of experimental animals is very inadequate because of a failure to specify adequately not only the composition of the diet, but also the lighting conditions, the time of day, the

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[2] USPHS Post-doctoral Fellow IFO2 CA-32836, 1968–1970.

period of food availability, and the duration of the regimen, in addition to all the usual specifications of age, sex, strain, and so on. The authors have moved from a practice of *ad libitum* feeding with controlled lighting and killing times to a position of advocating not only controlled lighting, but also controlled feeding schedules and an actual opposition to the practice of *ad libitum* feeding in experiments that emphasize biochemical parameters in contrast to behavioral parameters (*1, 5–15*). If behavioral studies involving the eating habits of rats are to be combined with biochemical measurements, they can be interpreted more readily in the light of data from experiments in which the feeding schedule was controlled. Elsewhere, the details of protocols have been summarized, and five objectives that may be furthered by the use of controlled feeding regimens have been listed (*8*).

Enzyme changes in metabolic transitions

In 1961 Potter and Ono reported their first explorations in the realm of controlled feeding experiments (*9*). A metabolic transition was defined as "the period during which an organism is changing its pattern of enzyme activity and enzyme amount in response to a change in its environment." Later, it was emphasized that "the living animal is adapting during every moment of life," and particularly in connection with the diurnal changes in light and darkness (*5*). In 1961 experiments were patterned after those of Tepperman and Tepperman in which animals were refed after fasting two or three days. Data on glycogen and glucose-6-phosphate dehydrogenase were provided ((*9*) Figures 1 and 2, and Chart 1). It was established that the adaptive increase in the dehydrogenase could not be accomplished during the first 12 hours of refeeding even with an optimum diet and could not be accomplished in the first 48 hours of refeeding with diets containing too little carbohydrate (91 per cent protein) or too little protein (2 per cent). Optimum responses were obtained with 20 to 60 per cent protein in the presence of 71 to 31 per cent glucose ((*9*), Figure 4). However, the dehydrogenase activity did increase on the high-protein diet by days 4 and 6 of refeeding after a failure to respond during two days of refeeding ((*9*), Figure 5). This delay during the high-protein diet was probably caused by the delay in certain components of the gluconeogenic system, possibly including some of the amino acid catabolizing enzymes. In 1961, a delay in the threonine dehydrase induction was reported, and in 1968 a delay in the serine dehydratase (possibly the same enzyme) response, but no delay was recorded in the tyrosine transaminase response (*12*).

Studies on benzpyrene hydroxylase

Benzpyrene hydroxylase is an adaptive enzyme that increases rapidly in the livers of animals exposed to a variety of foreign agents including pesticides, tranquilizers, hydrocarbons, and others (*2, 3, 5, 6, 13*). In terms of the concept of physiological cost (*5, 6*), it has been clearly established by Conney *et al.* (*3*) that the drug-hydroxylases also act on a variety of steroid hormones. These workers also urged that "further studies should be carried out to determine whether the chronic environmental exposure of humans to pesticides is sufficient to alter their metabolism of drugs and steroids..." since enhanced drug and steroid metabolism in man has already been demonstrated to occur in both man and animals after administration of known drugs (*3*).

Benzpyrene hydroxylase is present at low levels in the liver of male rats and at levels near zero in the liver of female rats. It is rapidly induced to high levels in both males and females after a single injection of methylcholanthrene ((*13*), Chart 1), in confirmation of earlier experiments by Conney, Miller, and Miller (cited in *13*). Experiments were then carried out to determine if the metabolic adaptation in question was influenced by the level of protein in the diet or the feeding regimen and it was found that, in fact, it appeared to

be quite independent of these influences ((*13*), Chart 2), in contrast to an earlier experience with glucose-6-phosphate dehydrogenase (*9*) or with tyrosine aminotransferase.

Studies on tyrosine aminotransferase (TAT)

Tyrosine aminotransferase (abbreviated as TAT, also known as tyrosine transaminase, and also EC 2.6.1.5) is an enzyme with a very short half-life. It is induced by a variety of dietary and hormonal manipulations that enable one to demonstrate the modulation of gene expression (Figure 1).

Experiments in the induction of TAT under carefully controlled conditions and correlating enzyme levels with the activity of the amino acid transport system have been of primary interest to the authors in recent years. In 1967, simultaneous measurements of the daily variation in rat liver glycogen, serine dehydratase, and TAT in comparison with plasma corticosterone in rats on 60 percent protein and an 8+16 regimen were reported ((*10*), Figure 3). These comparisons are of interest because they show that under the reported conditions, induction of this enzyme is not simply a feeding response: the increase in liver glycogen continues for at least six hours after the activity of tyrosine amino transferase starts to fall. The data also reveal an important clue to later experiments: the increase in TAT occurs at the time of a sharp metabolic transition, when

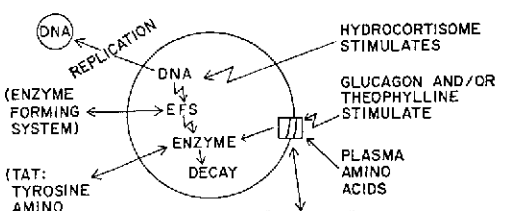

Figure 1. Schematic indication of variables and oscillating parameters studied in liver and plasma of rats on controlled feeding schedules. The sites of action for the hormones and for theophylline are still not fully established although theophylline is known to inhibit cyclic adenosine monophosphate (AMP) diesterase.

light turns to darkness, food is suddenly available, hyperactivity occurs, glucagon, epinephrine, and insulin may be involved and *glycogen levels are falling* even as the animals are ingesting food. This observation has been observed repeatedly in the laboratory and reported in connection with other observations whenever the observed time points included the first one or two hours after the onset of feeding and darkness (*12*). Moreover, when experiments were carried out with rats on a 12 percent protein diet, which does not induce tyrosine transaminase, administration of tryptophan, 5-OH tryptophan, or serotonin, decreased liver glycogen but induced TAT. Shortly after these experiments were completed, a study of tyrosine aminotransferase in relation to amino acid transport was undertaken.

Induction of the amino acid transport system (TS)

The induction of tyrosine aminotransferase is closely correlated to the induction of the amino acid transport system (TS) and this, in turn, is correlated with cyclic adenosine monophosphate (AMP) as suggested in Figure 2. However, at this time the exact sites of action and mechanisms remain to be established; multiple targets for various stimulating factors should be considered and seem obligatory from a theoretical standpoint.

It was established that tyrosine aminotransferase shows a daily oscillation in which the amplitude is determined by the protein level in the diet. Serine dehydratase levels are also determined by dietary protein, but instead of responding immediately they require several days to establish. The presence of the adrenals is not a requirement for these adaptations (*12*).

Since amino acid input was obviously of importance, study of the amino acid transport system and effects of various hormones on this parameter were undertaken, using both amino-iso-butyric acid (AIB) (*1*), and amino-cyclopentane-carboxylic acid (ACPC or cycloleucine) (*8*).

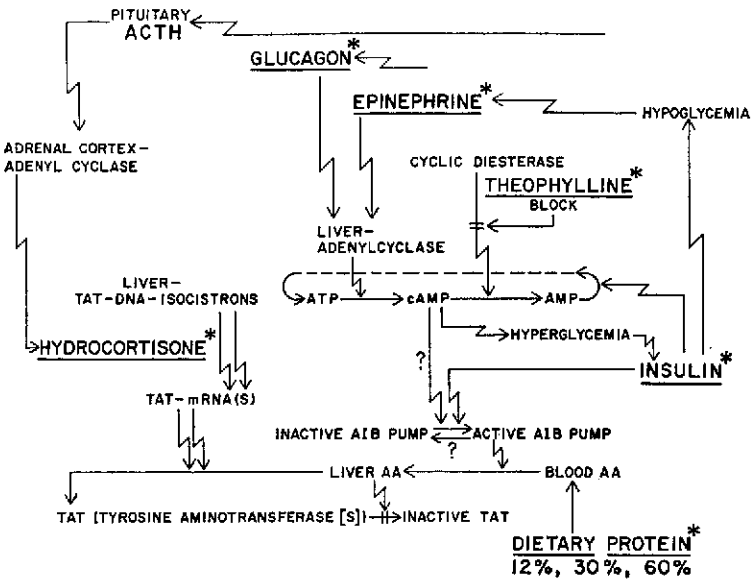

Figure 2. Schematic indication of possible sites of action for the systems mentioned in Figure 1. The same limitations apply.

Subsequent to the above reports, studies by Fuller and Snoddy ((*4*), and personal communication) in which theophylline and epinephrine-induced TAT in rat liver were noted. Meanwhile, Wicks *et al.* (*16*) published studies showing induction of TAT by theophylline, glucagon, and cyclic AMP. These investigators had not carried out any studies that included the amino acid transport system, so it was decided to coordinate studies on the two systems in the same animals using controlled feeding schedules. Under controlled conditions theophylline alone gave responses with close coordination in both TAT and TS that had not been seen previously ((*7*), preliminary data). Further studies are still in progress (*11*). It is shown that time after administration and dosages are critical (*11*), but that at all dosages and times there is a close correlation between TAT and TS (*11*). When AIB or ACPC is transported into liver, TAT rises phenomenally, but the plasma level changes relatively little, and the AIB or ACPC appear not to be coming from muscle or intestinal mucosa (*11*).

Conclusion

Reference is again made to Figure 2 to indicate the parameters under study and their possible relationships, and to emphasize the thought that nutrition cannot ignore the fields of endocrinology and the molecular events that underlie metabolic adaptations. But the real impact on the field of nutrition must lie in the practical applications of the changing concepts of normality, adaptability, and positive health, whether or not investigators are involved in studies at the molecular level.

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ADAPTABILITY AND AMINO ACID REQUIREMENTS

A. E. Harper

Concept of homeostasis

Claude Bernard (*5*) prior to 1878, concluded that homothermic organisms were little influenced by fluctuations in the external environment because they actually lived in their own *milieu intérieur*. He recognized that this freedom from the influence of a hostile and fluctuating external environment was accomplished, not by the organism being separated from it, but because it was highly responsive to changes therein, and could continuously and delicately compensate for changes that might otherwise affect it adversely. Among his examples he included the ability of higher organisms to tolerate a wide range of nutritional conditions.

In his classic studies of blood and body fluids, L. J. Henderson (*23*) was struck by the relative constancy of fasting concentrations of blood constituents despite the fluctuations that occurred under other conditions. His interest in the ability of the body to restore the blood to its standard state led him to recognize the phenomenon of adaptability as a basic biological phenomenon. He also recognized that if the external influence were too great, the body might be unable to restore the standard state and might have to compromise at something less than the ideal. He further realized that failure of the adaptive mechanisms would lead to inability of the organism to survive.

"The law of adaptation," Henderson stated ". . . seems to be quite as well established as the Second Law of Thermodynamics and almost equally serviceable . . . adaptation is relative. It involves a question, not of what is best, but of what is efficient under certain conditions, of what promotes survival in a particular environment. . . ."

The concept of the body as an open system in a steady state was developed by W. B. Cannon (*9, 10*). He was concerned with the mechanisms involved in the integrated responses required to restore the body fluids toward the standard state after disturbances of various types, especially the role of the nervous and endocrine systems. Cannon coined the term "homeostasis" (from similar and condition) which he defined as "the coordinated physiological reactions which maintain most of the steady states of the body."

The concept of homeostasis was examined in detail, especially from an evolutionary viewpoint, by Joseph Barcroft (*3*) who was led to the conclusion that, as one ascended the evolutionary ladder, the accuracy of regulatory mechanisms increased, leading to increasing stability of the internal environment. He concluded that this high degree of stability was essential for development of higher mental activity, and for intellectual development that permits the greatest freedom from the restrictions of a fluctuating external environment.

Today, as concern with metabolic regulation has developed, there is renewed interest in the concept of homeostasis. Understanding of the regulatory mechanisms underlying homeostasis

has increased greatly as knowledge of enzymatic mechanisms and biochemical regulation has accrued (*32*). Also, the requirements for stability of such regulatory mechanisms has become better understood as knowledge of the requirements for stable electronic feedback systems has progressed (*42*). Nevertheless, we are still far from being able to assess quantitatively the significance of homeostatic mechanisms in relation to nutritional requirements, and, in particular, to recognize the limits beyond which such mechanisms cannot prevent some permanent impairment of the organism exposed to nutritional deprivation.

Amino acids as essential nutrients

The nutritional essentiality of specific amino acids was demonstrated by Willcock and Hopkins (*46*) and Osborne and Mendel (*30*) during the first two decades of this century. Shortly after their discovery of threonine in 1935, Rose and his associates (*34*) demonstrated that adult man required protein as a source of eight indispensable amino acids, and as a source of additional nitrogen which could be provided in a variety of reduced forms. They then set about determining amino acid requirements quantitatively as did others.

Most of the quantitative studies of human amino acid requirements have been done on adults. The basic information available (*15*) is summarized in Table 1. It is striking how small the individual amino acid requirements are.

The sum of the amounts of indispensable amino acids required is only 6.35 gm for men and 4.35 gm for women. Whether this difference is a sex difference or merely a reflection of the different bases for estimation of minimum requirements is problematic (*15*). In any event, assuming that these amino acids, together with cystine and tyrosine, which are semi-indispensable, represent about half of the amino acids in high quality proteins, they would be provided in the required amounts by only from 9 to 12 gm per day of a protein with a net protein value (NPV) of 100.

Table 1

Minimal daily amino acid requirements of man

	Infant	Adult	
		Male	Female
	mg/kg/day	mg/day	mg/day
Histidine	32	—	—
Isoleucine	90	700	450
Leucine	150	1100	620
Lysine	105	800	500
Total sulfur A.A.	85	1100	550
Total aromatic A.A.	90	1100	1120
Threonine	60	500	305
Tryptophan	22	250	157
Valine	93	800	650
Total	727	6350	4352

From NRC *Publication* 711 (*15*).

If, on the other hand, requirements are calculated on the basis of obligatory nitrogen losses that must be replaced, as shown in Table 2, a value of 26.5 gm of protein is obtained (*12*). This is over double the amount of protein of high nutritional value needed to meet the estimated requirements for the indispensable amino acids determined individually. Since the indispensable amino acid pattern of total body proteins resembles quite closely that of the amino acid requirements (*47*), it is evident that the obligatory losses of nitrogen cannot come equally from dispensable and indispensable

Table 2

Calculated minimum daily nitrogen requirements of adult men [a]

	mg N/day
Endogenous urinary loss (2.0 mg N/kcal)	3,200
Metabolic fecal loss (0.57 mg N/kcal)	912
Daily skin loss (0.08 mg N/kcal)	128
	4,240

[a] Minimum daily protein requirement $= (4.24 \times 6.25) = 26.5$ gm

Based on *Recommended Intakes of Nutrients for the United Kingdom* (*12*).

amino acids arising from body protein breakdown, unless the values for individual amino acid requirements determined by use of highly purified diets differ greatly from those estimated from minimal protein intake to maintain nitrogen equilibrium.

Measurements of the amounts of food proteins required to maintain adult man in nitrogen equilibrium are in general agreement with the values estimated from obligatory nitrogen losses. Bricker, *et al.* (*7*) concluded that the average daily protein requirement of a group of college women consuming a diet in which 70 percent of the protein was from cereals was 31.7 gm. Thus, if requirements for indispensable amino acids are estimated from the amounts of natural proteins required to maintain adult man in nitrogen equilibrium, the values are considerably higher than those obtained in experimental studies in which the minimum requirements have been determined using amino acid diets. It may be important that the minimum requirements for individual indispensable amino acids have been determined with diets containing an amount of nitrogen, largely from glycine and diammonium citrate that would be provided by 38 gm (*25*), and usually closer to 60 to 65 gm of protein (*26, 34*), more than is provided by diets composed of foodstuffs that will maintain man in nitrogen equilibrium.

These two types of observations raise a question concerning the relationship between the indispensable amino acid requirements and the total nitrogen requirement of the adult. They imply that the total requirement for indispensable amino acids represents a much smaller proportion of the total nitrogen requirement for the adult than for the young growing organism, and that the indispensable amino acid requirements fall proportionately more than the total nitrogen requirement with maturation, or that indispensable amino acids are well-conserved by adult man, at least under the conditions that have been used for the determination of minimum amino acid requirements, that is, with very low intake of the amino acid under study and a relatively high nitrogen intake.

Romo and Linkswiler (*33*) and Clark *et al.* (*11*) indicate that in mature human subjects on relatively low nitrogen intakes (about 6.0 gm of nitrogen daily), nitrogen balance improves as the proportion of indispensable amino acids in the diet is increased (Figure 1). These observations, taken together with those on estimated minimum amino acid requirements, imply that to maintain individuals in nitrogen equilibrium on quantities of indispensable amino acids approaching those determined individually as the minimum requirements, total nitrogen intake must exceed that needed when the quantities of indispensable amino acids exceed the estimated minimum requirements. This means that requirements of adult subjects for indispensable amino acids increase as nitrogen intake falls. Observations by Scrimshaw *et al.* (*40*) on nitrogen retention of young men fed a diet providing from four to five gm of nitrogen per day, largely from whole egg, tend to support this view as their subjects were in negative nitrogen balance, even though the amounts of indispensable amino acids in the

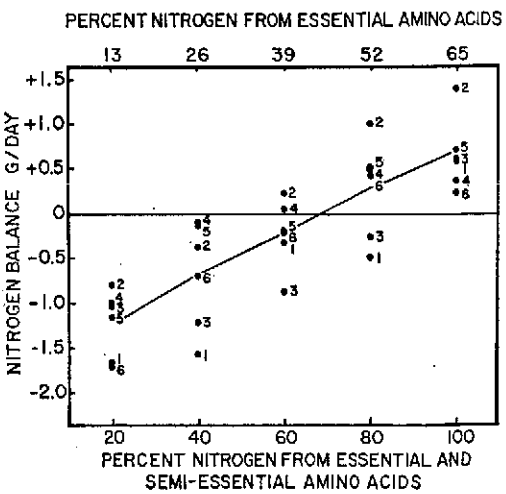

Figure 1. Effect of substituting indispensable amino acids for dispensable amino acids on nitrogen retention of young men consuming 6.3 gm of nitrogen daily from amino acids patterned as in maize. From Romo and Linkswiler (33).

diet exceeded the estimated minimum requirements. Interestingly, nitrogen balance of their subjects did not become appreciably more negative when part of the protein was replaced by glycine and diammonium citrate.

The infant and young growing subject require amino acids for the synthesis of new tissue as well as for maintenance, as illustrated in Figure 2 from Hegsted (*22*). If protein intake were inadequate, conservation of indispensable amino acids could permit redistribution within tissues and could increase efficiency of utilization of the available amino acids, but since about half of the amino acids in tissue proteins cannot be synthesized by higher animals, they must be provided in the diet, as such, if growth is to continue.

The rapidity with which growth failure occurs in animals or human subjects fed amino acid-deficient or low-protein diets, and the rapidity with which negative nitrogen balance occurs in adult subjects under such conditions, indicates that amino acids cannot be stored as reserves comparable to energy stores and that whatever increase in efficiency of utilization may occur when amino acid intake is low is limited. Further evidence for the lack of amino acid stores comes from studies of delayed amino acid supplementation in which all of the dietary

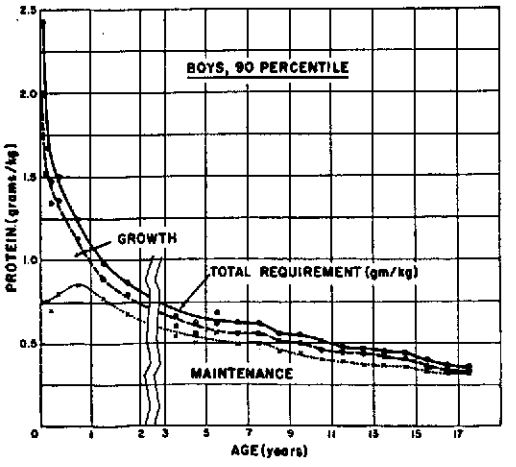

Figure 2. Estimates of protein required per kilogram of body weight per day for larger boys. From Hegsted (22).

components, except for one amino acid, are fed together in a single meal, with the missing amino acid being fed separately later (*13, 18*). A delay of a few hours in the provision of the missing amino acid results in growth failure, indicating that a surplus of amino acids is cleared rapidly from body fluids, and within a short time is no longer available for protein synthesis. There are differences among amino acids in this regard. As illustrated in Figure 3, lysine administered twice daily with a lysine-deficient diet fed *ad libitum* supports considerable growth, whereas isoleucine similarly administered is much less efficiently used (*43*).

The rapidity of clearance of amino acids that cannot be used for protein synthesis is illustrated by some observations of Haider and Tarver (*19*), reminiscent of earlier observations by Borsook (*6*), as shown in Figure 4. The survival time of ^{14}C-labeled amino acid injected intraperitoneally is very short indeed.

If there are not readily available reserves of indispensable amino acids, what is the situation regarding the so-called protein reserves—proteins from which amino acids can be redistributed to maintain essential structures during periods of amino acid deprivation. These appear not to be reserves in the usual sense of that term, but rather a reflection of the metabolic state of the organism, and to consist of functional body proteins that can be mobilized at different rates depending upon the nutritional needs of the organism (*21*). During starvation, when the primary need is for energy and glucose, many of the enzymes of amino acid degradation and gluconeogenesis increase in activity, despite the lack of a dietary source of protein, as illustrated for glutamate-alanine amino transferase (Figure 5) from a study by Rosen *et al.* (*35*). Yet, under conditions of simple protein deprivation, when glucose and energy are provided, but amino acids are lacking, and the primary need is for conservation of indispensable amino acids and nitrogen, many of these same enzymes fall to very low levels of activity. Thus, under one condition of protein deprivation this group of

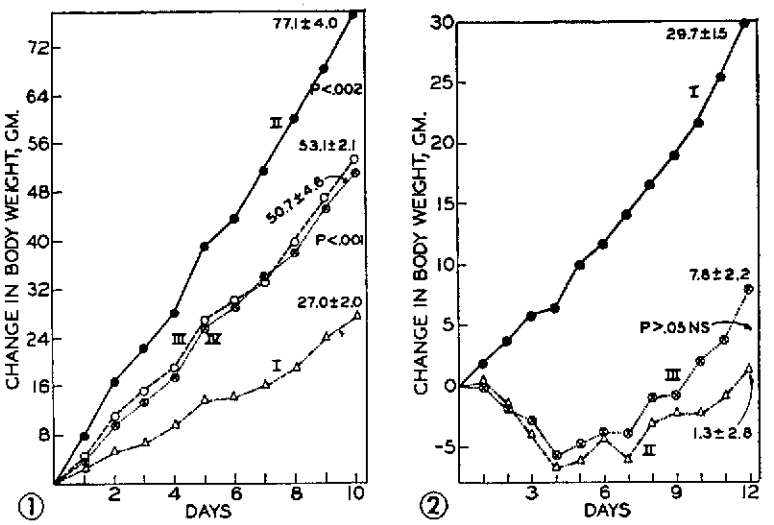

Figure 3. 1. Effect of lysine administered with the diet, or injected twice daily on growth of rats fed *ad libitum* a lysine-deficient diet. (I) 30 per cent wheat gluten (II) 30 per cent wheat gluten +0.4 per cent L-lysine-HCL; (III) 30 per cent wheat gluten +0.4 per cent L-lysine injected intraperitoneally; (IV) as for (I), but injected subcutaneously. 2. Effect of isoleucine and valine administered with the diet or injected twice daily on growth of rats fed *ad libitum* a diet deficient in isoleucine and valine. (I) Amino acid diet with quantities of amino acids in 9 per cent casein; (II) as for (I), but isoleucine and valine reduced by one-third; (III) as for (II) but isoleucine and valine equivalent to that consumed by (I) injected subcutaneously. From Spolter and Harper (43).

proteins represents part of the so-called protein reserves whereas, under another, they do not. Studies of muscle proteins, which appear to be dispensable under both of these conditions, do not reveal this distinction, but then the individual components of muscle have received much less attention than those of liver.

An excessive intake of protein appears to pose little problem for the healthy, well-fed organism. Substrate concentrations for amino acid-degrading enzymes are high when protein intake is high. Food intake may fall if the intake of amino acids exceeds the capacity of these enzymes to clear amino acids. But adaptive responses occur—the activities of amino acid-degrading enzymes rise (*2, 38*), as shown in Figure 6, for enzymes of histidine catabolism (*39*), and within a short time food intake is restored and the organism has achieved homeostasis at a higher level of nitrogen metabolism and functions well (*1*).

Dietary deprivation of amino acids is a more serious problem. There is no reserve of amino acids that can be mobilized to support the growth of the young organism subjected to severe deprivation. Even in the adult, despite such mechanisms of amino acid conservation as exist, there is an obligatory minimum nitrogen loss that must be replaced to prevent tissue wastage.

It would appear from the studies on amino acid and nitrogen needs of man discussed earlier, that a high intake of indispensable amino acids would be beneficial when nitrogen intake is low. But most low-protein diets are of plant origin, and these characteristically have a low ratio of indispensable to dispensable amino acids and therefore provide proportionately less indispensable amino acids than diets that contain high quality animal proteins (*20*). Also, ingestion of low protein diets is frequently accompanied by caloric inadequacy,

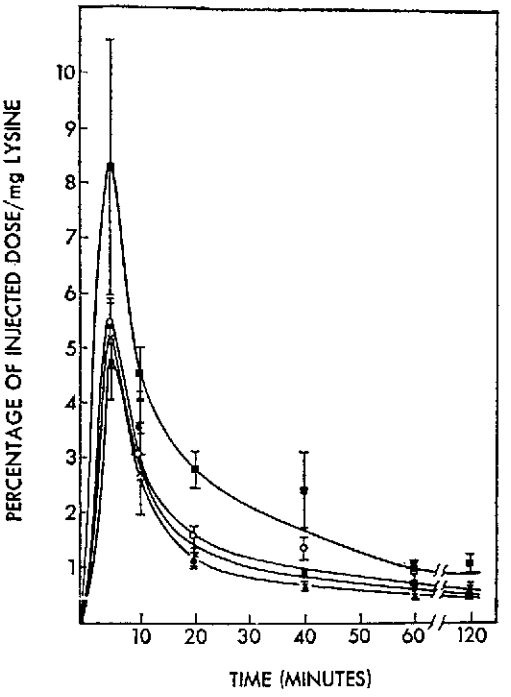

Figure 4. Disappearance of radioactivity from the liver after injection of ^{14}C-labeled lysine into mature rats. —O— laboratory diet —●— low protein —x— high protein —■—protein-free. From Haider and Tarver (19).

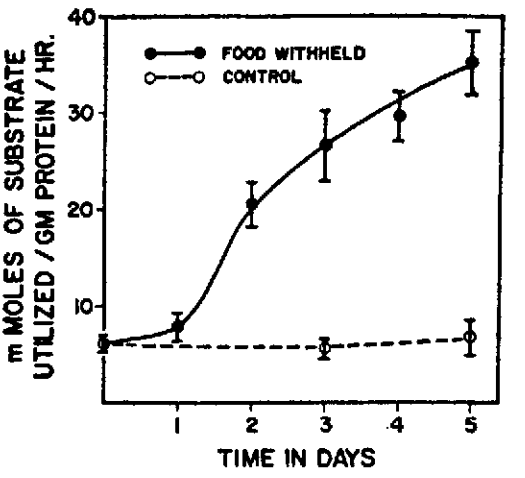

Figure 5. Effect of withholding food from rats on the activity of glutamate-alanine transaminase in liver. From Rosen *et al.* (35).

which results in utilization of a portion of the amino acids as a source of energy (*8*). Moreover, such diets tend to be high in carbohydrate, and an excess of carbohydrate in relation to well-balanced protein contributes to the adverse effects of a low protein diet (*31*), probably by stimulating fat synthesis in the liver (*49*) at a time when the capacity for fat transport to the periphery is limited (*14*). There is, thus, a great need for adaptive responses that will contribute to survival by increasing amino acid retention under conditions of limited protein intake.

One might suppose that a substantial reduction in protein turnover, with inhibition of degradation of tissue protein breakdown,

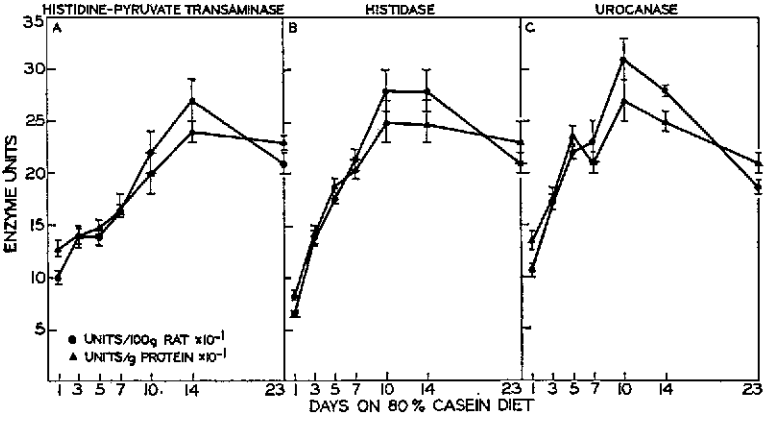

Figure 6. Changes in activities of histidine-degrading enzymes in liver with time after feeding rats a high protein diet. From Schirmer and Harper (39).

would provide such a mechanism. However, the phenomenon of protein turnover provides the organism with great adaptability, especially during periods of deprivation, permitting breakdown of less important tissue and organ proteins to provide amino acids for the maintenance of the essential ones—a mechanism with great survival value. Although there is evidence that turnover of some proteins may be slowed (*38*), general inhibition of this process would lead to rigidity of the system and lessen its ability to respond to changes in the external environment. Hormones and digestive enzymes, for example, must be synthesized continuously. It would, therefore, seem most appropriate for the body to depend upon increased efficiency of conservation of the indispensable amino acids arising from tissue protein degradation as the major form of adaptation when the dietary supply of these amino acids is inadequate, and to depend upon inhibition of protein turnover, with the restrictions this would impose, only as a last resort.

Adaptive (homeostatic) responses to low protein intake

A very early result of a low intake of an indispensable amino acid is a drop in the concentration of that amino acid in body pools. With low substrate concentrations for amino acid-degrading enzymes, the minimum obligatory oxidation of amino acids would be expected to fall. Observations by McLaughlan *et al.* (*27*) in Figure 7 illustrate the relationship between intake and plasma concentration for several amino acids. Since the Michaelis constants of amino acid-degrading enzymes are much above those for amino acid-activating enzymes, this should greatly favor incorporation of amino acids into proteins over oxidation.

The distribution of amino acid-degrading enzymes, although not altered by dietary alterations, would appear to be an evolutionary adaptation contributing to amino acid conservation. Most of the enzymes for degradation of the indispensable amino acids are confined to the liver and, therefore, have limited distribution. This is illustrated in Table 3 by observations of Miller (*28*), which provide a comparison of the capacity of the perfused liver and of the eviscerated rat carcass to oxidize individual amino acids. With this type of distribution, little degradation of most of the indispensable amino acids would occur in the periphery—a further factor that would favor conservation and reutilization of amino acids released as a result of tissue protein degradation.

Interestingly, the branched-chain amino acids are exceptions. However, even for these, only the aminotransferase for the first step in degradation is distributed in the peripheral tissues with the oxidase being confined almost exclusively to liver and kidney (*48*). Thus, conservation of these could also occur with the keto acids being readily reaminated in the peripheral tissues, as long as amino donors are available.

Further, in animals fed a low-protein diet, the activities of the enzymes for degradation of indispensable amino acids fall to very low activities. This is illustrated for threonine dehydratase (*37*), in Figure 8. Also the oxidase for the branched-chain amino acids falls sharply in animals fed a low protein diet (*48*) as shown by Figure 9.

With prolonged ingestion of a low-protein diet, enzymes for activation of amino acids rise

Table 3

Oxidation of ^{14}C-labeled amino acids by the perfused liver and by the eviscerated carcass

	% administered ^{14}C expired as $^{14}CO_2$	
	Eviscerated surviving rat	Isolated perfused liver
L-phenylalanine	0.14	25.5
L-histidine	0.44	30.0
DL-tryptophan	1.7	9.1
L-lysine	2.4	34.5
L-isoleucine	7.7	5.2
L-leucine	12.0	5.4
Glycine	7.1	19
L-glutamic acid	46.0	32

After Miller (*28*).

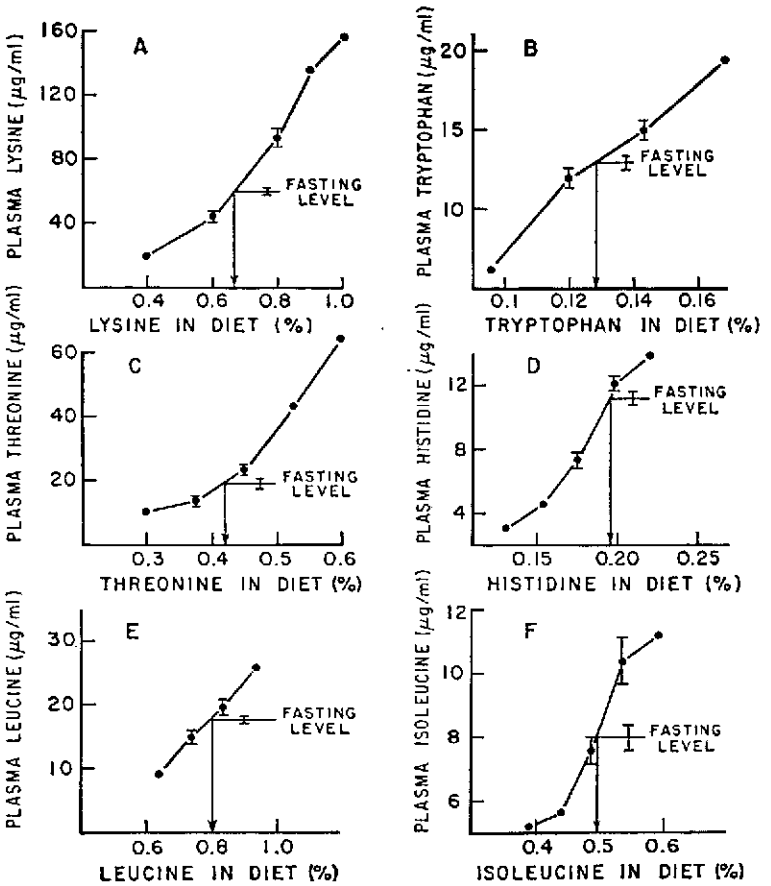

Figure 7. Plasma concentrations of amino acids in relation to the concentrations in the diet. From McLaughlan and Morrison (27).

(*16*), a further adaptation that should favor trapping and reutilization of available amino acids.

A low-protein intake, as was mentioned, is usually accompanied by a high-carbohydrate intake which would tend to stimulate insulin secretion. Munro (*29*) has shown that a protein-free diet stimulates tissue uptake of amino acids, probably by this mechanism. At the same time, enzymes for gluconeogenesis tend to fall when carbohydrate intake is high; hence, both of these responses would also tend to contribute to increased efficiency of utilization of available amino acids for tissue synthesis.

Lastly, although protein turnover contributes greatly to the ability of the organism to adapt to a changing external environment, as protein deficiency is prolonged, some flexibility may have to be sacrificed to prolong survival. Observations by Waterlow and his associates (*45*) indicate that protein synthesis in muscle is depressed within a short time in rats fed a protein-deficient diet, whereas that in liver is depressed only after more prolonged deficiency (Table 4). However, they also obtained evidence of substantial reutilization of amino acids released from tissues for serum and liver protein synthesis (Table 5). The high values for

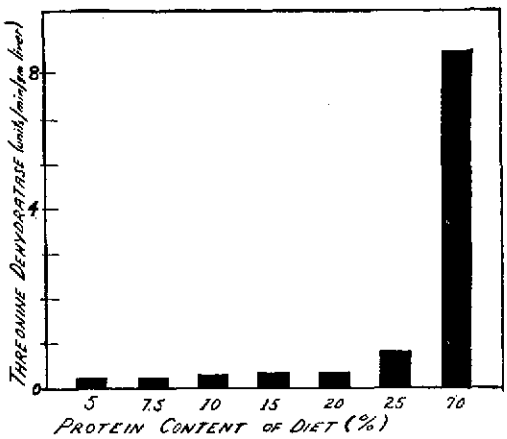

Figure 8. Effect of protein content of the diet of rats on serine-threonine dehydratase activity in liver. From Sando (37).

apparent half-lives of proteins in this study are the result of reutilization of amino acids. Evidence from isotopic studies of plasma albumin synthesis and catabolism in recovered and malnourished children indicate that the rate of synthesis of plasma albumin falls within a short time after protein intake falls and that after a few days the catabolic rate falls as well. Initially albumin was apparently transferred from the extravascular to the intravascular pool, then the concentration was maintained with a lower turnover rate—a highly effective adaptation as Waterlow (*45*) points out.

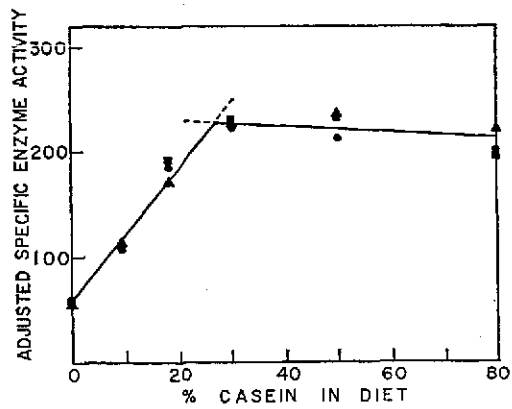

Figure 9. Effect of protein content of the diet of rats on branched-chain keto acid dehydrogenase activity in liver. From Wohlhueter and Harper (48).

Table 4

Synthesis rates of liver and muscle proteins in male rats under different dietary conditions measured by continuous intravenous infusion of ^{14}C-lysine

Condition	Synthesis-rate (% of control)	
	Liver	Muscle
Starved 2 days	105	69
Protein-free 3 days	113	65
Low protein 3 days	110	50
Low protein 10 days	125	48
Low protein 10 days + insulin	165	—
Low protein 5 weeks	80	56

From Waterlow (*45*).

Significance of adaptability in relation to amino acid requirements

It has long been recognized that human subjects can maintain nitrogen equilibrium with a wide range of nitrogen intakes and that if nitrogen intake is reduced gradually after a brief period of nitrogen loss, equilibrium is attained again at the lower intake (*29*). In other words, after a period of adjustment homeostasis is achieved with a reduced rate of nitrogen metabolism, the lowest nitrogen or protein intake at which equilibrium can be maintained being taken as a measure of the minimum requirement.

Evidence that this involves adaptation comes from observations that the capacity for amino acid degradation falls as nitrogen intake falls, and that in the protein-deficient organism this

Table 5

Half-lives of serum and liver proteins in the rat, measured with ^{14}C-arginine

	Half-life (T ½) in days	
	True	Apparent
Mixed serum-proteins:		
Control	2.0	2.7
Protein-depleted	3.3	5.5
Liver proteins:		
Control	1.9	5.5
Protein-depleted	2.3	9.2

From Waterlow (*45*).

is accompanied by increased efficiency of reutilization of indispensable amino acids and reduced rates of turnover of some proteins, as discussed earlier. Experiments discussed earlier also indicated the capacity for conservation of amino acids is not uniform. Further, Bender (*4*) found that the net protein utilization (NPU) of several amino acid mixtures, devoid of one amino acid, was not zero, as would be predicted (Figure 10), and Said and Hegsted (*36*) observed that the utilization of lysine-deficient proteins was greater than the predicted value when they were fed at low levels. It would therefore appear that the minimum amount of protein required to maintain nitrogen equilibrium would depend, not only upon the amount of the limiting amino acid present in the protein, but also, which amino acid is limiting, in view of the differential capacity of the organism to adapt to deficits of the different amino acids.

Questions raised regularly regarding estimates of minimum protein requirements needed to maintain nitrogen equilibrium in the adult, or normal growth of the young are, "Does this minimum deviate appreciably from the optimum?", and "Does a higher intake improve

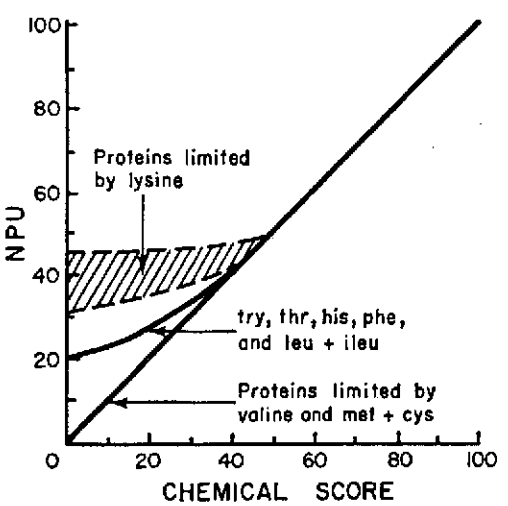

Figure 10. Relationship between chemical score and net protein utilization (NPU) of diets containing amino acid mixtures limiting in a single amino acid. From Bender (*4*).

nutritional status?" There are still differences of opinion about these, as there have been for decades, but there is little concrete evidence that extra protein, over and above requirements determined by these methods, confers additional benefit (*17*). This suggests that adaptive mechanisms function efficiently in enabling higher organisms to adjust from a high to a low-protein intake.

Nevertheless, despite the amazing adaptability of higher organisms to a reduced protein intake, reutilization of amino acids and nitrogen is never complete, and for maintenance of the adult, there are always minimum obligatory losses that must be replaced. In the young growing subject, the problem is more critical. Even if reutilization were complete, the requirements for growth would still remain. The immediate response of the growing organism to a shortage of protein is retardation of growth. This is obviously an adaptation with survival value, as Waterlow (*45*) has pointed out. But the question is, "Up to what point can growth retardation be considered an adaptation compatible with normal function, and beyond what point must it be considered an inability of homeostatic mechanisms to compensate for the nutritional deprivation?" This is also a critical problem in assessing the relationship between adaptability and requirements, for it is important to distinguish clearly between (1) a *low protein intake* which, with effective functioning of homeostatic mechanisms, will support not only survival but also a satisfactory rate of growth and efficient performance of the organism, and (2) *protein deficiency,* which represents a nutritional state in which the capacity of homeostatic mechanisms has been exceeded and deterioration of the organism progresses to a point of irreversibility (*44, 45*). It is a distinction that is and will undoubtedly remain difficult to define in view of the variety of factors that influence nitrogen and amino acid utilization and requirements.

Among problems that have been touched on in relation to this are the low values for amino

acid requirements determined individually in adult man with diets containing considerable surpluses of nitrogen and other amino acids. It seems quite possible that the amino acid in shortest supply is used particularly efficiently under these conditions as a result of a mass action effect, and hence the values obtained for minimum requirements with this procedure are unusually low. There is evidence that nitrogen retention of adult subjects, with low nitrogen intakes, improved as indispensable amino acids were substituted in increasing amounts for dispensable nitrogenous substances in the diet. However, there is also evidence from studies with both children (*41*) and adults (*24*) that retention of nitrogen by subjects with low nitrogen intakes can be improved by supplements of dispensable nitrogenous compounds. Unfortunately, information on this is quite limited, and there appears to have been no comparison, in a single experiment, of the effectiveness of these two procedures for improving a low-protein diet. Nevertheless, it is tempting to speculate that the minimum values reported for amino acid requirements are a reflection of experimental conditions that lead to highly efficient trapping of the amino acid that is limiting; that as total nitrogen intake falls, requirements for indispensable amino acids increase; and that as total nitrogen intake is increased, adaptive mechanisms come into play to increase efficiency of conservation of indispensable amino acids. This would imply that at the point where the capacity of adaptive mechanisms for conservation of amino acids is close to maximum, nitrogen retention could be improved either by increasing the indispensable amino acid content of the diet without increasing the total nitrogen content or by increasing the total nitrogen content without increasing the indispensable amino acid content.

It is not very satisfying to conclude without clarifying the relationship between adaptability and amino acid requirements, so I should like to close with the thought that, although efforts to assess minimum amino acid requirements under particular conditions have intrinsic value, it is time to focus more directly on such questions as, "What are the mechanisms of adaptation to a low protein intake?" "To what extent are the requirements for indispensable amino acids altered by adaptations?" "How can the line of demarkation between adaptation and breakdown be distinguished clearly?" Values for quantitative requirements for amino acids will become more meaningful when we have more adequate answers to such questions.

If the science of nutrition requires a challenge, investigation of mechanisms of adaptation to nutrient deprivation, of the limits of such adaptations, and of ways of supplementing such adaptive mechanisms as exist when they reach the limit of their capacity should provide challenges sufficient for a healthy future in the field.

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ENZYME SYNTHESIS AND DEGRADATION: EFFECTS OF NUTRITIONAL STATUS

Robert T. Schimke

There is an increasing wealth of examples of adaptation to alterations in the physiological and nutritional state of an animal, which involves striking changes in the levels of a number of enzymes in the liver (*11, 24*). Such changes do not reflect simply activation and inactivation of the enzyme protein, since agents that inhibit protein synthesis can prevent the increases in enzyme activity (*7*). More convincing are studies using combined immunologic and radioisotopic techniques in demonstrating both increased content of immunologically reactive protein, and an active, net uptake of isotopic amino acids into specific enzyme proteins (*8, 9, 22, 25*).

Most important, and central to an understanding of the dynamics of enzyme regulation in mammalian tissues, is the fact that changes in enzyme levels take place against a background of continual synthesis and degradation of protein, that is, turnover, as documented so elegantly by Schoenheimer and his co-workers (*26*) in the early 1940's, and studied more recently in other laboratories (*3, 30*). We may consider the continual degradation of protein as an answer to the problem of how to remove enzymes when no longer needed as part of an adaptive response to altered nutritional states. In a rapidly growing bacterium, an unneeded enzyme can be diluted by subsequent cell division. In the generally non-growing cell of an animal, intracellular degradation becomes increasingly important for this removal process and hence for controlling enzyme levels. It is of interest that the tissue with the most rapid mean rate of protein turnover is the liver, the tissue whose metabolic machinery, that is, enzymes, undergoes the greatest changes in response to dietary changes as part of its role in maintaining the internal *milieu* of the animal.

Properties of protein turnover in rat liver

Since regulation of enzyme levels takes place against a background of continual synthesis and degradation, certain properties of this overall process are presented as a basis for subsequent presentations.

Studies from various laboratories have shown that replacement of liver protein is rapid and extensive. One such experiment designed to answer questions about the rate and extent of turnover of total liver protein and one specific enzyme, arginase, is shown in Figure 1 (*22*). Rats were fed an amino acid diet containing ^{14}C-L-lysine of constant specific activity for up to 20 days. The rate and extent of protein turnover were determined from how rapidly and to what extent total cellular protein and arginase were replaced from the dietary source. The free lysine pool (counts soluble after trichloroacetic acid precipitation of protein) approached maximal labeling in 24 to 36 hours (Figure 1). Lysine incorporation into total liver protein

(CPM[1]/mg protein) was initially rapid and then slowed markedly after five to six days. Incorporation of lysine into arginase (total CPM in enzyme precipitated with a specific anti-arginase antibody) was slower.

A measure of the extent to which the cellular proteins are replaced can be obtained by comparing the specific activity of the lysine in protein with that of the dietary source as shown in Table 1. The specific activity of the lysine isolated from the original diet was 1105 CPM/μmole. After 20 days of labeling, about 76 percent of the lysine residues of total liver had been replaced, that is, the specific activity of protein lysine was 829 CPM/μmole. In addition, virtually all of the lysine of arginase had been replaced in this time. From this experiment we can conclude:

(1) The replacement of protein in rat liver is extensive and rapid. At least 50 per cent of the protein is replaced in four to five days. Similar conclusions have been made by Swick (*30*) and Buchanan (*3*).

(2) The majority of protein degradation is intracellular rather than involving the death of entire cells. The life span of cells in liver is from 160 to 400 days (*3, 13*). It follows then that since most of the protein is replaced within 20 days, the degradation that occurs must be largely intracellular.

(3) There is a marked heterogeneity of degradation rate constants of different cell organelles and individual intracellular enzymes. This is already evident from the experiment of Figure 1. Thus, arginase is replaced more slowly than a large portion of the total protein. The data summarized in Table 2 emphasize the heterogeneity of rate constants even more. Among cell fractions as defined by differential centrifugation, of particular interest is the finding that the proteins of the membrane fractions, smooth and rough endoplasmic reticulum, and the plasma (cell surface) membranes are in the greatest state of flux with a mean half-life of about two days (*1*). There is an even more marked heterogeneity of degradation rate constants among individual enzymes. There is no correlation between the intracellular localization of an enzyme and its half-life. Thus δ-aminolevulinate synthetase has a half-life of only one hour, but is localized in mitochondria (half-life=6 to 7 days). Likewise half-lives of soluble proteins range from 1.5 hours (tyrosine transaminase) to 16 days (lactic dehydrogenase isoenzyme 5).

[1] Counts per minute.

Table 1

Specific activity of ^{14}C-L-lysine isolated from total liver protein and arginase following 20 days of continuous administration of ^{14}C-L-lysine diet

Source of lysine	Radioactivity recovered as lysine	Lysine specific activity	% Replacement of lysine
	%	(CPM/μmole)	
Diet	98	1105	—
Total liver protein	95	829	75
Arginase	66	1106	91

The liver from three rats maintained on a ^{14}C-L-lysine diet for 20 days as described in Figure 2 was pooled and arginase purified as outlined elsewhere (*22*). ^{14}C-L-lysine of the purified arginase and a sample of all discarded protein (total liver protein), as well as of the initial ^{14}C-L-lysine amino acid mixture, was isolated following hydrolysis 6 N HCl by chromatography on Amberlite CG–50 (NH_4 + form) columns.

Theoretical formulation of changing enzyme levels [1]

In view of the fact that there is a continual synthesis and degradation of essentially all proteins of liver, any description of changing enzyme levels must consider both synthesis and degradation. Thus, a change of an enzyme level can be expressed by:

$$(1) \quad dE/dt = k_s - k_d E$$

where E is the content of enzyme (units × mass^{-1}), k_s is a zero-order rate constant of

[1] See Berlin and Schimke (*2*) for a more detailed development of this formulation.

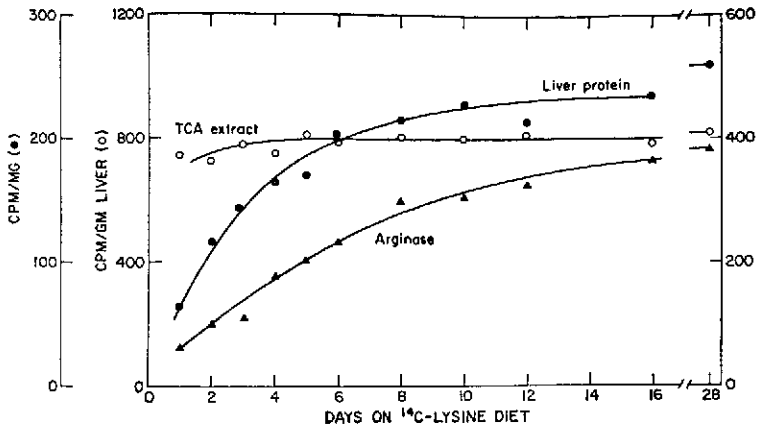

Figure 1. Incorporation of continuously administered ^{14}C-L-lysine into total protein, arginase, and trichloroacetic acid (TCA) soluble extracts of rat liver. Osborne-Mendel rats, weighing 250 to 275 gm each, were maintained for 7 days on a diet consisting of 25 per cent complete amino acid mixture. Following a 12-hour period without food, they were placed on a diet containing ^{14}C-L-lysine (specific activity, 1100 CPM per μmole). At the intervals specified, one rat was killed; the liver was then removed and divided into two weighed portions. One sample was made into acetone powder from which arginase was isolated by immunological techniques; the other was treated with 10 per cent TCA and divided into protein and supernatant fractions. Radioactivity of the trichloroacetic acid soluble counts is expressed as CPM per extract from 1 gm of liver, wet weight (○——○). Counts in total liver protein are expressed as CPM per mg of protein (●——●). Counts in the arginase represent the total number of counts precipitated (▲—·—▲). From Schimke (22).

Table 2

Half-life estimates of subcellular fractions and specific enzymes of rat liver

Fractions	Mean half-life, days		Reference
Nuclear	5.1		
Mitochondrial	6.8		
Supernatant	5.1		(*1*)
Smooth endoplasmic reticulum	2.1		
Rough endoplasmic reticulum	2.0		
Plasma membrane	1.8		
Enzymes		Localization	
δ-Aminolevulinate synthetase	0.04	Mitochondrial	(*15*)
Alanine aminotransferase	0.7 –1.0	Mitochondrial	(*31*)
Catalase	1.4	Peroxisomal	(*18*)
Tyrosine aminotransferase	0.06	Soluble	(*10*)
Tryptophan oxygenase	0.10	Soluble	(*5*)
Glucokinase	1.25	Soluble	(*16*)
Arginase	3 ⟩ (–5	Soluble	(*22*)
Glutamic-alanine transaminase	2 ⟩ (–3	Soluble	(*27*)
Lactate dehydrogenase 5	16	Soluble	(*6*)

synthesis[2] (units $\times$ time^{-1} $\times$ mass^{-1}), and k_d is a first-order rate constant for degradation[3] (time^{-1}). In general, there is little if any change in total mass of a tissue, for example, liver, during an experimental time period, and consequently an expression for a change in total tissue mass is not included.

In the steady state, that is, when $dE/dt=0$, then

$$(2) \quad k_s = k_d E, \text{ and}$$

$$(3) \quad E = \frac{k_s}{k_d}$$

Thus, in the steady state, the amount of enzyme is a function both of the rate of synthesis and the rate of degradation. An alteration in either rate can affect the level of E. The significance of this generalization as affected by nutritional variables will be the subject of the remainder of this paper.

Nutritional control of arginase levels in rat liver

In studying the mechanisms involved in changes of enzyme levels, the first question to be answered is whether the differences in assayable enzyme activity result from differences in content of enzyme protein. Once this has been established, the question of whether the rate of synthesis, the rate constant of degradation, or both have been altered, can be answered. We have attempted to answer these questions as directly as possible by isolating the specific enzyme protein in question. Generally this has involved the use of immunologic techniques as adjuncts to isolation and purification of the protein.

[2] The rate of synthesis of a specific protein is determined by a number of factors, including the number of ribosomes, amount of messenger ribonucleic acid (mRNA), levels of amino acids and transfer ribonucleic acid (tRNA), availability of initiation and transfer factors, etc. In this simplified model, the separate roles of such variables have not been factored, since they are largely unknown in mammalian tissues. Hence, all such variables have been included under a general notation of a rate of enzyme synthesis.

[3] The rate of degradation of a protein is expressed in terms of a first-order rate constant because in all cases studied in liver, the degradation of specific intracellular proteins has followed first-order kinetics. Rate constants of degradation are often expressed in terms of a half-life where half-life $= \frac{\ln 2}{k_d}$.

Steady-state conditions

As is the case with many liver enzymes that are involved in the catabolism of amino acids (*12*), a direct relationship exists between the levels of such enzymes and the caloric intake provided in the form of protein. Among such enzymes are those of the urea cycle, that is, carbamyl phosphate synthetase, ornithine transcarbamylase, arginino succinate synthetase, arginino succinase, and arginase (*21*). We have studied arginase, particularly, because it can be purified readily to a homogeneous state and is capable of eliciting precipitating antibodies when administered to rabbits. As shown in Table 3, there is a two- to three-fold difference in the specific activity of liver arginase between animals maintained 14 days on a diet containing either 8 per cent or 70 per cent protein by weight. That this difference in activity results from a difference in the amount of enzyme protein is supported by studies using an antibody specific for arginase. The arginase used to immunize rabbits was purified to the point of homogeneity as indicated by sedimentation-velocity studies in an analytical ultracentrifuge and by the presence of a single protein band on acrylamide gel electrophoresis. The anti-

Table 3

Effect of diet on steady-state levels, synthesis and degradation of rat liver arginase [a]

Diet	Activity	Half-life[b]	k_d	k_s
	μmoles/gm wet weight $\times 10^{-3}$	Days	Day^{-1}	Units/gm /day $\times 10^{-3}$
8% casein	20.2 $\pm$ 1.0	5.2	0.13	2.6
30% casein	36.7 $\pm$ 1.3	4.8	0.14	5.2
70% casein	56.1 $\pm$ 1.1	4.6	0.15	8.4

[a] From Schimke (*22*).

[b] See Figure 2.

serum obtained specifically precipitates only arginase.

Figure 2 shows a typical immunotitration of the antiserum with highly purified enzyme (5580 units/mg), as well as with crude liver extracts with specific activities varying by 4-fold (10 and 39 units/mg protein). These crude extracts were from livers of animals maintained on 8 per cent or 70 per cent dietary protein, respectively. The amount of enzyme activity neutralized (precipitated) by a constant amount of antiserum was the same, despite the fact that the total amount of protein added, varied over a 500-fold range in the three arginase preparations. Furthermore, as indicated by the amount of protein precipitated, the precipitation reactions were quantitatively similar with the highly purified enzyme and with the crude extracts of differing specific activities. These results, then, demonstrate that the differences in the amount of assayable enzyme activity in livers of animals maintained on diets

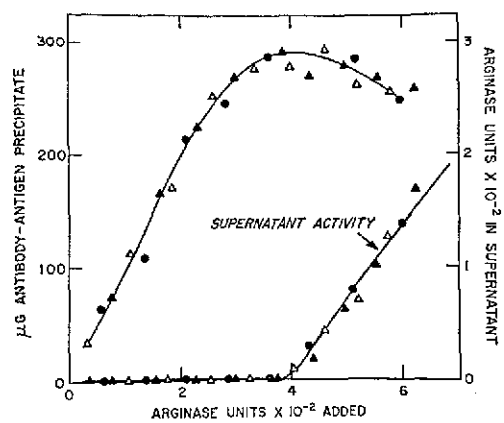

Figure 2. Quantitative precipitin reactions of purified arginase and liver extracts. Arginase preparations were of three sources: (a) purified arginase (specific activity, 5580 units per mg), ▲——▲; (b) a crude arginase preparation from animals maintained on 8 per cent dietary casein (specific activity, 10 units per mg), △——△; (c) a crude arginase from animals maintained on 70 per cent dietary casein (specific activity, 39 units per mg of protein), ●——●. Volumes and protein concentrations were made constant by equalizing the specific activity of all preparations by addition of suitable amounts of bovine serum albumin. From Schimke, (22). See same for details.

with differing protein contents represent differences in the amount of enzyme protein as determined immunologically.

The next question to be answered, then, is whether the difference in enzyme content resulting from maintaining the animals on diets with differing proportions of protein results from a more rapid rate of synthesis or a less rapid rate of degradation; that is, in Equation (3) above, is k_s or k_d altered? This question was answered by determining the values for k_d at three different steady-state levels of arginase. In this experiment, the details of which are given in the legend to Figure 3, animals were pulse-labeled with guanidino-^{14}C-arginine, and thereafter the decay of radioactivity of labeled total protein and arginase was determined with time. The degradation rate constant of arginase, here indicated by a half-life value, is essentially the same in the three steady states, that is, $t_{1/2} = 5$ days. The calculations of Table 3 indicate that variations in dietary protein content affect the rate of enzyme synthesis rather than the rate of degradation. It is of interest that Rechcigl (*19*), Rowe and Wyngaarden (*20*), and Majerus and Kilburn (*14*) have also found that differences in rates of synthesis determine steady-state levels of catalase, xanthine oxidase, and acetyl coenzyme A carboxylase, respectively, when rats are maintained on diets which affect steady-state enzyme levels.

Conditions of changing enzyme levels

During abrupt and extensive changes in nutritional status, differences in degradative rates also occur. Two experiments were performed: (1) The effect of starvation on arginase levels in rats that had previously been maintained on an 8 per cent protein diet; (2) The effect of change of animals maintained on 70 per cent dietary protein to a diet containing 8 per cent protein on arginase levels. Thus, during starvation after maintenance on an 8 per cent protein diet, there is a net increase of total arginase (Figure 4). This increase results from con-

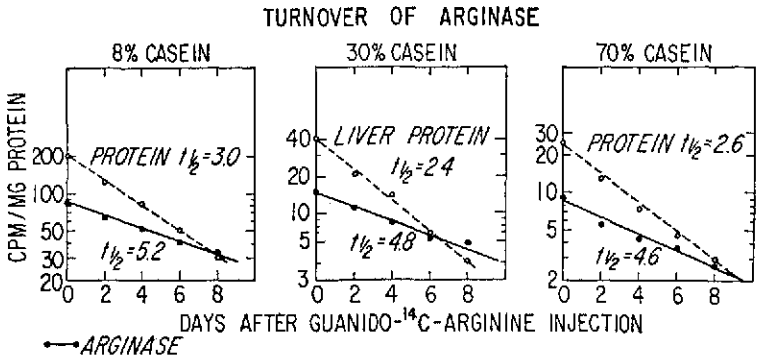

Figure 3. Turnover of total liver protein and arginase determined by the decay of radioactivity after single administration of ^{14}C-guanidino-L-arginine. Following maintenance of 20 rats for 14 days on each of three diets containing 8, 30, or 70 per cent casein, each rat was given a single intraperitoneal injection of 25 μc of ^{14}C-guanidino-L-arginine. One hour later, and at two-day intervals, four animals from each dietary group were killed and the four pooled livers were subjected to purification of arginase. The protein fractions discarded during the arginase purifications were pooled to constitute total liver protein. Results are expressed as CPM per mg of protein, ○——○, total liver protein; ●——●, arginase. See Schimke (22) for details.

tinued enzyme synthesis in the absence of any breakdown. Experimentally, this conclusion is based on the finding that there is no loss of total labeled arginase during subsequent starvation as opposed to the decay that occurs in the steady state. The stabilization of arginase occurs at a time when there is an extensive degradation of total liver protein. During change from a high-protein to a low-protein diet, there is a rapid decrease in total arginase over a nine-day period. This decrease results from altered rates of both synthesis and degradation (Figure 4). During the first three days of diet shift, there is a decreased rate of enzyme synthesis and an accelerated rate of degradation. Enzyme synthesis virtually ceases during the second three-day period whereas degradation continues. During the third three-day period, the rates of synthesis and degradation approach those characteristic of the new steady state of animals maintained on 8 per cent protein.

Nutritional control of acetyl coenzyme A carboxylase

The pattern of independent regulation of the rates of synthesis and degradation of specific enzymes is further illustrated by recent studies by Majerus and Kilburn. Fatty acid synthesis is inhibited by starvation and is accelerated when rats are placed on fat-free diets. Numa *et al.* (*17*) have shown that the first enzyme in the fatty acid synthesis pathway, acetyl coenzyme A carboxylase, is rate-limiting, and its activity as measured by *in vitro* assays fluctuates during fasting and refeeding. Majerus and Kilburn (*14*) have extended these studies by combined use of radioisotopic and immunologic techniques similar to those used for studies of arginase described above to assess the relative contributions of altered rates of synthesis and degradation in producing the altered enzyme levels. These results are summarized in Table 4 and Figure 5.[4] When rats are fed a fat-free diet for 48 hours, the hepatic activity of acetyl CoA carboxylase is 14.0 milliunits/mg liver protein, whereas the corresponding value for rats maintained on a high-fat diet containing 45 per cent vegetable oil (not shown in Table 4) is 0.6 milliunits/mg protein. Maintenance on Purina rat chow results in 6.0 milliunits/mg protein, and animals fasted after maintenance

[4] I am indebted to Dr. Philip Majerus for his permission to describe his results in this paper.

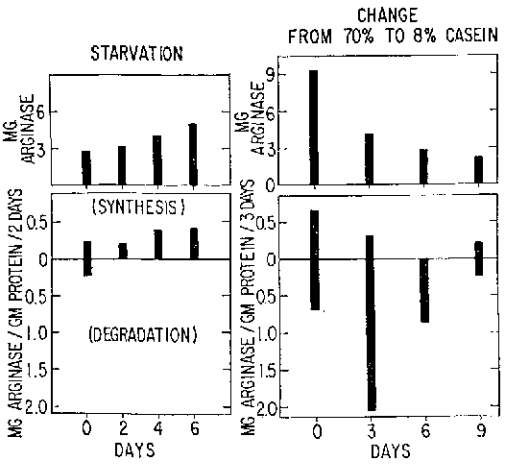

Figure 4. Rates of synthesis and degradation of rat liver arginase during fasting (left) and change from 70 to 8 per cent casein diet (right). Animals were maintained on diets containing 8 per cent (starvation) or 70 per cent casein for 14 days prior to the experimental period. The animals were given single administrations of ^{14}C-guanidino-L-arginine one hour prior to change in dietary status. The loss of isotope, both total and specific activity, from arginase was followed with time. The upper set of bars indicates the total milligrams of arginase in the pooled sample of four livers (starvation) or three livers (change from 70 to 8 per cent dietary protein). The lower set of bars shows the rates of synthesis and degradation expressed as milligrams of arginase synthesized and degraded per gram liver per observational period. The loss of total radioactivity in arginase is a measure of degradation. The decrease in specific radioactivity is a measure of the rate of synthesis. The basis for these calculations, as well as experimental details, are given in Schimke (22).

on a fat-free diet for 60 hours show 0.9 milliunits of acetyl CoA carboxylase activity/mg protein.

The rates of enzyme synthesis were markedly affected by the diet. Thus, animals maintained on the fat-free diet synthesized acetyl CoA carboxylase at a rate 10 times greater than animals starved for 48 hours relative to total liver protein (Table 4). However, the precipitous decline in enzyme activity that occurs during fasting is due not only to a decrease in the rate of enzyme synthesis, but also to an acceleration in the rate of enzyme degradation. Thus, as shown in Figure 5, pre-labeled enzyme decays with a half-life of 48 hours in the fat-

Table 4

Synthesis and degradation in nutritional control of acetyl CoA carboxylase[a]

Nutritional status	Enzyme activity (milliu/mg protein)	Relative[b] k_s	Half-life[c]
Fat-free diet	14.0	3.0	48 hrs
Purina rat chow	6.0	(1.3)[d]	48 hrs
Change from fat-free diet to fasting—48 hours	1.9	0.3	18 hrs

[a] Data from Majerus and Kilburn (*14*).

[b] Relative rate of synthesis = total CPM precipitated by specific antiacetyl CoA carboxylase antibody/CPM/mg total protein. ^{3}H-leucine was injected intraperitoneally and animals were killed after 40 minutes.

[c] Half-lives determined as described in Figure 3.

[d] Relative K_s calculated from known enzyme level and K_d as per Equation (3).

free dietary state, whereas during starvation the enzyme is inactivated far more rapidly (half-life of 18 hours). The difference in level of enzyme between fat-free diet and Purina rat chow under steady-state conditions (14.0 versus 6.0 milliunits/mg protein), in contrast, would appear to be related only to an effect on enzyme synthesis, since Majerus and Kilburn found in experiments comparable to Figure 5 that the half-life of the enzyme is the same (48 to 50 hours) with both the Purina chow and fat-free diets. Hence the difference in enzyme level must be related to the rate of enzyme synthesis from Equation (3), (page 24).

Discussion

The pattern that emerges from these studies is one of continual change in which the rate at which the steady-state complement of specific molecules of a particular enzyme is replaced varies from several hours to many days. This fact leads to a remarkable ability to alter selectively the amount of particular protein by altering the rate of synthesis or degradation or both. That these rates undergo marked changes in response to nutritional conditions is shown by the results reviewed in this paper. In the case of

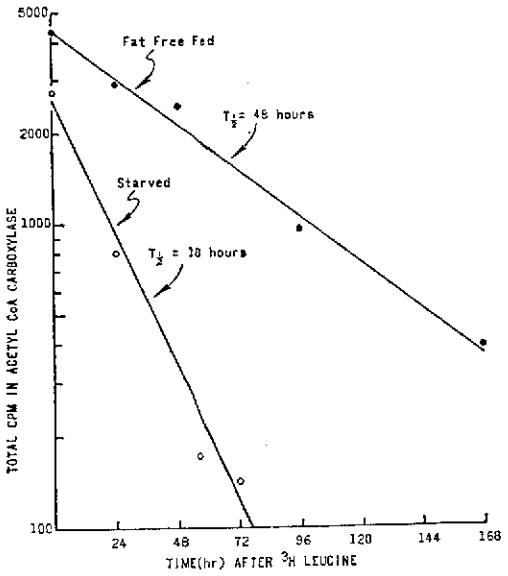

Figure 5. Acetyl CoA carboxylase turnover. Rats weighing 150 to 170 gm were used in these experiments. Rats fed a fat-free diet for 48 hours were given intraperitoneal injections of 250 uCi of ^{3}H-leucine, and were subsequently killed in groups of three, 40 minutes after injection (zero time), and at the subsequent times indicated. In a second experiment, rats fasted 48 hours were given intraperitoneal injections of 500 uCi of ^{3}H-leucine; the rats were killed in groups of four at the times indicated. Acetyl CoA carboxylase was purified on diethylaminoethyl (DEAE)-cellulose columns, and the radioactivity incorporated into the enzyme was determined by precipitation with a specific antibody. The results are reported as the total radioactivity incorporated into acetyl CoA carboxylase in each group. See Majerus and Kilburn (*14*) for details.

both arginase and acetyl CoA carboxylase, steady-state enzyme levels as affected by diet were determined by rates of synthesis. Clearly the stimulus is different, since with arginase, it is the protein content of the diet, whereas with acetyl CoA carboxylase, it is the fat content that is variable. During starvation there were also marked differences in both enzyme contents, but in opposite directions and by different mechanisms. Thus arginase activity actually increased, a result of continued enzyme synthesis, but with a *decreased* rate of degradation. With acetyl CoA carboxylase, enzyme activity fell rapidly, and resulted from both a *decreased* rate of synthesis and an *increased* rate of degradation.

These results, then, show that regulatory mechanisms are carefully poised to maintain enzyme activities appropriate to the nutritional condition of the animal. Thus we can look upon the increased levels of arginase and other urea cycle enzymes (*21*) and an entire host of enzymes involved in amino acid catabolism (*12*) that result from high-protein diet as adaptive responses to high-protein diet and the requirement for conversion of amino acids to sources of energy. Likewise the effects of fat on acetyl CoA carboxylase (*14, 17*) and of dietary carbohydrate on levels of liver glucokinase (*16, 28, 29*) are other examples of such adaptation.

Such results raise two types of fundamental questions at a symposium of this kind. One is the scientific question of the intimate molecular mechanisms involved in regulating the rates of synthesis and rates of degradation of specific enzymes. Unfortunately, an understanding of mechanisms in the depth and clarity known for microbial systems is not available at present for animal tissues. This is in large part due to difficulties in using intact animals as experimental systems, difficulties which include the differences in cell population, the complex interrelationships between nutritional variables and multiple hormones, and the lack of suitable mutants. Many of the possible mechanisms have been discussed in two recent reviews (*23, 24*) to which the interested reader is referred.

The other question that is raised by these studies, and one which is particularly important to nutritionists, involves why a continual degradation of protein is necessary in higher organisms. Unfortunately again, there is no answer to this nor to the contention that continual protein degradation is wasteful of energy. My own speculations on this stem from an evolutionary argument concerning possible needs and mechanisms for the breakdown of proteins: thus, during starvation, where massive protein degradation must occur, some mechanism is

required for relatively indiscriminate degradation which can be turned on and off rapidly. Out of such a requirement might have developed the lysosomal system (*4*). However, such comparatively indiscriminate degradation of cell constituents would not be appropriate for that protein degradation associated with selective and relatively specific removal of enzymes no longer required. Several "solutions" to selective protein degradation are possible. Specificity could have evolved through the elaboration of proteins specific for the removal of each protein. Such specific inactivating proteins may well exist in certain cases. However, such a solution would be "expensive" in a genetic and evolutionary sense, since it would require the existence of a large number of specific inactivating proteins as well as the regulatory apparatus required to recognize when such proteins were to be synthesized. An alternative solution is that specificity for degradation exists in the enzyme itself or in its interaction with ligands. Only a few non-specific degrading enzymes would be required. We can conceive, then, that during evolution it became necessary to provide an ability for some proteins to fluctuate in content more rapidly than others. Thus, for those proteins whose levels need not change, mutations in peptide sequence leading to increased stability *in vivo* would be selected. Conversely, for those proteins whose levels must change, mutations leading to an increased susceptibility to degradation would have been selected. Thus, although the solution involving the continual synthesis and degradation of proteins is wasteful, this solution would appear to have been the predominant one to emerge in the mammal.

Obviously, if we could prevent the continual degradation of protein by suitable nutritional manipulations or drugs, the saving of protein foodstuffs would be enormous in terms of total world food requirements. Clearly this is an area that deserves far more research and attention.

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DISCUSSION

Chairman Cohen: What has been revealed this morning is that the liver in particular, and the whole organism to one degree or another, are constantly undergoing change, and that this state of continuing change is very responsive to dietary influences.

Soberón: I have a question for Dr. Potter in regard to the rhythm of tyrosine transaminase. It has been proposed that the cyclic variation of polysome aggregation might have something to do with the cyclic variation of tyrosine transaminase. However, I well remember that in Dr. Potter's 8+40 schedule there are two distinct peaks in tyrosine transaminase, and I do not know how to reconcile the observations by Munro of the polysome aggregation and the enzyme activity of tyrosine transaminase described by Dr. Potter.

Potter: Dr. Soberón is referring to the fact that in the animals on the 8 plus 40 regimen, where they are fed for eight hours and fasted for forty hours, there is a peak of second synthesis which occurs when there is no food intake. About the only thing that I can say to your question is that we know that the second peak is dependent upon the status of the adrenals. It requires the intact adrenal glands, whereas the first rise does not. The first rise will occur equally well in the absence of the adrenal gland. We think that it is due to the flux of amino acid, so we believe that we can separate the effect of the amino acid flux from the status of the adrenal. We have not personally studied the polysome pattern at this point.

Neel: I should like first to compliment the three speakers on the excellence of their presentations. I noted that one of Dr. Potter's slides was headed "We can influence gene activity," a heading that doesn't seem to have carried over into the material reproduced for the Symposium. It is true that we can influence gene activity, but I wonder if we could have a few comments on the extent to which, on the other hand, genetic differences are setting individual limits to the activities that we wish to influence. More specifically, I missed the basis for the curves we saw, such as the numbers of animals involved, and to what extent inbred strains were used.

Some of the presentations attached errors to the points on the curve. Were these standard errors or standard deviations? If some of them were standard errors of the means they imply, the standard deviations must have been very large indeed.

We do have knowledge of some extreme cases of individual differences in this field: the inability to metabolize phenylalanine that we recognize as phenylketonuria or vitamin D resistant rickets, which can be overcome with doses of vitamin D about 100 times the normal intake. But what do we begin to know about lesser, but possibly meaningful individual variations in the emerging field we have been discussing this morning?

Potter: With regard to the caption on the slide, and not in the text, it was not any kind of a retreat from a position, but it is a fact that the text has a legend with it, and so the caption would be redundant.

The implied question, however, is whether we are or are not limited by the inherited genome. On that point I am sure that there is not any difference between us. We certainly recognize that we can only modulate gene activity within limits. In other words, the genome provides the potential for the individual and we can only modulate within the potential which the genome contains, but we recognize, as you do, that individuals may vary in the genes that

regulate the response of other genes, and not just in those regulating the enzymes we study. Actually, I am most concerned with the question of achieving the optimum for any individual genome. That is what we should all be striving for, and not merely what is the minimum nutritional requirement for a hypothetical average man.

Now, with regard to the question about the standard error: it is indeed the standard error and not standard deviation. The variation is very great because, in general, most experiments involved only three animals per point, so the standard error is a very rough kind of a figure (since $n-1=2$). However, we believe that the straight statistical analysis of a situation in terms of the variance at a given point has to be looked at in relation to all of the other data in the experiment, so that if you have a time curve, or a curve expressed as a function of per cent protein in the diet, the average of three animals or four animals does not stand by itself alone, but is buttressed by the three animals to the left of it, and the three animals to the right of it on the curve. In some of our curves which you saw, we had standard errors in both directions so that it is buttressed in two dimensions. The places where you see the large brackets are frequently at transition points. It gets too complicated on a graph of this kind to show all the individual animals.

Now, with respect to your question about the inbred strains. We have been using the Charles River rats for a great many of our experiments. We have also used Buffalo rats which are inbred. We used the Holtzman rats which are not strictly inbred, but we have made comparisons between Buffalo rats and Holtzman rats and we got quite similar responses. Perhaps Dr. Neel's questions regarding individual variability could be answered by a more detailed discussion of the data involving two parameters. We have strong indications that the variations are not experimental error alone, but truly represent individuality in the animals. This can be seen when we attempt to correlate one parameter with another.

Waterlow: I would like to comment on some of the last remarks of Dr. Schimke. He gave us details about turnover of liver protein and liver enzymes and indicated in his final remarks that he attached particular importance to the turnover of enzymes in the liver as a part of the regulatory mechanism. I am sure that this is true because, after all, the liver is the first to receive the amino acids. On the other hand, from the point of view of the nutrition of the whole animal, we must recognize that the turnover of other proteins, particularly those of muscle, is quantitatively very important. Recent results from our unit (Millward, in press) show that in the rat the turnover rate of the myofibrillar protein has a half-life of about eight days—only four times as long as the average liver protein. This is the highly organized structural muscle protein. Quantitatively there is so much of it that it accounts for as much turnover as the total protein of the liver, if not more. Furthermore, it is extremely sensitive to dietary changes. Fasting will rapidly increase its catabolic rate and reduce its synthetic rate. What seems to me very mysterious is how the muscle knows what to do. How does this information get to it? Is it in some way controlled by the liver?

From the point of view of the whole animal, the flux of amino acids from internal turnover represents a very large supply of substrates for energy production, and, therefore, a vulnerable one when there is a shortage of food. Dr. Potter's slide showed that in the rat, after food is removed, liver glycogen fell from about 50 mg per gm to nearly zero. In a 100 gm rat, this would correspond to about 250 mg glycogen. The turnover rate of adipose tissue in the rat is said to be about 10 per cent per day, equivalent to about 1 gm fat per 100 gm rat. The turnover of protein is about 2.5 gm per 100 gm body weight per day (Waterlow, 1968). Thus the amounts of energy available from *internal* turnover in the post-absorptive state would be roughly: From liver glycogen 1 kcal (muscle glycogen is much less reduced than that of liver, and, therefore, I have ignored it);

from fat 9 kcals; from protein 10 kcals, or half the total. If one supposes that the body tends to use whatever fuel is most readily available for energy production, then the rapid response of the catabolic enzymes, such as the tyrosine transaminase described by Dr. Potter, is extremely important for the amino acid economy of the animal. In this respect, I am inclined to think that the main problem is not so much the conservation of amino-N as the conservation of the carbon skeletons of the essential amino acids.

Schimke: Perhaps I could go back for a moment to Dr. Neel's comments. He mentioned inbred rats. We have explored in the last several years the use of inbred mouse strains for various studies, not so much on nutrition, but on regulation of enzyme levels in animal tissues. Although the rat has been the standard animal for any number of studies, I think inbred mouse strains represent a tremendous source of material for this type of study because of the potential interplay of nutritional aspects with genetics. As I am sure many of you know, there are a number of genetic defects that can be explored in inbred mouse strains.

I would also like to know about the mechanisms and control, in general, of the breakdown of proteins. I did not want to give the impression in my presentation that the only protein breakdown of significance takes place in the liver, because, obviously, muscle is where the major breakdown occurs. How breakdown is regulated is very difficult to study, and I do not think, even now, that it is exactly clear what the normal process for breakdown of protein is. Is it simply a hydrolytic cleavage of the peptide bond? Does it occur only in lysosomes? Is there any conservation of the energy in the form, for example, of amino acyl-t-RNA being an intermediate? These are some of the questions that arise. Exactly how a muscle knows when to break down proteins is again part of the general question of what regulates protein breakdown. My own feeling would be that it is not unlikely that there is a hormonal influence here just as there is in any number of interactions of different tissues, but exactly what, I would not know.

Neel: I did not refer to the use of inbred rat or mice strains with any great approbation but merely as a question of fact. In using these inbred strains one, of course, has sacrificed a great deal of the variability within inbred strains. It is very interesting to speculate on how much greater the variability might be if one used crossbreeding strains.

Chairman Cohen: One point relevant to the whole purpose of this discussion has emerged and that is the considerable variation of enzyme levels in individual animals. This deserves emphasis since one has a considerable degree of control of the selection and condition of the experimental animals and the conditions under which assays are carried out. On the other hand, there is a popular impression, widely accepted in some areas, that a fixed set of values for nutritional standards for humans exists, which can be used to determine whether individuals or groups are "well" or "malnourished."

Potter: I am addressing myself to Dr. Neel again with regard to the liver. Of course, my main interest is the cancer problem, and we have a whole series of what you might call inbred strains of hepatomas, which are serially transplanted and supplied to us by Dr. H. P. Morris. They are almost like mutant livers, without commenting on the connotations of that remark. However, the fact is that we have hepatoma lines, which are permanently set at a high level of amino acid transport and at a high level of tyrosine aminotransferase. We have another series of hepatoma lines at the other end of the spectrum and we have a whole series in between. We can take a hepatoma that is at the low end of the spectrum—almost zero, with respect to the enzyme tyrosine aminotransferase, and at a very low level of amino acid transport. It does not respond to a 60 per cent protein diet. It does not respond to hydrocortisone. We recently published a paper on "the induction of a previously non-

inducible enzyme" in this hepatoma line by stimulating it with glucagon under certain conditions. We can turn on the "pump" and turn on the synthesis of the enzyme, so this gets back to the question of individual variations in animals: How much is due to built-in levels of repression due to action of regulatory systems? There are animals set at a lower level, such as we saw in the hepatoma, that have greater need, shall we say, for more glucagon or the like.

Hoffenberg: Dr. Schimke has shown that all the urea cycle enzymes are reduced in relation to low protein diets. Now my question is, "Does he have any information about the time sequence of these enzyme reductions?" I ask this because a primary reduction in carbamyl phosphate synthetase, for example, might result in less available substrate for later enzyme action, so that reduction of late urea cycle activity might be seen as a secondary effect. Conversely, if one has a primary effect on later enzymes, one might expect build-up of earlier substrates and induction of their enzymes. Is there any evidence of time change?

Schimke: The sequence of change is that the first four enzymes change in about the same fashion, so the major change occurs in three days. Thus, it is not as if CPS increases in a day, or two, and the others change three or four days later. Arginase, the fifth, and last enzyme of the urea cycle, changes the least of the enzymes, and changes the most slowly. I would guess, although we have not done any experiments, that this might be related to the fact that arginase is turning over less rapidly than the other urea cycle enzymes.

Chairman Cohen: We have found that the half-life in a different animal system is of the order of 60 hours for carbamyl phosphate synthetase (CPS) and 90 hours for ornithine transcarbamylase. We are now in the midst of studies to determine the half-life of the other enzymes of the ornithine-urea cycle in tadpole liver. The point that needs to be made is that the rate-limiting concentrations of enzymes for the overall process of urea biosynthesis are determined by the concentrations of CPS and arginino succinate synthetase. The other enzymes are present in relative excess and therefore their variations, within rather wide limits, are going to have a lesser effect on the overall process than the levels of these two enzymes.

Waters: In one of Dr. Harper's slides, he indicated increased levels of the liver enzyme urocanase associated with increased protein intake. We (Waters, Mollin, and Dawson, unpublished observations) have noticed a marked increase in the urinary excretion of urocanic acid after oral doses of histidine in women with the clinical syndrome of Anorexia Nervosa, which is a self-imposed dietary restriction. As you know, this also occurs in patients with kwashiorkor, and in some patients following partial gastrectomy with a kwashiorkor-type syndrome. Does this indicate a fall in the level of urocanase in the liver of such patients? and secondly, Has Dr. Harper had the opportunity to measure the level of urocanase in the liver under these conditions?

Harper: We haven't had an opportunity to measure the levels of these in human tissues. Under conditions of starvation, as I remember, they are not as responsive as some of the other enzymes of amino acid degradation.

Turning to the problem raised earlier, one should recognize that people studying nutrition *do* take note of individual variation. Even in the early studies of amino acid requirements, Rose noted that, although the average tryptophan requirement was about 150 milligrams per day, one individual appeared to require 250 milligrams. Moreover, studies at Cornell by Nesheim on strains of chickens have shown quite substantial differences in the arginine requirement of two strains of chickens; these chickens also respond differently to an antagonism between lysine and arginine.

Finally, in relation to Dr. Waterlow's comment about the relatively high total amount of energy that could become available from turnover of amino acids, the question is: To what

extent do the amino acids that are turning over rapidly serve as a source of energy, and to what extent are they merely reincorporated into the proteins without escaping from the cell?

Waterlow: I think that the extent to which they serve as a source of energy depends on the level of protein intake and, of course, on the rest of the diet. Our evidence with N-15 amino acids in humans shows that of the total amount of amino acid entering the pool—mainly from internal turnover—the proportion which is oxidized varies from about five per cent on a maintenance level to about 30 per cent on a high protein intake.

As regards the second point, the scanty data available suggest that in the rat, on a normal protein intake, about 50 per cent of the amino acids liberated by catabolism in the liver is immediately reutilized, probably in the same cell (Aub, M. R., and J. C. Waterlow. *J Theor Biol* 26: 243–250, 1970). In muscle, this figure is about 10 or 20 per cent less. The difficulty is that at the moment I do not think it is really possible—I am sure Dr. Schimke would agree with this—to distinguish exactly between reutilization in the same cell of amino acid, which has not yet penetrated into the general circulation, and reutilization of amino acid which has circulated from one tissue to another. The former is calculated by us on the assumption that the amino acid pool in the cell is homogeneous; this assumption is undoubtedly wrong, but how wrong, is anybody's guess.

Schimke: It appears that in HeLa cells one finds as much as 80 per cent reutilization of amino acids; this would be in a situation where the amino acid is not getting outside the cell, so that reutilization can be extremely extensive within the same cell.

Arroyave: To return to the question of individual variability, there is a difference between animal and human experiments. In the first instance, investigators can largely control conditions, such as light and dark, and breeding strains. With human subjects, it is much more difficult to standardize a sample. Dr. Harper mentioned the work of Rose on amino acid requirements, and it is a good example. Recently, we have tried to estimate protein requirements of children between two and three years of age. The studies involved four normal children who were completely free of obvious clinical pathology. In a longitudinal design, we put the children in a metabolic ward on diets of differing amounts of egg protein, and under very standardized conditions, as one would when performing a rat experiment. After three months, we found a large variability in the estimated protein intake required to maintain normality in certain biochemical parameters and growth. In examining some of the "hidden" data that had been registered, it was discovered that those children who had more repeated infections during the study were the ones who also needed more nitrogen, that is, more protein, to meet their protein requirement, judging from the variables measured. This was something we could not control. Again, two of the children were sick more often than the other two under the same conditions of metabolic unit care and this greatly increased the variability. Although some variability may be genetic, there was obviously a very large uncontrollable environmental component in the experiment. An additional problem in human experiments is that they usually involve a very small number of individuals—two or three in each group—which makes the standard statistical estimates of variability more difficult to interpret.

DIETARY AND HORMONAL EFFECTS ON LIVER GLUCOKINASE[1]

Hermann Niemeyer

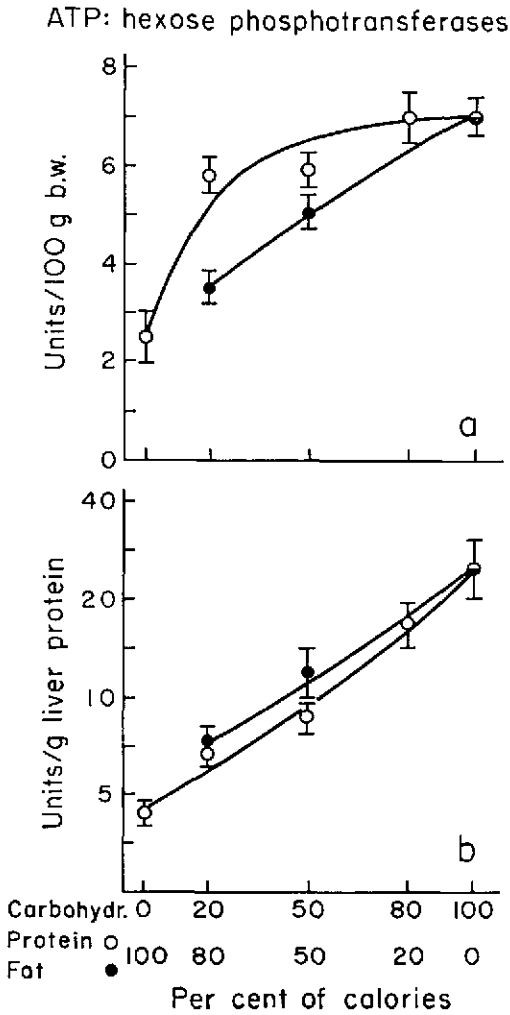
The general concept that the enzyme pattern of rat liver is modified by changing the nature and the amount of food offered to the animal seems to be well-founded. Normal activities of several enzymes, for example, depend on the appropriate supply of either protein, carbohydrate, or both. This observation derives in part from experiments performed in our laboratory, in which only one nutrient was given at a time, or two nutrients were fed in different proportions to adult rats during a constant six-day period (*27*). All the experimental diets contained the same amounts of salts and vitamins per calculated 100 calories. Intakes were restricted to approximately isocaloric values by giving 50 kcal of the diet daily.

From these studies, adenosine triphosphate (ATP): hexose phosphotransferase appears to be a very sensitive enzyme with regard to the supply of carbohydrate, inasmuch as maximal activities are observed only when this nutrient is present in the diet (*27, 39*) (Figure 1a). When proteins are the sole source of calories, a marked reduction in enzyme activity is observed. If fat substitutes for protein in varying proportions, a more accentuated diminution is attained (*27*). The enzyme activities are in terms of 100 gm of body weight, and the body weight selected for reference is that of the animals in the last common condition, that is, when they start the experimental diet. There is a very good correlation between the specific activities of the phosphotransferase (units per gram of liver protein) and the proportions of carbohydrate in the diet (Figure 1b). In fact, the specific activities are about the same whether carbohydrate is replaced either by protein or by fat, in spite of the great difference in total liver proteins under these two dietary conditions.

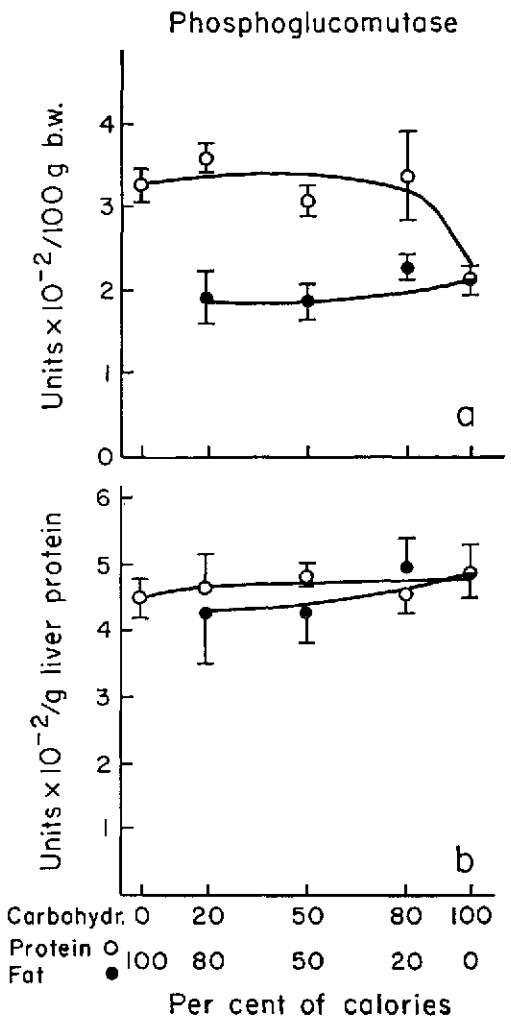
α-Glucan phosphorylase follows the same pattern as hexose phosphotransferase (*23, 27*). Other enzymes whose levels appear to depend on carbohydrate supply, such as uridine diphosphate glucose (UDPG), glucan transglucosylase (*19*), and pyruvate kinase (*14*), and perhaps phosphofructokinase, could most probably be included in the same group.

Phosphoglucomutase is an example of an enzyme that requires a certain amount of protein in the diet in order to be maintained at normal levels (*27*). If carbohydrate or fat substitute for up to 80 per cent of the calories contributed by protein, the same levels of activity as in the livers of rats fed the 100 per cent protein diet are observed (Figure 2a). The specific activities remain approximately the same under the various dietary conditions (Figure 2b). This behavior has been considered to be the result of a nonspecific effect of dietary proteins (*27*), since enzymes closely follow the total liver proteins, which increase when the amount of dietary protein augments (*16, 27*). The effect may result from a general activation

[1] The work reported in this paper was supported in part by research grants from the University of Chile, the Rockefeller Foundation, and the United States Public Health Service.



Figures 1a and 1b. ATP: hexose phosphotransferase activities in liver as function of diet. Rats were fed for six days isocaloric diets with variable proportions of protein, carbohydrate, fat, and constant amounts of vitamins and minerals. From Pérez, Clark-Turri, Rabajille, and Niemeyer (27).

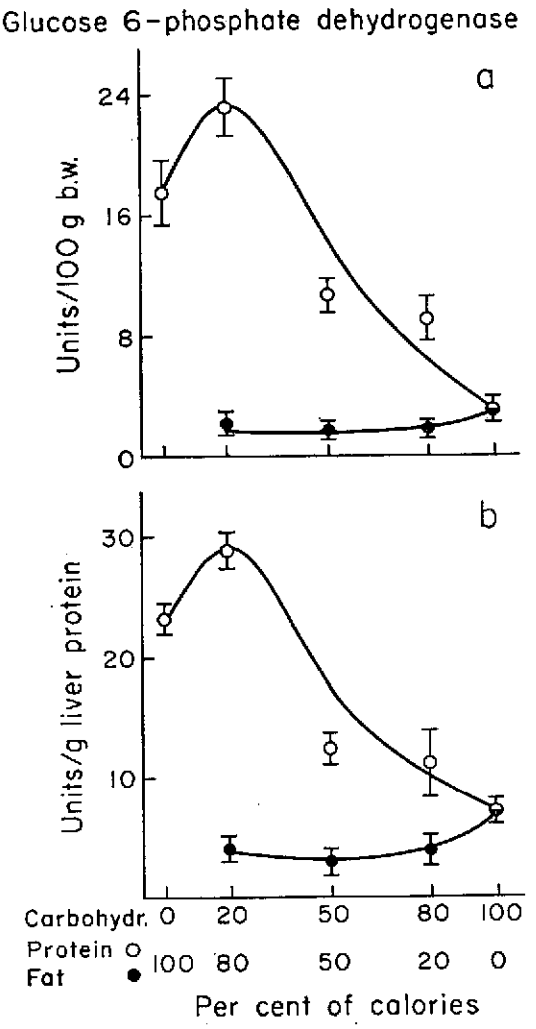


Figures 2a and 2b. Phosphoglucomutase activities in liver as function of diet. The same animals as in Figures 1a and 1b were used. From Pérez, Clark-Turri, Rabajille, and Niemeyer (27).

of protein biosynthesis depending on the cell amino acid pool. Glucose-6-phosphatase and few other enzymes so far studied behave similarly (*16, 27*).

The specific activities of a rather large group of enzymes concerned with the metabolism of amino acids increase markedly as the protein in the diet increases (*16, 27, 33, 35*), and appear to be the result of a specific effect of dietary proteins (*27*). Several oxidoreductases follow the same pattern (*15, 16, 40*).

Glucose-6-phosphate dehydrogenase and phosphogluconic dehydrogenase are also dependent on the protein supply (*27*). However, carbohydrates represent a valuable complement, inasmuch as the maximal activities are obtained with a diet composed of both protein and carbohydrate (Figure 3a). When fat substitutes for either protein or carbohydrate, the enzyme



Figures 3a and 3b. Glucose-6-phosphate dehydrogenase activities as function of diet. The same animals as in Figures 1a and 1b were used. From Pérez, Clark-Turri, Rabajille, and Niemeyer (27).

activities reach very low levels. However, the specific activities of both enzymes are augmented as the protein content of the diet increases to 80 per cent of the calories when the complementary nutrient is carbohydrate (Figure 3b). We are not aware of other enzymes that follow the same pattern.

The total content of several other liver enzymes (per 100 gm of body weight) is not significantly altered by changes in the nature of the diet, as is the case of glucosephosphate isomerase (*27*), and probably as is the case in the sequence of enzymes responsible for the conversion of 3-phosphoglycerate into pyruvate (*39*).

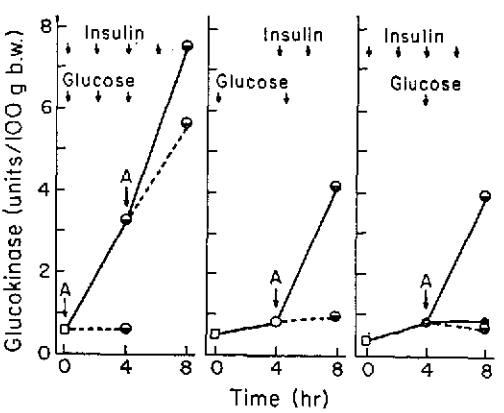
A method that appears to fulfill the requisites for the detection of specific effects of dietary compounds is the administration of a single food (or a combination of two or more in different proportions) to animals that have been stabilized on another diet not containing that particular substance, or simply to animals fasted for several days. This approach seems to be especially adequate when enzyme activities change rather abruptly. With this procedure it has been demonstrated very clearly that only glucose is required for glucokinase (*17, 21, 27*) and phosphorylase (*23*) induction after a carbohydrate-free diet or after fasting, whereas both protein and carbohydrate are necessary for the induction of glucose-6-phosphate dehydrogenase after fasting (*27, 32*). The participation of amino acids in the induction of serine dehydratase after protein depletion has also been studied by this procedure (*29*). For the sake of simplicity, the term induction will be used to indicate a selective increase in enzyme activity that appears associated with a *de novo* synthesis of protein, and that may result from changes in the relative rates of synthesis and degradation of the enzyme.

Actually several mechanisms responsible for the maintenance of the various enzyme levels may be superimposed, making the interpretation of the effect of dietary constituents on enzyme activities difficult. In fact, the actual mechanisms of enzyme regulation in mammals are poorly understood at present. As recognized very early in our excursion into this subject (*17*), an outstanding complication is introduced in multicellular organisms by the existence of the endocrine glands. Changes in the diet evoke alterations in the balance of the endocrine system, promoting the secretion of some hormones and blocking the secretion of others. It is now established, on the other hand, that disturbances

of the endocrine balance bring about changes in the enzyme content of the liver. In addition, the presence of an endocrine gland is necessary sometimes to permit changes in the enzyme pattern to occur after the administration of a nutrient. In other cases, however, the effect of the nutrient appears to be rather independent of the endocrine system. The participation of the endocrine system appears complicated by the fact that very often more than one hormone acts as an inducer of a particular enzyme, probably through the operation of different mechanisms and other hormones act antagonistically.

The following discussion will be concentrated on the interactions of hormones and dietary glucose in the induction of glucokinase in rat liver. Glucokinase is one of four isoenzymes that catalyze the phosphorylation of glucose in the liver (*7, 8, 13*) and appears to be the single one affected by modifications in the diet (*8, 34, 36, 41*). Carbohydrate deprivation causes a rather slow fall in glucokinase activity that is quickly reversed by glucose (*18, 21*) and to a lesser extent by other carbohydrates (*20, 30, 36*). Changes in the supply of carbohydrate lead to disturbances in the endocrine balance. Thus, glucose stimulates insulin release (*3, 9, 12*) and, in fact, not only glucose, but also insulin is essential to maintain glucokinase activity and to initiate the induction of the enzyme (*2, 6, 22, 34, 36, 37, 41*). The crucial problem that emerges is to have unequivocal evidence of whether the agent that causes the modification in the enzyme content is the nutrient or is the hormone. Glucose and insulin seem to act together at the transcriptional level, since neither the substrate nor the hormone alone appear to permit the accumulation of a postulated messenger ribonucleic acid (RNA) (*18*). This can be deduced from the following observations made in alloxan diabetic rats (Figure 4): Actinomycin D elicits a complete block of glucokinase induction when administered to the animals at the initiation of the induction promoted by the simultaneous supply of carbohydrate and insulin; however, the antibiotic inhibits only slightly the rate of induction when injected after several hours of induction (Figure 4a). The same type of response has been observed in normal rats when given carbohydrate after a carbohydrate-free diet (*20*). The injection of actinomycin after four hours of the separate action of glucose or insulin blocked completely the subsequent induction of glucokinase by insulin or glucose, respectively (Figures 4b, 4c). These experiments seem thus to indicate that during the interval of several hours of separate action of either glucose or insulin, there was no accumulation of messenger RNA, which could be active at the second period when both agents, substrate and hormone, were present. If glucose or insulin had acted alone as inducer at the transcriptional level, and the complementary agent only at the translational level, one would expect that actinomycin did not block the induction completely when insulin or glucose were given after a previous treatment with these two, respectively.

Interrelations between glucose and insulin effects may explain peculiarities of the kinetics of glucokinase induction by glucose in normal animals. When glucokinase induction is elicited



Figures 4a, 4b, 4c. Effect of separate or simultaneous treatment with carbohydrate and insulin on glucokinase induction in liver of alloxan-diabetic rats fed a carbohydrate-free diet. Influence of actinomycin D injected intraperitoneally at times indicated by arrow A. □ = controls without glucose and insulin; ○ = rats treated only with carbohydrate; ● = animals treated only with insulin; ◒ = animals that received both insulin and carbohydrate. From Niemeyer, Pérez, and Rabajille (18).

by a load of concentrated carbohydrate solution repeatedly given by stomach tube, a detailed study of the kinetics of the induction shows that after several hours of enzyme increase there is a detention for a few hours (*5*). Thereafter, the induction continues until the final steady state is reached (Figure 5). This interruption of the induction is interpreted as the consequence of a transient exhaustion of the pancreas β-cells caused by the load of carbohydrate. The enzyme induction would reassume when newly synthesized insulin was available. In support of this interpretation is the fact that the transient detention of glucokinase increase is prevented by the administration of exogenous insulin together with the carbohydrate (Figure 5). The assay of blood and pancreas insulin is certainly needed to place this hypothesis on a more rigorous basis.

Glucocorticoids and perhaps other hormones modulate the rate of glucokinase induction. Thus adrenal glands are not required for the induction (*20, 41*), but the rate of enzyme increase is lower in adrenalectomized than in sham-operated rats (*20*), and can be brought to normal values by the administration of cortisol (Figure 6). The importance of glucocorticoids in the modulation of the adaptive response of several liver enzymes to diet has been recently pointed out (*25, 26*). After hypophysectomy, a decrease of liver glucokinase is observed compared to sham-operated animals (*1*), and it has been briefly reported that the adaptive increase in glucokinase after glucose feeding does not occur in hypophysectomized rats (*28*). When physiological doses of cortisol, thyroxin, and testosterone are given in order to compensate for the atrophy of the respective secreting glands due to hypophysectomy, normal values of glucokinase are, however, maintained. Animals so treated show a decreased glucokinase activity after being fed a carbohydrate-free diet, as occurs in intact animals or in the sham-operated controls, and glucokinase induction can be elicited by administration of glucose (Figure 7). Although the final levels of glucokinase in the hypophysectomized rats (supplied

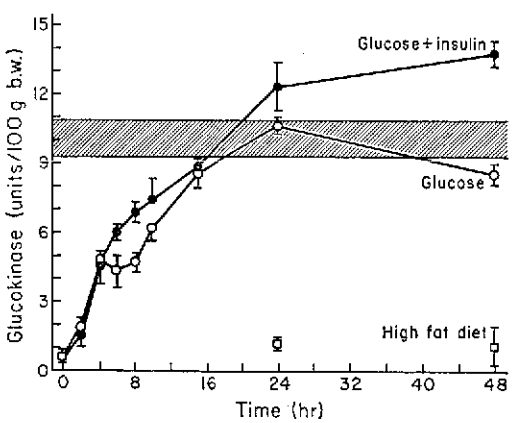


Figure 5. Kinetics of glucose-mediated glucokinase induction and effect of exogenous insulin. ○ = rats fed a carbohydrate-free diet for six days were given a 20 per cent glucose and 30 per cent dextrin solution by stomach tube every four hours. ● = In addition, rats similarly treated received crystalline insulin intraperitoneally. From Chamorro and Schillkrut (5).

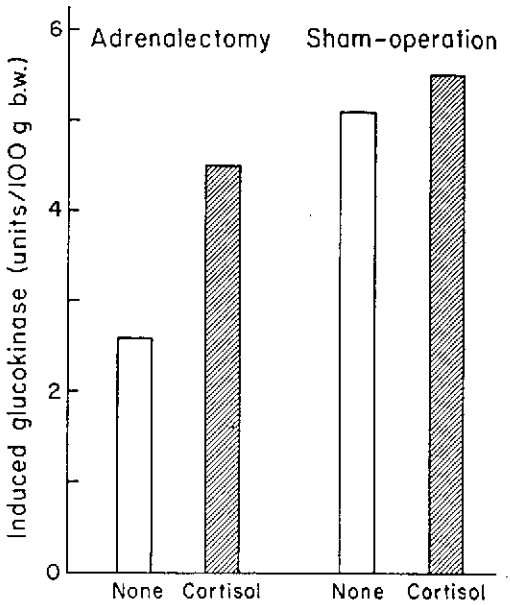


Figure 6. Effect of adrenalectomy and administration of cortisol on glucokinase induction. The animals were fed a balanced diet for six days after surgical operation and a carbohydrate-free diet for a subsequent period of four days. Some rats were injected with cortisol during the last period. Carbohydrate was given by stomach tube three and six hours before the rats were killed. From Niemeyer, Clark-Turri, Pérez, and Rabajille (20).

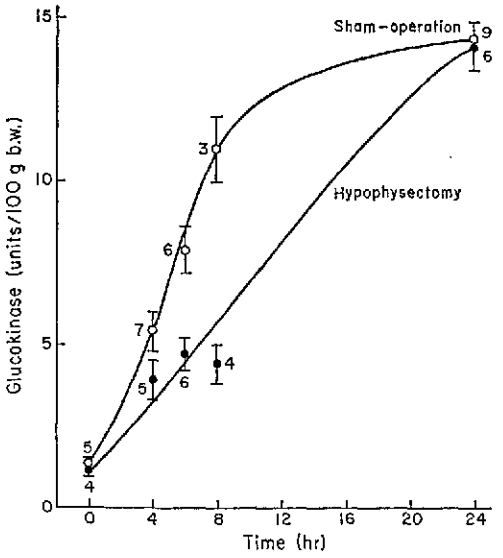


Figure 7. Effect of hypophysectomy on glucokinase induction. Hypophysectomized and sham-operated rats were kept on a stock diet for four days after surgical operation, and then fed a carbohydrate-free diet for six days. The hypophysectomized rats received physiological doses of cortisol, thyroxin, and testosterone daily. Glucokinase induction was elicited by the administration of a carbohydrate solution every four hours. From Niemeyer, Pérez, Zamorano, and Rabajille (unpublished).

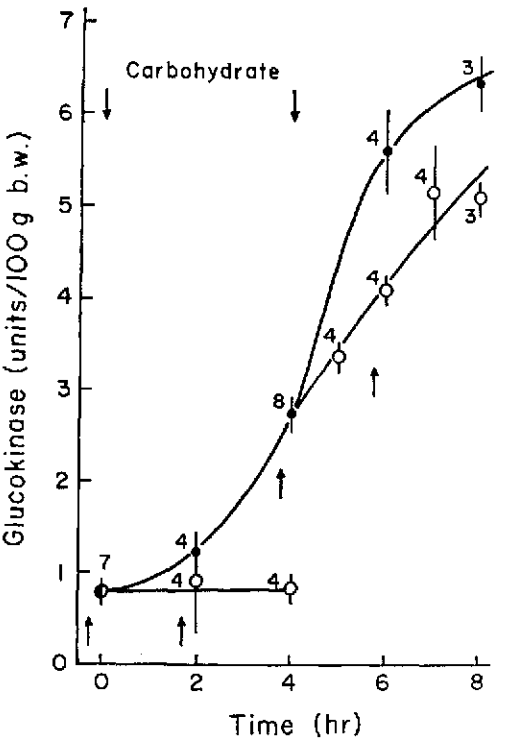


Figure 8. Effect of epinephrine on glucokinase induction. Rats fed for six days with a carbohydrate-free diet received carbohydrate by stomach tube every four hours. ○ = rats treated with epinephrine subcutaneously, starting at the initiation or after four hours of induction. From Ureta, Radojković, and Niemeyer (38).

with cortisol, thyroxin, and testosterone) are normal, the rate of induction is slower than in the sham-operated animals (Figure 7), suggesting that somatotrophin is another modulating hormone for glucokinase induction.

Glucagon and catecholamines (epinephrine, norepinephrine, isoproterenol) inhibit glucokinase induction (*24, 30, 38*). This inhibition is complete when the hormones are administered at the initiation of induction, but not if given when induction is already in progress when only a slight decrease in enzyme increase is observed (Figure 8). The effects mimic those of actinomycin D referred to above. These results may be interpreted as hormonal effects at the transcriptional level of protein synthesis. The effects of glucagon and catecholamines are most probably mediated through cyclic AMP, since the dibutyryl derivative of the nucleotide prevents the induction of glucokinase (*28, 38*) (Figure 9). From the work of several investigators, it can be seen that glucagon and catecholamines, as well as cyclic AMP can act as inducers of several enzymes in addition to their role in regulating enzyme activity (*42, 43*). The inhibition of glucose-mediated glucokinase induction constitutes, perhaps, the first example of the selective repression of enzymes by those agents.

Glucagon and catecholamines can be considered to act antagonistically to insulin, and it is reasonable at present to accept the fact that this antagonism in the metabolism of the target cells may be exerted through the participation of cyclic AMP. In the case of the regulation of enzyme synthesis, there appears also to exist an antagonism between insulin, on one hand, and glucagon and catecholamines on the other.

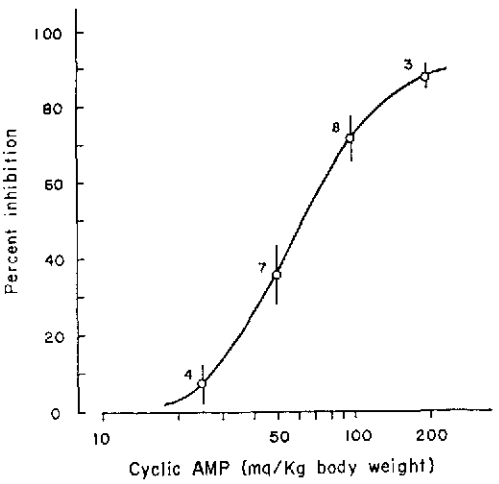


Figure 9. Inhibition by various doses of dibutyryl cyclic AMP of glucose-mediated glucokinase induction. Rats fed for six days with a carbohydrate-free diet were given a carbohydrate solution every four hours and killed eight hours after initiation of this treatment. Some of the animals were injected with a single dose of the cyclic AMP derivative by the intraperitoneal route. From Ureta, Radojković, and Niemeyer (38).

Thus glucagon, catecholamines, and cyclic AMP (or the dibutyryl derivative) act as inducers of phosphoenol pyruvate carboxykinase, and insulin blocks their actions acting as a repressor (*42, 43*). Their effects are the opposite in the case of glucokinase. There is one exception, and that is liver tyrosine aminotransferase, which is induced, probably through different mechanisms, by glucagon, epinephrine, and cyclic AMP, as well as by insulin (*10, 11, 42*).

The mechanism of the interplay of the three hormones in the case of glucokinase induction is complicated in the intact animal by the fact that there are interactions of the hormones in the liver cells as well as in the pancreas β-cells. The secretion of insulin is under the control of blood concentrations of glucose, epinephrine, and glucagon (*3, 9, 12, 31*). Under normal conditions, epinephrine inhibits insulin secretion acting via α-receptor interaction, its effect being prevented by α-adrenergic blocking agents (*31*). On the other hand, glucagon, the activation of β-receptors, and various experimental conditions that lead to an increase in the intracellular concentration of cyclic AMP, prompt insulin secretion (*12, 31*). As insulin is an obligatory requirement for glucokinase induction, it could be postulated that part of the repressive effect of catecholamines is mediated through a mechanism involving a blockade of insulin secretion (*38*). Insulin counteracts about 50 per cent of the inhibition by epinephrine of glucokinase induction (*38*). On the other hand, the inhibition caused by glucagon (*24*), or by dibutyryl cyclic AMP (*38*) is not reversed by insulin. If catecholamines and glucagon, on one side, and insulin on the other, interact in the liver cell to regulate the synthesis of specific proteins through modifications in the concentration of cyclic AMP, it is puzzling that insulin does not counteract the inhibition of glucokinase induction by glucagon and does not reverse completely the inhibition by epinephrine. The fact that the inhibition caused by dibutyryl cyclic AMP is not counteracted by insulin would be in line with the idea that there is no neutralization of the nucleotide action by insulin or by any substance produced under the hormone influence, as has been postulated to explain other observations (*4*).

In view of the extreme complexity of the whole animal that makes the interpretation of several observations very difficult, it appears highly desirable to test hormones and substrate in a more simple system, such as the isolated, perfused liver, or cells in culture. Unfortunately our efforts, as well as those of others in the field, have been unsuccessful until now in developing *in vitro* systems capable of inducing glucokinase.

Several years ago, studies of the adaptation of animals to variable diets at the enzyme level were begun, and attempts to understand the underlying mechanisms have involved us in the interaction of hormones in the regulation of enzyme synthesis.

Acknowledgments

The work reported in this paper was made possible by the cooperation of many persons

who are co-authors of various cited reports, published and unpublished. The author thanks Miss Elsa Rivera for the drawing of the illustrations.

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THE PHYSIOLOGICAL SIGNIFICANCE OF TISSUE ENZYME ACTIVITIES AS AFFECTED BY DIET

Guillermo Soberón

Introduction

Living organisms take substances from the environment and submit them to sequential transformations through chemical reactions catalyzed by enzymes. The products of the transformation are eventually excreted into the surrounding medium. As a result of such a process —intermediary metabolism—the energy contained in chemical bonds is released and partially trapped by specific mechanisms, thus becoming available for use when required by the biological system. In addition, some of the metabolites formed serve as the building blocks used by the organisms to construct their own material. Hence, nutrients taken from the outside have great importance for the growth, functioning, reproduction, and survival of living organisms.

A given chemical reaction takes place in a biological system depending on the presence of a specific enzyme. The enzyme, in turn, can be produced by the system if it possesses the corresponding information in its genome, and if this can be expressed. Thus, the genetic information indicates a potentiality to carry out certain chemical reactions, but does not, by any means, imply that such reactions will be performed. This possibility is under the command of mechanisms belonging to the process known as metabolic regulation. The process involves, in addition to the transcription of the genetic information, other levels of control where a key role is played by metabolites and nutrients that give rise to them. Metabolic regulation is mandatory for a biological system to function efficiently and harmoniously.

During the past two decades, many outstanding contributions have been made which are advancing our understanding of how biological systems operate. Some basic concepts pertaining to metabolic regulation have been worked out employing microorganisms. These have been utilized in view of their relative simplicity compared to higher organisms. However, there has been an all too frequent tendency to extrapolate indiscriminately concepts developed from microorganisms to explain adjustments in the highly complicated machinery for the regulation of the intermediary metabolism of higher organisms.

Schimke (*12*), among others, has repeatedly introduced a note of caution in this respect. He has objected to the careless use of terms such as *induction* and *repression* to indicate increases and decreases of enzyme activity, without being sure, in many cases, that they obey mechanisms well-established in bacterial physiology. There are, for example, significant differences between the prokariot and eukariot organisms. Prokariots have their naked genome in direct contact with the cytoplasm; during their exponential growth, proteins do not turn over, they are synthesized, and the "excesses" are diluted by the increasing cell mass and numbers;

the messenger ribonucleic acids (mRNAs) are generally short lived; in many instances the genes responsible for the synthesis of enzymes participating in a given metabolic path are clustered in the genome. In contrast, eukariots have their genome mainly confined to the nucleus (functioning deoxyribonucleic acid (DNA) has been localized in other subcellular particles); their protein turnover and excesses are disposed of by catabolic breakdown; the ribonucleic acids (RNAs) generally have a long life, and clusters of genes linked in metabolic paths are not frequent. In animal cells there is a rapid turnover of intranuclear RNA and DNA has become redundant to a great extent. Cell differentiation has brought about homeostasis, therefore, it has been necessary to put into operation supracellular mechanisms of metabolic regulation, namely, the central nervous, and the endocrine systems. It suffices to say, in connection with the matter being discussed, that the increasing complexity built up by biological evolution has made it mandatory for higher organisms to resort to other levels of regulation and organization than those found in simple microorganisms. Hence, even though the information obtained from these cannot be directly extrapolated from the former, it can provide solid grounds for a starting point for a better knowledge of mechanisms involved in their metabolic control.

There is another difference between microorganisms and higher organisms that it is important to point out. Microorganisms take up nutrients from an environment to which they are constantly exposed, while higher organisms have developed a pattern of intermittent feeding. Thus, they have to cope with the stress of a load of nutrients to which they are periodically subjected.

The changes in enzyme activities of living organisms provoked by influences originating in the environment are referred to as metabolic adaptation. It is well-substantiated that the quality and quantity of ingested food produces changes in the enzyme activities of different tissues and organs. It is beyond the aim of this paper to review them extensively. Instead, consideration will be given to some aspects of the variations of tissue enzyme activities, bearing more on their physiological significance, and on the limitations of interpretation of *in vitro* studies toward an understanding of the function of the intact animal. It should be kept in mind that the effects of a lack of or an excess of nutrients and composition of diet are intimately interconnected with influences exerted by neural and hormonal factors and these are difficult to distinguish. The direct participation of neural factors in the regulation of tissue enzyme activities is proved by the work of Shimazu and Fukuda (*119*), who found that the stimulation of the sympathetic system caused an increase in the activities of hepatic phosphorylase and glucose-6-phosphatase, while the excitation of the parasympathetic system increased the activity of glycogen synthetase. Both systems counteract each other with respect to the effects produced. The indirect participation of the central nervous system, through its connection with the endocrine system, is an obvious one, since hormones are effective agents in the control of enzyme activities. We will have more to say regarding hormonal influences, particularly in so far as they might affect a given dietary action; however, we will restrict ourselves fundamentally to the effects of the quality and quantity of the diet on enzyme activities.

The review made in 1956 by Knox, Auerbach, and Lin (*55*) brought together information available at that time and caused a great deal of interest in the subject. Again, Knox and Greengard (*56*) made another thorough revision of the matter in 1965. Very recently Schimke (*112*) collected information relevant to the control of enzyme levels in animal tissues, thus bringing into sharp focus the problems faced at the present time. These three papers constitute an excellent reference for those who wish to learn more on the topic.

Dietary stimulus capable of producing changes in enzyme activities

Modifications in the composition of the diet by increasing or decreasing its constituents cause alteration in some enzyme activities. Another experimental model that has been extensively studied is the effect of starvation followed by refeeding. Some changes observed under these particular conditions will be described before considering the effects of variations in dietary protein, carbohydrate and fat, vitamins and minerals.

Starvation and refeeding

When an animal is starved or fed with a protein-free diet, there is a small fraction of the total body protein that is rapidly lost and rapidly regained when fed with an adequate diet. Such a fraction has been called labile body protein (*77*). It has been recognized that liver, pancreas, and intestinal mucosa are important sites of labile protein deposition, while other organs such as muscle do not respond as readily to protein deprivation, and still others, such as brain, are almost unaffected (*77*). Liver is directly exposed to the flow of nutrients absorbed from the intestine, therefore it is subjected to more stimulus than other tissues and has a greater need of metabolic adjustments. Since it has been more extensively studied than other sites, reference will be made mainly to hepatic changes.

Fasting causes a rapid mobilization of liver protein content which tends to level off after 24 hours (*124*), perhaps at a time that coincides with the adjustment of urinary nitrogen output to the endogenous level established when dietary protein is completely withdrawn (*77*). There is another period of rapid loss of protein just prior to the death of an animal that inevitably ensues if food is not allowed (*124*).

The mobilization of protein is accompanied by a removal of RNA and phospholipids; endoplasmic reticulum becomes less abundant. These changes can be seen a few hours after the initiation of fasting, and they are, moreover, restored quite rapidly in relation to the elevated intracellular concentration of amino acids and other nutrients after food consumption (*77*).

Catalytic proteins should be part of the labile protein, since enzyme activities decrease sharply during starvation and a protein-free diet. More careful analysis has shown that during fasting, not all enzyme activities decrease at the same rate: some seem to be spared and others are, in fact, augmented (*11, 124*). By the same token, when an animal is refed, not all enzyme activities are restored at the same rate; some do not increase, but, in fact, decrease during the refeeding period (*11, 124*). Of note is the case of glucose-6-phosphate dehydrogenase, whose activity upon refeeding reaches levels much greater than those seen in a normal animal (*15, 134, 135, 136, 137*).

No attempt will be made to mention every enzyme whose variation in activity has been detected during starvation and refeeding. A few changes that seem to follow from the particular metabolic condition introduced by the stress of starvation and refeeding will be mentioned. For instance, those enzymes whose activity has been considered as determining the flow of metabolites from glucose to pyruvate (glucokinase, phosphofructose kinase, and pyruvic kinase) diminish during fasting and increase upon refeeding, while the activities of enzymes involved in shifting the flow of metabolites from pyruvate to glucose (pyruvic carboxylase, phosphoenol pyruvic carboxylase, fructose-1-6-diphosphatase, and glucose-6-phosphatase) increase by fasting. These changes are in accordance with the predominance of gluconeogenesis and diminution of glycolysis during fasting, and the reversal of this situation is noted when the animal is refed (*104, 117, 145, 146*).

Enzymes intervening in lipogenesis also change in a similar fashion, that is to say, they decrease during fasting and recover upon refeeding. In addition to the change in glucose-6-phosphate dehydrogenase already mentioned, a similar variation has been noted in 6-phospho-

gluconic dehydrogenase, citric acid cleavage enzyme, acetyl CoA carboxylase, fatty acid synthetase and long-chain fatty acid desaturase (*2, 35*). The driving force in lipogenesis of nicotinamide adenine dinucleotide phosphate (NADPH) generation by the two first enzymes has been emphasized (*136, 137*). Concomitant with the changes described, it is found that there is lipid deposition in the livers of animals refed after a starvation period (*124*). Similarly, it should be mentioned that hepatoma 7794-A does not respond to the stimulus of fasting and refeeding with respect to the increase in glucose-6-phosphate dehydrogenase seen in normal liver (*145*).

Starvation causes an increased protein and amino acid catabolism. Correspondingly, there is an augmentation in the activities of carbamyl phosphate synthetase, ornithine transcarbamylase, arginino succinic synthetase, arginino succinase and arginase—enzymes that participate in urea biosynthesis (*108, 109*). There also is an augmentation in the activity of glutamic oxaloacetic transaminase (*124, 141*) and glutamic pyruvic transaminase (*79, 101, 124*)—enzymes ancillary to the urea cycle. The feeding of casein hydrolysate to the starved rat greatly increases the activity of serine dehydratase (*89*). Glutamic dehydrogenase changes in a manner parallel to the change in liver weight (*42*).

The recovery of enzyme activities that occurs when the starved animal is refed has been judged, in many instances to be due to *de novo* protein synthesis, because inhibitors of this process prevent the observed increase in enzyme activity. The use of actinomycin D, puromycin and ethionine, as well as the reversal of the effect of the latter by methionine, have been widely used (*27, 34, 35, 104, 117, 136, 147*).

First, the quality of the dietary protein used to feed the fasted animal is of great importance in determining the pattern of recovery of the enzymes affected. Secondly, the nutritional status of the animal is fundamental for a deficient diet to have an effect on the recovery of enzymes under conditions of rapid liver growth. These two statements are substantiated by a study carried out in our laboratory where the activity of eight hepatic enzymes was followed under two different anabolic conditions: rapid liver growth obtained when a previously starved animal is refed (*124*), and liver regeneration after partial hepatectomy (*105*). In both situations, the effect of feeding zein, a protein of low biological value, deficient in tryptophan and lysine, was explored in comparison with the feeding of casein, a good protein. In the depletion-repletion system it was found that all enzyme activities recovered in relation to protein content. Thus, although the total activity was lower in animals fed with the deficient diet, the specific activity expressed as given units per milligram of protein, was the same in both groups. The deficient diet prevented the liver lipid deposition seen with the casein diet. No difference whatsoever was detected in the regenerating livers of the animals fed either diet. It was necessary for the animals to be fed the deficient diet prior to hepatectomy in order to see differences. These findings indicate that in the event of limiting amino acids, hepatic enzyme activities recover to about the same extent, in relationship to one another, as they do under conditions where amino acids are not limiting. It is likely that the amino acids missing in the diet can be made available to the liver from the catabolism of proteins from other tissues. Indeed, it has been demonstrated that protein is redistributed among different tissues depending on the prevailing metabolic situation. Mobilization of muscle labile protein, with a flow of amino acids toward the liver and consequent deposition of labile hepatic protein, follows the administration of catabolic hormones (thyroxin and corticoids) while the reverse flow is favored by anabolic hormones (growth hormone and androgens) (*77*).

Other tissue that readily responds to the stress of fasting is adipose tissue. Under this condition the incorporation of labeled acetate into fatty acids is diminished and there is a decrease in RNA synthesis—refeeding restores both ca-

pacities to normal values (*34*). Jejunal glycolytic enzymes change during starvation in the same way that the corresponding liver activities change (*127*).

Variations in dietary protein, carbohydrate, fat, vitamins, and minerals

Protein-free diet mobilizes labile protein, therefore, the changes in enzyme activities resemble somehow those seen in starvation, although they are less pronounced. The administration of carbohydrate, however, has a definite specific effect on protein tissue redistribution, mediated probably through the release of insulin, hence, the flow goes from liver to muscle (*77*). A decrease in urea biosynthesis enzymes is observed after the administration of a protein-free diet (*30, 109, 110*); glutamic dehydrogenase and glutamine synthetase also diminish (*30*). The effect of lack of protein in the diet is very striking on xanthine oxidase which reaches very low levels of activity (*73*). A low-protein diet (containing 2 per cent casein) increases the activity of 3-phosphoglycerate dehydrogenase and phosphoserine phosphatase (enzymes involved in the phosphorylated path of serine formation), while decreasing the activity of serine dehydratase (*23, 24*).

A high-protein diet produces an augmentation of digestive proteolitic enzymes (*40, 67*) with little or no effect on lipase and amilase, which has been attributed to increased synthesis and secretion by the pancreas (*67*).

A high-protein diet also produces an increase in several activities involved in amino acid catabolism: serine dehydratase (*23, 43, 87, 88*), glutamic oxaloacetic transaminase (*65, 79, 87*), glutamic pyruvic transaminase (*43, 65, 79*), tyrosine transaminase (*65, 85, 87, 88*), tryptophan pyrrolase (*88*), ornithine transaminase (*46, 85, 88, 130*), carbamyl phosphate synthetase (*110, 111*), ornithine transcarbamylase (*108, 110, 111*), arginino succinic synthetase, arginino succinase, and arginase (*109, 110, 111*), histidine pyruvate transaminase, histidase, and uricase (*114*). There is also indirect evidence that the increased resistance to ammonia intoxication of rats fed with a high-protein diet might be due to an increase in amino acid catabolic enzymes (*154*). Other enzymes related to carbohydrate metabolism like glucose-6-phosphatase, fructose-1-6-diphosphatase, malic dehydrogenase and sorbitol dehydrogenase, although less responsive than the above-mentioned, still increase after feeding the animal with a high-protein diet (*43*).

Some individual amino acids have specific effects: tryptophan increases the activities of tryptophan pyrrolase and tyrosine transaminase (*53, 112*). Tryptophan analogues and other indol compounds have the same effect (*39, 57, 100*). Serine increases the activity of serine dehydratase (*128*), an effect more readily seen employing a mixture of amino acids from casein hydrolysate (*86, 88, 90, 91*). Serine and glycine diminish the activity of 3-phosphoglycerate dehydrogenase (*128*). Ornithine increases the activity of ornithine transaminase (*12*). Creatine, a nitrogenous compound derived from amino acid metabolism, decreases the activity of glycine transaminase (*142*).

A high-carbohydrate diet increases pancreatic amilase activity while it decreases the proteolytic activity, but it does not have any effect on lipase (*40*). Such a diet increases the activities of liver pyruvic kinase (*59, 133*), glucose-6-phosphate dehydrogenase (*136*), and citrate cleavage enzyme in previously fasted rats (*58*). Effects of specific sugars have also been noted. The administration of glucose causes an augmentation of glucokinase and of fructose-1-6-diphosphatase (*80, 90, 104*), and increases the incorporation of leucine C^{14} into tissue beta plus pre beta lipoproteins, with a corresponding change of the electrophoretic pattern of plasma lipoproteins (*21, 22*). The feeding of sucrose or fructose and of glycerol increases the activity of hepatic pyruvate kinase, glucose-6-phosphatase, and fructose-1-6-diphosphatase while decreasing the activity of glucokinase and phosphofructokinase (*121, 122, 147*). Glucose, fructose, or sucrose increase glucose-6-phosphate dehydrogenase in the

starved animal (*15*). Glucose interferes with the enhancing effect of casein hydrolyzate on serine dehydratase in the starving animal (*88, 90*).

Individual sugars in the diet also have specific effects on certain jejunal glycolytic enzymes: fructose increases fructokinase and glucose increases glucokinase and hexokinase (*102, 127*).

The administration of a fat-free diet to starved rats increases the activity of fatty acid synthetase (*10*) and acetyl CoA carboxylase (*72*). A high-fat dietary content causes a lowering of the activities of pancreatic amilase and lipase but proteolytic activity is not affected (*40*). The feeding of cholesterol (*123*) or ubiquinone (*60*) greatly decreases the synthesis of this compound by interfering in the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonic acid.

It has been noted that vitamin-deficient diets have definite effects on the corresponding enzymes. Pyridoxin deficiency causes a diminution of the activities of threonine dehydrase (*38*), glutamic pyruvic transaminase, glutamic tyrosine transaminase (*79, 100*), cysteine sulfinate decarboxylase, cystathionase and aromatic decarboxylase (*56*). Riboflavin deficiency produces a decrease in xanthine oxidase and D-amino acid oxidase (*9*) and flavokinase (*25*). Biotin-deficient rats have a decreased content of carcass cholesterol, but not liver cholesterol; esterified fatty acids are also lowered, particularly in adipose tissue, and in the carcass (*17*). The effect of vitamin K deficiency on the synthesis of prothrombine and other clotting enzymes is well substantiated (*81, 82*). It is well recognized that a given vitamin deficiency affects differently enzymes that function with the corresponding coenzyme derived from that vitamin.

The administration of pyridoxin causes a doubling of tyrosine transaminase (*39*), nicotinamide increases the tissue content of pyridine nucleotides, but the activities of glutamic dehydrogenase, lactic dehydrogenase and glucose-6-phosphate dehydrogenase are not affected (*42*).

A dietary deficiency of Mn^{++} lowers arginase activity which is not restored by the addition of the metal *in vitro* (*5*).

Iron deficiency causes an increase in the copper tissue concentration and a decrease in monoamide oxidase (*131*).

Effect of the feeding schedule

After digestion of the ingested food and absorption of the nutrients from the gut, these become metabolites that flow through the organism. Changes in the metabolic machinery render it able to cope with the transient load of nutrients which, by means of complex homeostatic mechanisms, are returned to the concentrations prevailing in the steady state of the postabsorptive period. Adjustment to new metabolic conditions applies, of course, to enzyme activities, the entities responsible for transforming the metabolites.

Therefore, it is easy to understand that food ingested periodically by animals should produce differences in tissue enzyme activity as compared with animlas fed *ad libitum*. Studies carried out in the laboratory of Potter (*92, 93, 94, 95*) clearly show that such is the case. Rats kept under very careful conditions of constant temperature and well-timed alternate periods of light and dark are trained to feed according to different schedules: 8 hours and 16 hours, 12 hours and 36 hours, or 8 hours and 40 hours, respectively, for alternate periods of feeding and fasting. Under these conditions, variation in several enzyme activities and other biochemical parameters has been shown. However, the oscillations are not necessarily coincident as one might be led to believe as though they were dependent on the same factor. To make this point clear, it is worth describing the findings of the 8+40 schedule. Stomach weight (including content), liver weight, and glycogen deposition show increases correlated with food intake; influx to and aflux from the liver of cycloleucine (probably an indication of variations in the amino acid pool), tyrosine transaminase activity, and the concentration of plasma corticosterone are related to each other, but not clearly to food intake; serine dehydratase, glucose-6-phosphate dehydrogenase, and citrate

cleavage enzyme oscillate similarly among themselves, but differently in relation to the other parameters described; synthesis of RNA and of its precursors seems to be related to the pattern of food intake. By feeding training it has been possible to shift the peak of maximal activity of certain enzymes (*143*).

The daily variations of tyrosine transaminase have attracted the interest of several research groups. The enzyme activity cycle is clearly seen in the animals fed *ad libitum* (*33*); it persists in constant darkness with unchanged amplitude (*4*), but a rhythm with reduced amplitude remains after exposure to one week of constant light (*4*). The peak of activity persists after 10 hours of fasting in rats fed *ad libitum* (*4*), however, in the 8+40 feeding schedule of Potter (*92*), there are two peaks of activity, one clearly corresponding to the fasting period. It is of interest that no rise in activity appears if the period without food is extended. Protein-free diet prevents the cyclic variation of tyrosine transaminase, and apparently tryptophan is involved, since the feeding of an amino acid mixture devoid of tryptophan also suppresses the rhythm of enzyme activity (*159*). It has been suggested that the oscillation of the enzyme activity corresponds to a daily cycle of increase of polysome aggregation which depends on amino acid influx, principally tryptophan (*29*). This proposition is difficult to reconcile with the second peak of activity during the fasting period of 8+40 feeding schedule of Potter (*92*). There has been a great deal of discussion concerning the "true circadian" nature of tyrosine transaminase cyclic variations. In this respect, Potter (*92*) has stated, "The shifts in tyrosine transaminase activity can only be looked upon as a response to an ordered succession of cyclic changes in the levels of amino acids, corticosterone, glucagon and insulin, and possibly epinephrine and norepinephrine, associated with behavioral responses to the light-dark cycle. It seems likely that the circadian rhythms in actual life are a blend of internal and external factors."

Another experimental model has been studied which clearly shows the type of metabolic adjustments produced by intermittent feeding, that is, the meal-fed rats, whereby the animals are trained to ingest food for one or two hours daily (*64, 132*). Infrequent feeding develops hyperphagia, that is to say, the ability to consume large amounts of food within a short time interval (*6*). Other changes that follow are: hypertrophy of the digestive tract which accounts for greater digestive capability and enhanced intestinal absorption (*63*), high respiratory quotient after carbohydrate ingestion (*6*), and increased ability of hepatic and adipose tissue to synthesize fatty acids *in vitro* (*6, 62*). The hyperlipogenesis is accompanied by an increased activity of many enzymes involved in the conversion of glucose to fatty acid and glycerophosphate (*13*). Rats fed two hours daily or refed after fasting have more lipocytes in adipose tissue; the DNA content of these cells is greater, and they incorporate more thymidine $2C^{14}$. There is an increased sensitivity of adipose tissue to insulin and epinephrine, and the adenyl cyclase activity of lipocyte ghosts is augmented (*6*). Meal-fed rats also have a greater capacity to accumulate glycogen in muscle and in adipose tissue than nibbling animals which is reflected in the activity of the glycogen-synthesizing enzymes in both tissues (*158*).

Some factors influencing the effect of diet on tissue enzyme activities

In studying the changes in enzyme activities provoked by dietary variations, it is necessary to take into account some factors that may alter the response. It would seem to be desirable to mention them at this point.

Endocrine nutritional interactions

It has been previously stated that the endocrine system plays a very important role in the metabolic regulation of higher organisms. Some of the changes described previously are also under the command of hormones, therefore dietary stimulus might depend on the endocrine status, and conversely a given effect caused by

a hormone might depend on the nutritional conditions.

Glucocorticoid increases the amount of arginase (*3*), glutamic pyruvic transaminase (*115*), tryptophan pyrrolase (*11, 57*), tyrosine transaminase (*48, 49*), ornithine transaminase (*85, 130*), glucose-6-phosphatase, fructose-1-6-diphosphatase, phosphoenol pyruvic carboxylase, and pyruvic carboxylase (*146*). Insulin increases the activities of uridine diphosphate glucose (UDPG)-glycogen synthetase (*139*), glucokinase (*80, 104*), phosphofructokinase, and pyruvic kinase (*146*), tyrosine transaminase (*49*), and glucose-6-phosphate dehydrogenase (*135*). Glucagon increases the activities of serine dehydratase (*44*), tyrosine transaminase (*49*), adenyl cyclase, hence through cyclic adenosine monophosphate (AMP) formation, it increases phosphorylase (*129*). Adrenalin acts similarly on the latter system (*129*). Thyroid hormone increases the activity of L-glycerophosphate dehydrogenase, and malate dehydrogenase (*61*).

The endocrine-nutritional interactions are illustrated by the work of Freedland: the effect of glucocorticoids on amino acid metabolizing enzymes and a few others depends on the dietary protein (*43*). Conversely, in general, adrenalectomy and hypophysectomy diminish the amplitude of the response of enzymes that normally increase or decrease with the feeding of a high-protein diet (*31*). Other enzymes that normally do not vary with the high-protein diet have rather erratic responses after endocrinectomy (*31*).

Liver malate dehydrogenase (decarboxylating) increases more and is more sensitive to thyroxin in rats previously fed with a high-carbohydrate diet than in those fed with a high-fat diet (*41*). The fall in enzyme activities as a result of starvation is more striking in hypophysectomized rats (*146*). Similarly, the fasting of diabetic rats has a more pronounced effect on ribonucleic acid (RNA) synthesis, and lipogenesis (*34*), and on those enzyme activities directly committed to gluconeogenesis (*153*). The effect of cortisol and starvation on tyrosine transaminase depends on the basal activity of the enzyme which, in turn, is related to the previous dietary experience. The basal activity is high when the animal is fed with a high-protein diet and does not increase after cortisol. Starvation produces a decline in the enzyme activity of rats previously fed a high-protein diet, but produces an augmentation in those previously fed a high-carbohydrate diet (*75*).

Glutamic pyruvic transaminase requires adequate stores of pyridoxal phosphate *in vivo* to obtain maximal response when increased by cortisol, however tyrosine transaminase has normal levels and can be increased readily in rats severely depleted of pyridoxin (*79, 100*). Tyrosine transaminase increases by exposing the fasted animal to the cold, but it does not increase by applying the same stimulus to the recently fed animal (*27*).

Glucose alone increases glucokinase, citrate cleaving enzyme, and glucose-6-phosphate dehydrogenase in the intact animal, but glucose and insulin are required to produce the same change in the diabetic animal (*88*).

Adrenalectomy prevents the effect of refeeding by the increase of glucose-6-phosphate dehydrogenase in the fasted animal (*94*).

Species and strain

Taking labile body protein as representative of the type of response that can be obtained by challenging the animal with a dietary stress (starvation or a protein-free diet), the following considerations made by Munro (*77*) are pertinent to emphasize the diverse results that might be observed in different species: "The relative importance of labile body protein in relation to other aspects of protein metabolism is likely to differ in species of different body size . . . it was concluded that the proportion of body protein which is labile is about the same in the rat as in man. The evidence we shall now consider shows that the intensity of total protein metabolism in the rat is five times greater than in man. Consequently, labile body protein will assume greater importance in the economy of protein metabolism in man than in the rat."

To illustrate the point of species differences, it can be said that the administration of xanthine to mice causes an increase of 70 per cent in xanthine oxidase activity, but rats respond rather poorly to the same stimulus (*19*). There are also differences due to the strain of the experimental animals used. The administration of a diet containing 25 per cent cooked, dried egg produces kidney damage, esteatosis, hyperlipemia, and hypercholestoremia in the BHE rats but not in the Wistar rats.

Age of the experimental animal

The level of enzyme activities varies with age, so does their responsiveness to change under the influence of different stimuli. For instance, tryptophan pyrrolase increases in the mature rat so that at 400 days of age the activity has doubled in comparison to the activity found in animals 38 days old (*65*). The fasting levels of liver tyrosine transaminase are lower in senescent mice than in young ones. The same enzyme increases after the feeding of a diet containing five per cent tyrosine in rats of seven weeks of age, but mature animals of 15 weeks of age do not respond at all (*53*).

The importance of tissue enzyme activities as they change with age in relation to longevity has been emphasized in a recent study by Ross (*103*). He determined the pattern of change of four enzymes: Alkaline phosphatase, histidase, ATPase, and catalase in relation to age from 25 to 995 days. A correlation was found between the activities on one side and life expectancy and the incidence of disease on the other. Very importantly, when the activity of the enzymes was varied by dietary means so that they approached the pattern seen in the young rat, a significant increase in life expectancy was also observed.

Organ differences

It is well known that starvation and protein-deficient diets have a differential effect; whereas great changes are produced in liver (*124*), brain is less influenced (*140*). Starvation and diabetes cause inhibition of fatty acid synthesis in liver but do not affect the heart. Neither have any effect on the nicotinamide adenine dinucleotide/NAD (NADH/NAD) ratio that regulates heart lipogenesis, nor on the products of the process. Hence the heart mitochondrial system that accounts for 90 per cent of fatty acid synthesis is regulated by different mechanisms and is insulated from nutritional and metabolic conditions which profoundly influence overall lipogenesis in the whole animal (*156*).

Liver, brain, and kidney contain 3-phosphoglycerate dehydrogenase and the other enzymes for the phosphorylated path of serine production. D-Glycerate dehydrogenase, the enzyme of the non-phosphorylated path, is present only in liver and kidney. The elevation in 3-phosphoglycerate dehydrogenase obtained by feeding a diet containing 2 per cent protein is seen only in liver and corresponds to a diminution in the cysteine, methionine, and taurine content that appears only in liver. Supplementation with methionine or cysteine removes the change (*24*).

It has been found that the ratio of aldolases, the one acting on fructose-1-6-diphosphate to that using fructose-1-phosphate as substrate, remains close to one in the liver of animals subjected to different experimental conditions, whereas in jejunum, the ratio can be changed to be less than one by dietary means (*127*). Ubiquinone inhibits sterol synthesis by affecting the reduction of β-hydroxy-β-methylglutaryl CoA of the liver, but has no effect on kidney or intestine (*60*). Liver glucokinase and pyruvate kinase differ in physical, kinetic, and regulatory properties from that found in other tissues (*112*). The rate of degradation of lactic dehydrogenase isozyme-5 varies in liver, skeletal muscle, and heart muscle (*32*).

Although many more examples of organ differences may be given (*61*), those mentioned above should suffice to illustrate the point that different tissues may contain different isozymes, and also that the same protein in different surroundings may be subjected to different regulatory mechanisms.

Mechanisms of changes in enzyme activities caused by diet

Higher organisms have the peculiarity that their proteins turn over. Thus, a variation in a given enzyme activity may be a consequence of a change in the steady-state concentration of the specific protein being considered to a different steady-state concentration. The shift may be the result of a modification in the rate of synthesis, or in the rate of degradation, or a combination of both (*112*). The variation of activity might also be due to other factors, such as the concentration of activators, inhibitors, and coenzymes which affect the catalytic action (*124*). Redistribution of enzymes among subcellular fractions should also be considered (*18, 124*).

The relation of enzyme synthesis and degradation to the nutritional status will be dealt with here, but rather briefly, because the subject has been covered thoroughly by Dr. Schimke. It is conceivable that diet may alter enzyme activities in several ways.

Non-specific effects on protein synthesis

Amino acids supplied in the diet may serve as building blocks for the synthesis of protein. Although many of the 20 species that participate in protein synthesis can be readily produced by intermediary metabolism, there are several, the indispensable amino acids, that have to be provided by the ingested food. Under certain circumstances, the amino acid pool becomes limited when certain enzymes are increased by the administration of hydrocortisone (*43*), as suggested by the increasing effect on the activity of higher dietary protein content. Protein synthesis is a highly endergonic process, therefore it should be hampered by a shortage of calories.

Muno (*78*) has shown that a flow of an adequate mixture of amino acids to the liver is determinant in preserving the protein biosynthetic machinery in working condition. The feeding of a deficient mixture causes a loss of heavier polysomes with accumulation of oligosomes and also of ribosome subunits. These smaller particles are more suitable to the action of ribonuclease, which, though remaining latent in the intact particles, becomes activated upon disintegration. The administration of a complete amino acid mixture causes aggregations of ribosome sub-units and of oligosomes to produce polysomes. It has been further demonstrated that the deficiency of tryptophan is fundamental for causing polysome disaggregation (*78*). However, Shaw and Fillios (*118*) have noted that soon after the administration of a free- or low-protein diet one perceives an increased incorporation of labeled amino acids into protein as well as an augmented activity of RNA polymerase Mg^{++} dependent, while the Mn^{++} dependent remains unchanged. Furthermore, a shift in the ribosomal profile characterized by the aggregation of monosomes to produce polysomes, and an increased incorporation of labeled orotic acid into RNA was noted. This type of response was rather transient, since at a later time the situation was found to be similar to that referred to above (*78*). At any rate, in both cases, a correlation between amino acid incorporation and the status of polysome aggregation was found.

Specific effects on protein synthesis and degradation

When a change of enzyme activity is detected, the first question is whether the variation corresponds to an alteration in the amount of a specific protein. In answer, it can be shown by direct proof that more specific protein has been synthesized. Purification of the specific protein permits its quantification. Alternatively, purification of protein allows the preparation of a specific antibody for the isolation of the antigen antibody complex and by this means the quantification of the given protein. In both cases it is possible to determine the incorporation of labeled amino acids into the specific protein in comparison with the general incorporation in all proteins, information that is also relevant to the specificity of given changes.

It has been demonstrated that among the enzymes that are changed by dietary stimulus,

increases in the activities of arginase (*108, 111*), ornithine transcarbamylase (*108*), acetyl CoA carboxylase (*72*), tyrosine transaminase (*48, 100*), glutamic alanine transaminase (*116*), tryptophan pyrrolase (*26, 113*), serine dehydratase (*44*), and glucose-6-phosphate dehydrogenase (*161*) correspond to increases in the absolute amount of the corresponding proteins.

An increase in the amount of a given protein can be a consequence of either an increase in the rate of its synthesis or a decrease in the rate of its degradation (*112, 115*). Indeed, it has been found that both levels are operative in animal tissues. For instance, the increase in arginase activity induced by a high-protein diet is due to an augmented rate of synthesis, but the increase caused by starvation is induced by a drop in the rate of degradation to essentially zero (*112*). The shift from a high- to a low-protein diet lowers the amount of arginase by the combination of both a decreased rate of synthesis and an increased rate of degradation (*112*). Similarly the difference of acetyl CoA carboxylase in animals fed a fat-free diet or starved is due to a combination of both factors; increased rate of synthesis in the former and increased rate of degradation in the fasted without any apparent connection between them (*72*). The case of tryptophan pyrrolase has been clarified in different laboratories (*3, 26, 39, 54, 113, 126*). The increase observed after glucocorticoid administration is the result of an increased rate of synthesis, but the augmentation produced by the administration of tryptophan is caused by a decreased rate of degradation. Knox (*54*) has proposed the following scheme for the regulation of the enzyme. The association of L-tryptophan with the apoenzyme renders it more suitable to combine with hematin. Interestingly enough, there appears to be a reciprocal control in the synthesis of the tryptophan pyrrolase and heme. The increased synthesis of L-amino levulinic acid synthetase (which enhances heme formation) by the administration of allylisopropyl acetamide produces an increase in the tryptophan pyrrolase apoenzyme. Conversely, concomitant with the increase in apoenzyme that follows tryptophan administration, there is an increase in the activity of L-amino levulinic acid synthetase (*74*).

The effect of glucocorticoids on tyrosine transaminase and glutamic pyruvic transaminase appears to be limited only to the rate of their synthesis (*49, 50, 51*).

The increase of serine dehydratase that follows the intubation of casein hydrolysate in protein-depleted rats is due to an augmentation of the rate of synthesis (*44*). The suppressor effect of glucose results from the avoidance of the amino acid effect on the rate of synthesis, and also from an increased rate of degradation of existing serine hydratase (*44*). Glucagon also enhances the enzyme activity by increasing the rate of synthesis, an effect that is probably mediated by cyclic AMP (*45*). A similar action of glucagon on tyrosine transaminase has been described (*157*).

The decreased activities of glycine transaminase after the feeding of creatine (*142*), and the prevention of the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid by cholesterol (*123*) or ubiquinone (*60*) represents two instances of control of a metabolic path by a final product in higher organisms. To our knowledge, no others have been described. However, the operating mechanisms have not been elucidated yet, although it has been suggested that the synthesis of the enzyme is hampered.

It has been inferred, from the effect of inhibitors of protein synthesis that the change of many other enzyme activities are due to *de novo* protein synthesis. Actinomycin-D and puromycin have been greatly favored, since it is known that they act respectively at the levels of transcription and translation of the genetic information. These ideas follow the concept of the model proposed by Jacob and Monod, which has been found to operate in microorganisms. However, the interpretation of experiments where these inhibitors are employed should be looked upon very cautiously considering, first

of all, that they have toxic effects, and, therefore, other extraneous factors might come into play (*112*). In addition, the behavior of some systems where inducing agents and protein synthesis inhibitors have been employed cannot be easily understood in terms of the Jacob-Monod model. Hence, it is necessary to look for alternate explanations. Tomkins *et al.* (*138*) have proposed a model where the control is exerted by a post-transcriptional repressor, which by combining with mRNA prevents its translation and renders it susceptible to degradation.

One of the main features derived from the Jacob-Monod model is that of the coordinated variation of the enzymes clustered in the same operon. In higher organisms, coordinated variation has been found in several groups of enzymes connected metabolically. A clear example of such is that of the urea biosynthesis enzymes. Schimke (*108*) found that by varying the amount of protein in the diet, the activities of carbamyl phosphate synthetase, ornithine transcarbamylase, arginino succinic synthetase, and arginase varied in a coordinated form. In fact the variation of arginase, although following the same trend, did not quite change in the same proportion as the other enzymes did (Figure 1). The same type of coordinated variation in starvation has been found in feeding rats with a diet containing zein, and during liver regeneration after partial hepatectomy (*30*). The same type of coordinated variation in urea biosynthesis enzymes was found in our laboratory (*76*) in the steady-state concentration of animal species with a wide variety of nitrogen excretory patterns (Figure 2). Again, a very good correlation was found among the relative variations of carbamyl phosphate synthetase, ornithine transcarbamylase, and the arginine synthetase system (arginino succinic synthetase and arginino succinase assayed together); arginase, following the same tendency, somehow varied in a different

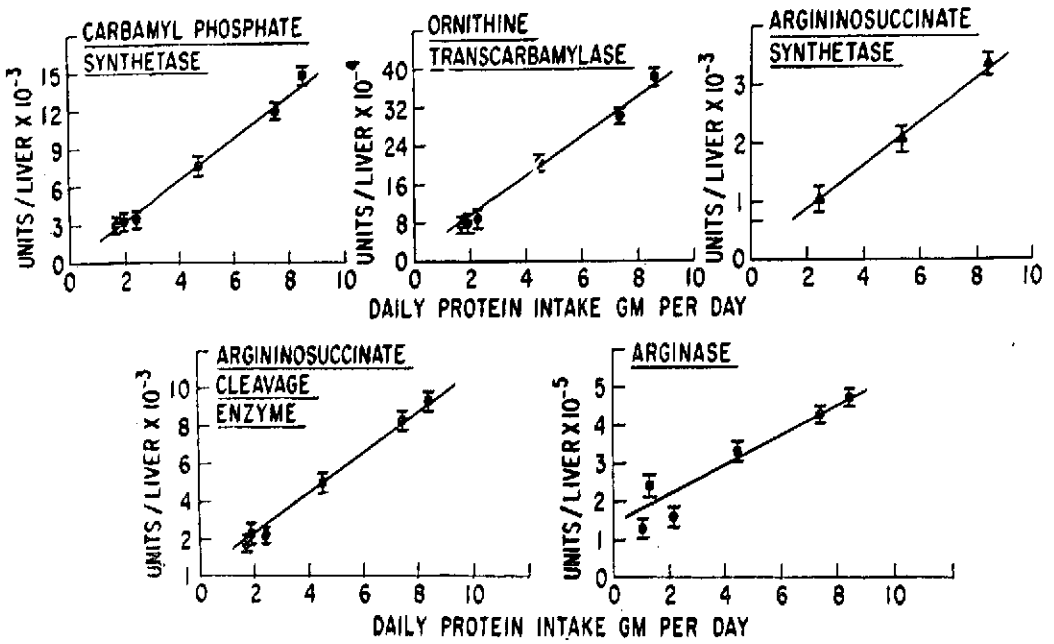

Figure 1. Relationships of total liver content of urea cycle enzymes to daily protein consumption. Protein intake in grams per day is plotted against enzyme content in units per liver. Brackets indicate ± 4 standard errors. The circles represent animals initially weighing 50 to 60 gm each, and the squares indicate animals weighing 140 to 150 gm each at the start of the experiment. Triangles indicate a separate group of rats weighing 140 to 150 gm each, which were initially assayed only for arginino-succinate synthetase. From Schimke (108).

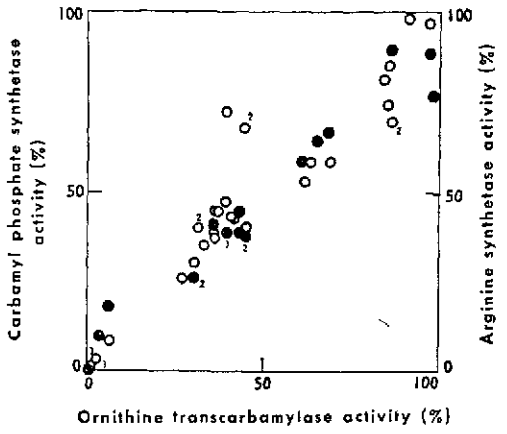

Figure 2. Relation between the activities of carbamyl phosphate synthetase, ornithine transcarbamylase and the arginine-synthetase system among species with different nitrogen excretory patterns. The values are expressed as the percentages of the highest activities: 21,000 units/gm wet weight for ornithine transcarbamylase, 300 units/gm for carbamyl phosphate synthetase and 100 units/gm for the arginine-synthetase system. ○ = Carbamyl phosphate synthetase ornithine transcarbamylase plot; ● = arginine synthetase system ornithine transcarbamylase plot. The numbers on the figure denote numbers of overlapping points. From Mora *et al.* (*76*).

proportion than the others (*76*). It is pertinent to the case being discussed that for *E. coli* (*66*) and for *N. crassa*, the enzymes that participate in the biosynthesis of arginine map in different regions of the genome. Moreover, there are experimental conditions where the variation of the urea enzymes is not coordinated: in the metamorphosis of the tadpole (*7*), the development of the rat (*47*), the administration of carcinogenic dyes (*70*), carbon tetrachloride (*30*), ethionine (*68*), and glucagon, alloxan derivatives and alloxan derivatives with insulin (*69*). It would appear that urea enzymes under certain circumstances are under the mandate of the same regulatory mechanism which operates at a level other than genetic transcription and also that there are situations where other factors operative in their regulation come in to play. Another case, if not of coordinated variation, at least of simultaneous variation in the same direction of groups of enzymes metabolically linked, concerns the processes of glycolysis and gluconeogenesis. Glucokinase, phosphofructokinase, and pyruvic kinase catalyze reactions that caused metabolites to proceed in the direction of glucose to pyruvate, while pyruvic carboxylase, phosphoenol pyruvic carboxylase, fructose-1-6-diphosphatase, and glucose-6-phosphatase catalyze reactions determining the metabolic flux in the opposite direction. The other enzymes of the glycolytic path are bifunctional and catalyze the corresponding reactions in either direction. Because the variations by dietary stimulus or by the administration of certain hormones affect the enzyme as the defined groups, Weber (*151, 152, 153*) postulated the existence of three "functional genetic units," one for each group of enzymes. However, the administration of fructose or glycerol which bypass glucokinase and phosphofructokinase, creating a triose load, causes an increase in pyruvic kinase, glucose-6-phosphate, and fructose-1-6-diphosphatase, and a diminution in glucokinase and phosphofructokinase (*121, 122*). These findings do not lend support to the concept of functional genetic units. It has been suggested that diversified mechanisms of regulation in higher organisms are responsible for the described behavior which offers more degrees of freedom and versatility for metabolic adjustments (*121, 122*). Nevertheless, there exists an instance where, by a single mutation in mice, the activities of three enzymes involved in pyrimidine catabolism are affected (*16*)—a situation that might correspond to the existence of an operon in higher organisms (*112*).

Another reason for variations in enzyme activities might be their redistribution among cellular fractions. Such is the case of the increased liver lysosomal enzyme activities elicited by means of starvation and their diminution upon refeeding (*18*).

The activity of a given enzyme may also be altered by its interaction with metabolites which might be nutrients supplied in the diet. Such change of activity can determine the direction of flow in metabolic paths. To illustrate this point, it is pertinent to mention how different molecules influence the activities of enzymes

engaged in glycolysis and gluconeogenesis. The situation is presented in Table 1.

The conversion into each other of certain metabolites is catalyzed by two different enzymes which may be regulated by the presence of important metabolites (Table 1). For instance, acetyl CoA activates pyruvic carboxylase and inhibits pyruvic kinase; guanosine monophosphate (GMP) activates phosphoenol pyruvic carboxylase and inhibits pyruvic kinase. AMP inhibits F-1-6-diphosphatase (DPase) and activates phosphofructokinase. Some of the metabolites produced by the glycolytic path feed back by inhibiting enzymes pushing the flow in the direction of their formation: free fatty acids inhibit glucokinase, phosphofructokinase, and pyruvic kinase; acetyl CoA inhibits glucokinase, and pyruvic kinase; phosphoenol pyruvic inhibits glucokinase, and NADH inhibits phosphofructokinase, and pyruvic kinase. Other metabolites at the beginning of the glycolytic path signal ahead favoring the flow in this direction: glucose-6-phosphate, fructose-1-6-diphosphate, and glyceraldehyde-3-phosphate increase the activity of pyruvic kinase. Some amino acids inhibit pyruvic kinase, and hence glycolysis. Several nucleotide diphosphates act as acceptors of pyruvic kinase, and the corresponding nucleotide triphosphate, product of the reaction, inhibits the enzyme.

Then, by a complicated interplay of inhibition and activation exerted by metabolites, glycolysis, and gluconeogenesis might function alternatively, in an on and off way, avoiding unnecessary recycling (*147, 150*).

It is easy to understand that the concentration of coenzymes and metals directly affects the enzyme activities. It has been mentioned above that the deficiency of vitamins as well as minerals causes a decrease in the enzyme activity which is not always restored by the addition of the missing factor to the assay mixture, which means that the amount of apoenzyme is affected by the concentration of the coenzyme or metal. Moreover, it has also been noted that the response to hormonal stimulus in some cases is af-

Table 1

Effect of different metabolites on the activity of enzymes of carbohydrate metabolism

	Glycolytic enzymes			Gluconeogenic enzymes			
Metabolite	GK	PFK	PK	G6Pase	F1-6DPase	PEPCase	PCase
Free fatty acids	↓	↓	↓				
Acetyl CoA	↓		↓				↑
ADP				↓	↓		
ATP	↑	↑	↓				
CTP, UTP, TTP			↓				
AMP		↑			↓		
GTP			↓			↑	
G6P			↑				
F-1-6-DP			↑		↓		
g3P			↑				
NADH		↓	↓				
L-Alanine			↓				
L-ø-alanine			↓				
PEP	↓						

Abbreviations: GK: glucokinase; PFK: phosphofructokinase; PK: pyruvic kinase; G6Pase: glucose-6-phosphatase; F1-6DPase: fructose-1-6-diphosphatase; PEPCase: phosphoenol pyruvic carboxylase; PCase: pyruvic carboxylase; g3P: glyceraldehyde-3-phosphate; other abbreviations corresponding to metabolites are of standard use or are implied in those used for the enzymes. Upward arrows mean activation; downward arrows mean inhibition. From data obtained by Weber *et al.* (*144, 151*).

fected by the concentration of coenzymes (*114*) and that the regulation of the biosynthesis of heme influences the biosynthesis of apotryptophan pyrrolase (*74*).

Adjustment in the metabolic machinery can be effected by altering the enzyme activity without modifying the amount of the enzyme, that is, by means of metabolic effectors which, on interacting with the specific protein, thus can affect its catalytic properties. Accordingly, it has been proposed that this particular type of control constitutes a sort of *acute adaptation* since it rapidly comes into play, while the change in the amount of the specific protein may be considered a *chronic adaptation* because protein synthesis should take place and the process cannot be adjusted as immediately (*153*). The first type of regulation cannot be evidenced, in many instances, by the *in vitro* assays of enzyme activities because the incubation system usually puts the enzymes in the so-called optimal conditions, whereby substrate, coenzymes, metals, and known activators are included in non-limiting concentrations. In addition the pH of the system corresponds to sites at which the catalytic activity is higher. These conditions, of course, may hide the fact that *in vivo* a given metabolic effector (activator or inhibitor) was operating. The situation could be better estimated by determining the efficiency of a metabolic flux where the enzyme involved participates. Another important consideration is that the conditions of an *in vitro* assay can sometimes be far removed from those prevailing *in vivo*. What is the effective substrate concentration in the proximity of the active site of a given enzyme? What is the pH at interphases affecting the dissociation constants of functional groups at the active, inhibitor, and allosteric sites? Finally, how is the conformation with other macromolecules changed from what it is *in vivo*? Without answering these questions, it can at least be said that caution should be exerted in extrapolating from *in vitro* assays to *in vivo* functioning.

The physiological significance of changes in enzyme activities

From the above, it is clear that tissue enzyme activities do change under the influence of dietary variations. Henceforth, changes in enzyme activities as produced by alteration in the amount of the specific protein will be noted, and it will be assumed that although the relation between activity and amount of protein has been proven directly in only a few cases, this relationship often holds. Several questions can be raised regarding the meaning of those changes with respect to the specific functions of the organism that are related to the corresponding enzymes.

Urea biosynthesis enzymes will be used whenever possible to illustrate questions that will be considered, since having worked with several aspects of their regulation and functioning over the past ten years, we are more familiar with them. In addition, urea biosynthesis constitutes a very interesting model for gaining insight into the nature of an adaptive device. Indeed, urea biosynthesis allowed living organisms to adapt to terrestrial life, and urea cycle enzymes readily change under conditions demanding increased removal of ammonia from body fluids (*125*). Ammonia is highly toxic for higher organisms, hence it must be kept at very low concentrations in tissues and biological fluids. Animals living in an aquatic environment can readily excrete ammonia, but land animals had to develop other mechanisms to dispose of it, namely, ureotelism and uricotelism. Urea biosynthesis also operates as an osmoregulatory device in some sea water fishes (*8*). Interestingly enough, the ureotelic *Rana cancrivora* that lives in the brackish waters of tropical mangrove in Southeast Asia had to develop a kind of "superureotelism" in order to cope with the salinity of the environment (*36*). Urea cycle enzymes, as already stated, readily respond to the stimulus of starvation and change proportionally to the protein content of the diet (*108*).

Are the changes in enzyme activities that occur in higher organisms adaptive?

By "adaptive" is understood those changes that make sense in coping with altered environmental conditions able to produce metabolic disturbances in the organism. For instance, the feeding of the animal with a high-protein diet brings about an increased protein and amino acid catabolism. This situation, as already explained, is accompanied by an increase in several enzyme activities that participate in amino acid catabolism, in gluconeogenesis, and in the conversion of ammonia into urea.

The "purpose," on the other hand, of increased lipogenesis observed in the refed animal after a period of starvation is not very clear. The affected process, in fact, rebounds to such an extent that it causes liver lipid deposition, an obviously abnormal condition.

It might be argued that a change in enzyme activity might be responsible for or is a consequence of a particular metabolic disturbance. One may wonder, for instance, what is the case regarding the increase in glutamic oxaloacetic and glutamic pyruvic transaminases seen after high-protein diet, starvation, alloxan diabetes, and administration of cortisone or adenocorticotrophic hormone (ACTH), conditions all characterized by enhanced gluconeogenesis, increased protein catabolism, and the elevated amino acid pool in the liver (*56*). It has been postulated that in each case elevated enzyme activities might be the response to some primary change in a physiological state of the tissues, or rather, the cause of some of the secondary physiological changes (*56*). Thus, according to Knox and Greengard (*56*), "The functional inter-relatedness between cause and effect may present a problem; for example, does the increase in certain enzymes cause the increased protein catabolism, or does the increased catabolism cause the enzyme increases? Biologists generally refrain from asking such questions because the answer may involve them with the problem of purpose: that the final result can 'cause' the effect. With our present familiarity with feedback mechanisms, this problem is no longer one of metaphysics, but one of information transfer. Before we can even discuss the chemical mechanisms of control, we must identify the systems which control."

It may also happen that a change in a given enzyme activity is not related directly to the variations introduced by a metabolic disturbance, but rather by a reflex of new metabolic events taking place and thus could hardly be counted as a truly adaptive change since the variation of activity is not directed to the preservation of homeostasis. Thus, serum alkaline phosphatase is an entity composed of isozymes originating from different sources: bone, liver, kidney, leukocytes, and placenta, the first two making the greatest contribution. They can be distinguished by their different heat stability. It has been demonstrated that the decreased serum alkaline phosphatase activity observed in chronic human protein malnutrition is at the expense of the osteal enzyme and also that it is related to the cessation of growth, evidenced by X-ray diagnosis.

Enzymatic changes corresponding to metabolic adaptations should be reversible, that is to say, once the environmental stimulus causing the change has ceased, the original situation is restored. Mechanistically, it could be said that the steady-state concentration of an enzyme can be adjusted to the prevailing metabolic conditions, as already noted, by altering its rates of synthesis or degradation under the influence of the concentration of metabolic effectors.

Nevertheless, it might well be that at times the changes in enzyme activity are not of an adaptive nature but rather an expression of "damage" caused by faulty nutrition. Indeed, the methemoglobin reductase, glutathion reductase and adenosine triphosphatase (ATPase) of the rat erythrocyte are affected by fasting the animal and are not reestablished by refeeding, which is in accordance with the lack of biosynthetic capacity of these cells. Glucose-6-phosphate dehydrogenase and acetyl cholinesterase

are not affected by the treatment mentioned (*106, 107*).

Is it necessary to change all activities involved in a metabolic path to produce an increased output of the metabolic flux?

The functioning of a group of enzymes linked in a metabolic path defines the steady-state concentration of the metabolites being converted. It has been repeatedly said that enzyme activities, by and large, are in excess in relation to the existing concentrations of substrates, that is to say, substrate concentrations are usually well below the K_m values of the enzymes transforming them (*120*). It would appear, then, that the enzyme machinery is apt to cope with loads of metabolites several times the concentration found in the steady-state condition. In other words, the rate of conversion of metabolites in a metabolic path can be increased just by increasing the concentration of substrates. However, the rate would also be increased by elevating the enzyme activities whether or not these are far from being saturated. The fact remains that the latter type of response seems to be widespread, and thus it appears that higher organisms favor handling their metabolites in a condition of "excess" of enzymes.

Siebert, Pfaender, and Kesselring (*120*) followed a kinetic approach to relate enzyme rates to the flow of metabolites in metabolic paths. They reasoned that the extrapolation of the rates of enzymes competing for a common substrate, as determined *in vitro*, would allow defining the rates and flows of metabolites in branching points of metabolic sequences. Their approach took into consideration the expected velocity corresponding to the sizes of the metabolic pools, the K_m of each enzyme, and its intracellular location. They fully recognized that the predictions that can be made necessarily introduced many assumptions and met almost insurmountable difficulties. Indeed, as previously stated, *in vitro* assays cannot deal with factors inherent to the cell organization which undoubtedly influences enzyme activities. Among such factors should be mentioned compartmentalization of substrates, supramacromolecular structure, channeling, and others already considered, such as the effective substrate, metabolic effectors and hydrogen ion concentrations, that is to say, their concentration in the immediate surroundings of the functional sites at the enzyme surface. To illustrate the point being discussed it may be recalled that axolotl liver arginase is very unstable when the tissue is homogenized, therefore a system containing citrulline, aspartate, adenosine triphosphate (ATP), Mg^{++}, and liver homogenate accumulates arginine, and no urea is formed if the homogenate is preincubated. However in the presence of Mn^{++} and some other divalent cations, arginase is greatly preserved and urea is produced. Moreover, if the flow of conversion of citrulline to urea is allowed, at least some arginase keeps functioning, since urea is efficiently produced (*84*).

Another example of the importance of cell organization is the metabolic fate of the guanidino group of arginine in higher vertebrates. Figure 3 illustrates the situation. The amidino group is transferred and the same C-N bond is split during the catalytic action of the two enzymes. When arginase intervenes, water is the acceptor molecule and urea is formed; when glycine transamidinase participates, glycine is the acceptor molecule, and glycocyamine is produced. In both cases ornithine is the product of the reaction. Although in many species the two enzymes are present in different organs, human liver contains both. In a free competition of the two enzymes by the common substrate arginine, glycine transamidinase would be at great disadvantage because of the high arginase activity and the fact that ornithine is an inhibitor of the former. Therefore, the only possibility for their coexistence and functioning is compartmentalization so the competition cannot take place.

That changes in enzyme activities really determine the metabolic flux can be substantiated by the variations in key enzymes involved in the conversion of glucose to pyruvate and

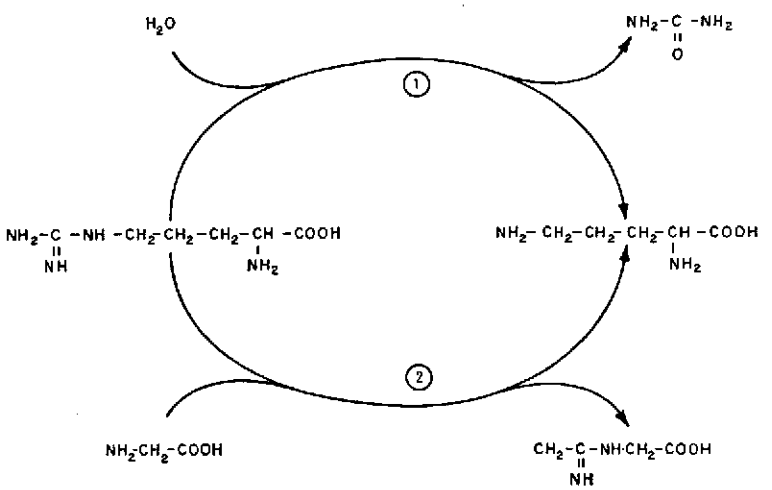

Figure 3. Fate of the guanidino group of arginine: (1) denotes the activity of arginase; (2) denotes the activity of glycine transamidinase.

vice versa. Glucokinase, phosphofructokinase, and pyruvic kinase activities push the flow in the direction of glycolysis whereas pyruvic carboxylase, phosphoenol pyruvic carboxylase, fructose-1-6-diphosphatase, and glucose-6-phosphatase determine gluconeogenesis.

The glycolytic flux is regulated through the activity of three enzymes, the gluconeogenic flux by means of the activity of four enzymes. There are other enzymes (hexose isomerase, aldolase, triose isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, and enolase) that do not change the direction of the flux in either way and usually are not affected by dietary or hormonal influences that favor glycolysis or gluconeogenesis.

However, in many other cases all enzymes involved in the metabolic path change under the influence of metabolic disturbances that claim a greater output of the metabolic flux. Such is the case with the urea cycle enzymes when the animal is fasted or fed with a high-protein diet. Therefore, the question that immediately arises is whether the change in all enzymes was necessary or would it have sufficed to change the activity of one of them to achieve the same increase in urea production.

To gain insight into this problem, it is pertinent to recall the activities of the enzymes involved in the livers of different ureotelic species (Table 2).

It can be readily seen that while the activities of carbamyl phosphate synthetase, arginino succinic synthetase, and arginino succinase are low, those of ornithine transcarbamylase and arginase are rather high. Moreover, it has been repeatedly stated that arginino succinic synthetase, having the lowest activity, constitutes the pacemaker of the urea biosynthesis path. What is the meaning of this statement? Again, it is difficult to predict from *in vitro* enzyme assays how the enzyme acts when it is a part of a highly organized metabolic machinery. The fact remains that the advent of ureotelism may correspond to an augmentation of all urea cycle enzymes (metamorphosis of the tadpole), to an increase in carbamyl phosphate synthetase (*Xenopus laevis* in hypertonic medium or during estivation), to an increase in arginino succinic synthetase (birth of the rat), to an elevated ratio of glutamic oxaloacetic transaminase to glutamic pyruvic transaminase (estivation of the lung fish), or it may even occur without any apparent change in the enzyme activities (metamorphosis of the Mexican axolotl) (Table 3),

Table 2

Activities of the enzymes in the livers of ureotelic species

Common name	Binominal name	Carbamyl phosphate synthetase	Ornithine trans-carbamylase	Arginine-synthetase system	Arginase
Rat	*Rattus norvegicus*	260	18000	72·0	56400
		275	21600	78·0	50400
		247	18000	72·0	46800
Mouse (Ajax DBA strain)	*Mus musculus*	180	14500	66·0	79000
		180	14500	67·0	76800
		165	13000	60·0	68500
Frog	*Rana montezumae*	225	18000	90·0	80000
		237	18000	90·0	96000
		211	9450	42·0	51000
		211	9450	42·0	51000
Semi-aquatic turtle	*Pseudemys scriptae*	140	7570	46·0	45600
		144	8100	43·0	60000
		120	7500	43·0	55000
		95	6500	40·0	50000
Semi-aquatic turtle	*Kinosternon hirtipes*	133	8900	45·0	37800
		128	6850	35·0	38000
		139	8750	—	—
		124	7500	—	—
		292	20200	90	57600

From Mora *et al.* (*76*).

Table 3

Enzyme activities

System	Observations	Reference
Earthworm during fasting	Increase of incorporation of HCO^-_3 into urea. Increase 2–4 fold in activity of the enzymes of the cycle.	Bishop and Campbell (*7*)
Metamorphosis of Tadpole of *Rana catesbeiana*	Natural and induced by thyroid hormones. Increase 8–30 fold in activity of the enzymes of the cycle; *de novo* synthesis demonstrated for CPS [a]	Cohen (*22*)
Xenopus laevis during estivation	Increase 6-fold in activity of CPS. Reversible.	Balinsky *et al.* (*4*)
Xenopus laevis in hypertonic medium	Increase 5-fold in activity of CPS. Reversible.	Janssens and Cohen (*41*)
Metamorphosis of the Mexican axolotl	Induced by thyroid hormones. No increment in enzymes of the cycle. Coupling of Arginase? Decrease in NH_3 diffusion?	Soberón *et al.* (*78*)
Lung fish during estivation	Synthesis of urea from HCO^-_3. Decrease in NH_3 excretion. Increase 10-fold in the ratio GOT/GPT [b]	Janssens and Cohen (*41*)
Birth of the rat	Increase in the enzymes of the cycle, especially of arginino succinate synthetase. Depends on hormonal control.	Raiha and Suihkonen (*69*)

[a] CPS (carbamyl phosphate synthetase)

[b] GOT (glutamic oxaloacetic transaminase)/GPT (glutamic pyruvic transaminase)

From Soberón *et al.* (*125*).

(*125*). This situation suggests that the possibilities for the regulation of the enzyme cycle are rather versatile and different biological systems may utilize one or another. Furthermore, the metabolic flux can be directed by a change in just one of the enzymes of the metabolic path.

However, all urea cycle enzymes vary in relation to the protein content of the diet. In addition, a good correlation has been found between arginase activity and urea excreted (*154*).

Does this mean that the increase of arginase activity is required to augment the output of the cycle? Let us review some facts relevant to the physiological role of arginase. A high activity is found in the liver of ureotelic animals which has led to the conclusion that there is an excess of arginase. When labeled citrulline is administered to a rat, kidney protein arginine becomes labeled, but not liver protein arginine; however, if labeled arginine is administered, it is incorporated into liver proteins (*98*). In this respect it should be recalled that the K_m of arginase and the arginyl RNA synthetase are of different orders of magnitude, 10^{-3}M (*37*) and 10^{-6}M (*1*), respectively. It has recently been demonstrated that liver arginase hydrolyzes preferentially endogenous arginine, the one originating from citrulline and aspartic acid over that artificially generated by the hydrolysis of hippuryll-l-arginine (*83*). This evidence, together with the low concentration of arginine found in the liver of ureotelic animals, has led us to postulate that arginase is physically integrated with the arginine forming sites (*125*). Because it is possible to diminish the arginase activity to 2 per cent of the original, and still arginine is converted efficiently to urea, it is likely that not all arginase molecules present in liver tissue are integrated with the arginine forming sites. A logical inference is that an increase in the arginase molecule would not be required for a greater output of urea. Therefore, we may reach the conclusion that the change in arginase activity is rather a consequence of the action of a regulatory mechanism that affects all urea cycle enzymes.

Is there a direct relation between enzyme activities and output of the corresponding metabolic path?

It has been said before that even though the enzyme activities are in excess in relation to the substrate concentrations, an increase in enzyme activity will produce an augmentation of the metabolic flux where they participate. There is an increased rate of conversion of C^{14} uracil to radioactive CO_2 observed in a mutant of mice characterized by the elevated activity of three enzymes involved in pyrimidine metabolism (*16*). There is an increased lipogenesis and glycogen deposition in conditions where the activities of the enzymes participating in those processes are elevated (*158*). The same may be inferred from the work of Wergedal, Ku, and Harper (*154*), who found an elevated toxicity of amino acids administered to rats previously fed a high-protein diet for several days. The animals also had an elevated ammonia concentration in the blood. These findings were interpreted as indicative of an augmented amino acid catabolism in due correspondence to an elevated activity of the intervening enzymes as assayed *in vitro*, which supported the concept that these increased activities represent an adaptive response of physiological significance *in vivo*.

The relation between increased enzyme activity and function is not as clear in other cases. The administration of L-methyl tryptophan, which increased the activity of tryptophan pyrrolase produced a higher conversion of $2C^{14}$ L-tryptophan (ring-labeled) to radioactive CO_2 (*71, 160*). However, the administration of cortisone that also elevates the same enzyme activity did not produce augmentation in the conversion of labeled side-chain tryptophan to radioactive CO_2 in the perfused rat liver (*52*). In the latter study, greater velocity of conversion of labeled tyrosine to radioactive CO_2, in spite of the fact that the hormone administration causes an elevation of tyrosine transaminase activity, was also not observed (*52*). By the same token there is evidence that glycogen depo-

sition is not related to the increased activity of gluconeogenic enzymes (*96*).

The converse situation, namely, the effect on the output of a metabolic path when the intervening enzymes have a diminished activity also deserves great attention. To gain insight into the problem posed, a study was made on the capacity of the urea cycle enzymes to clear *in vivo* an ammonia load under various experimental conditions. First of all, it was necessary to determine the distribution of the ammonia load in the normal rat (*99*). It was found that the following sequence of events seemed to be operative: initially, the ammonia was rapidly removed from the circulation by muscle uptake, then glutamine synthesis took place, mainly in liver and brain, and finally, urea synthesis took over utilizing the ammonia gradually released from muscle, and from the enzymatic hydrolysis of glutamine. The results obtained are in accordance with the findings of Duda and Handler (*20*), who followed the metabolic fate of N^{15} ammonia administered to the rat. The urea cycle enzymes, and glutamine synthetase, and glutamic dehydrogenase that were also studied, were decreased by dietary means (protein-free

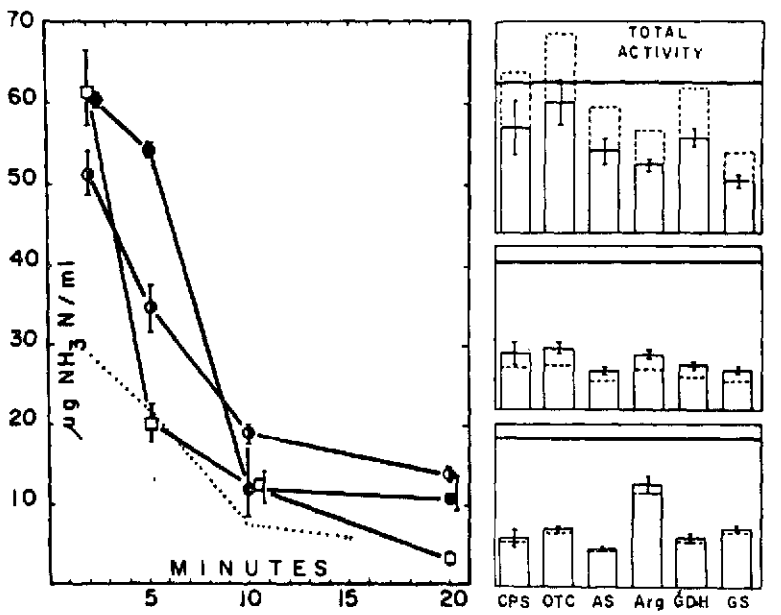

Figure 4. The left side illustrates the ammonia uptake in animals starved for four days (□), fed a protein-free diet (◑), and fed a diet that contained only zein as a source of protein (●). At zero time, 500 μmoles of ammonium chloride were administered by injection into the porta. At the indicated times after injection, blood was removed from a hepatic vein for ammonia determination. Vertical bars represent standard error of the mean. The broken line indicates uptake of ammonia by the normal animal. The right side illustrates the enzyme activities in the liver of rats starved for four days (upper), fed with a protein-free diet (middle), and fed with a diet that contains zein as a source of protein (lower). The activities of the enzymes studied represent relative values compared to the activities found in the normal animal (taken as 100 per cent, and indicated by the gross horizontal line). The results are expressed as total activity (units for total liver). One unit of enzyme is defined as the amount of enzyme that converts one micromole of substrate in one minute under the conditions of each assay. Broken lines indicate values that would be expected if the livers had the same weight as the normal ones. Vertical bars represent standard error of the mean carbamyl phosphate synthetase (CPS); ornithine transcarbamylase (OTC); arginine synthetase (AS); arginase (ARG); glutamic dehydrogenase (GDH); glutamine synthetase (GS). From Flores *et al.* (30).

diet or zein-containing diet), by surgical removal of the liver and by the administration of CCl_4. It was found that only the rats treated with the hepatotoxic agent were hyperammonemic to begin with. The rats fed with deficient diets or partially hepatectomized, although showing an impaired capacity to clear the ammonia load in comparison to the normal controls, still retained enough capacity to return the blood ammonia concentration to near normal values in about 20 minutes. The rats treated with CCl_4 had the lowest enzyme activities and could not clear the ammonia load (Figures 4, 5, and 6).

Interestingly enough, anoxic animals failed completely to clear the ammonia load, which could be explained by a drop of 90 per cent in the ATP liver content. These results suggest that a diminished enzyme activity could still suffice to cope with the main physiological task of the path where they intervene—ammonia detoxification in the case presented—and that only when metabolic disturbances impose more stressing conditions upon them does the diminished capacity become apparent. The following facts support this concept. It is possible to decrease the activity of glucose-6-phosphatase even to 20 per cent of the normal value without any impairment of its main function, namely, the maintenance of glycemia (*144, 148, 149*). Xanthine oxidase can reach very low values by the feeding of protein-deficient diets without any diminution in the excretion of allantoin and uric acid (*155*). Even though the feeding of a cooked dried egg to BHE rats caused a decrease in hepatic glucose-6-phosphate dehydrogenase, the CO_2 production from the first carbon of glucose was augmented. With respect to the last, the suggestion was made that the enzyme of other organs of the intact animal might be responsible for the discrepancy (*14*).

In conclusion, the changes in enzyme activities provoked by dietary means constitute one aspect of the very complex problem of metabolic regulation in higher organisms—a field that is beginning to be understood and awaits important contributions in the years to come. With respect to the physiological significance of such changes, the data now available permits speculation more than anything else.

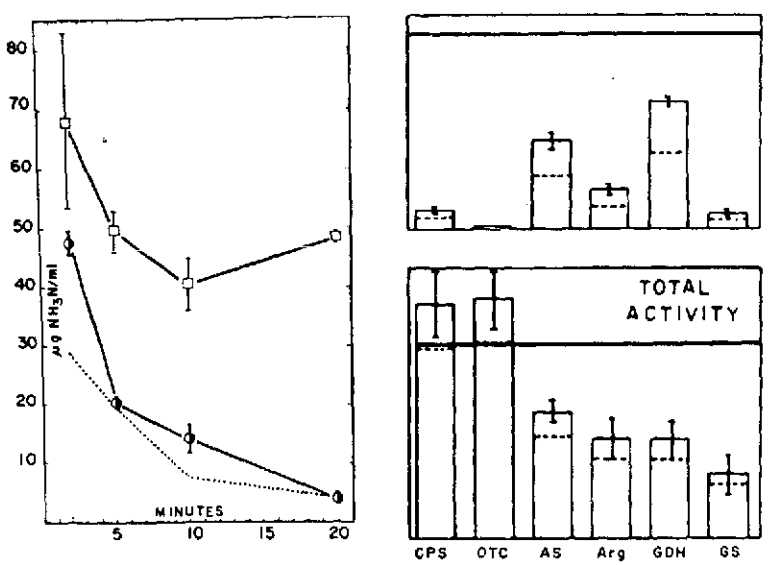

Figure 5. The left part represents the ammonia uptake in animals acutely (◑), and chronically (□) intoxicated with CCl_4. The right side represents the enzyme activities in the same groups of animals. (Upper, acute intoxication; lower, chronic intoxication). See Figure 4 for other indications. From Flores *et al.* (*30*).

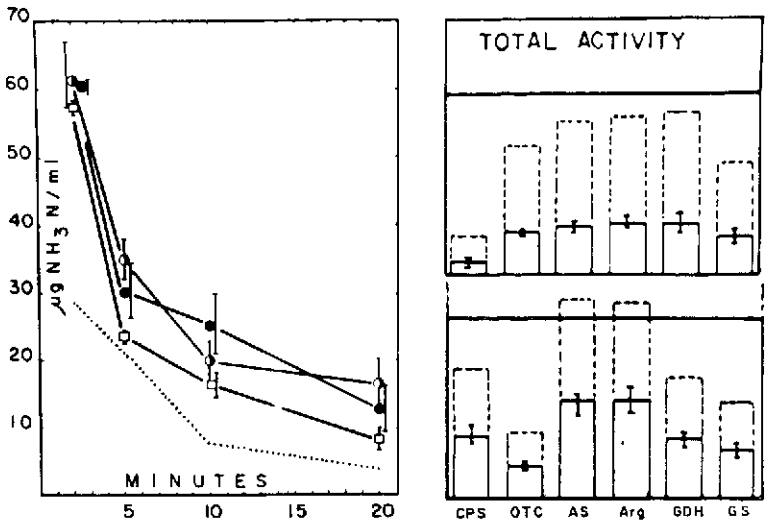

Figure 6. The left part represents the ammonia uptake of partially hepatectomized animals 12 (□), 18 (◑), and 48 (●) hours after surgery. The right side represents the enzyme activities found in the liver remnant after partial hepatectomy, 12 (upper), and 24 (lower) hours after surgery. See Figure 4 for other indications. From Flores *et al.* (30).

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DISCUSSION

Potter: I was very much interested in the first question raised by Dr. Soberón. Are the changes in enzyme activities which occur in higher organisms of an adaptive nature? From a philosophical standpoint, as well as from a physiological standpoint, we could answer that question by saying, "it makes adaptive sense if the organism is in the environment in which the species evolved to its present state." A great many responses are occurring under conditions in which the species never found itself during its evolution, and if we interpret the nonadaptive responses in those terms, they begin to be understandable. In other words, should the individual member of the species be in a situation which is totally abnormal or strange—one in which the species didn't evolve—then some response which is detrimental to the animal might occur.

Soberón: I agree with the position taken by Dr. Potter. However, it is difficult to interpret all changes occurring under the stress of variations in the environment in terms of homeostatic adjustments. There are some cases where changes do occur without it being possible to correlate them with the activity of the enzyme that is changing.

Chairman Cohen: I think Dr. Potter raised a very important point and one might ask the question whether man, who has become adapted to a relatively constant environment over a period of 100,000 years, or so, is now faced with the dilemma that his environment is changing far too rapidly for him to be able to adapt to it.

Harper: It seems clear, as Dr. Soberón pointed out, that for many, perhaps even most of the enzymes in tissues, the activities are considerably higher than the organism ordinarily needs to handle its metabolic load.

In any adequately functioning system, it is important to have a capacity well beyond what the normal load is in order to contend with unexpected emergencies, and one can look at the influx of individual meals as such emergencies. Many animals eat once a day, twice a day, or three times a day, so there can be large influxes in relatively short periods of time when extra enzymes may be beneficial. Many of the adaptations that we measure would seem to represent mainly an increase in the safety factor as we approach conditions in which the capacity of the system is in danger of being exceeded. The animal can handle a large load without adaptation, but after adaptation, the total capacity of the system has so increased that an emergency load that would have exceeded the capacity of the original system can now be handled.

Chairman Cohen: What has been emphasized is that there are regulatory factors, not only in terms of what Dr. Potter alluded to earlier, such as the access of nutrients into a particular cell by transport mechanisms, but also the hormonal factors Dr. Niemeyer, in particular, stressed and which Dr. Mönckeberg will later talk about in relation to a more complex situation. While the rate-limiting factor properly focuses on the catalytic capacity of enzymes, the other regulatory factors that the cell and the organism utilize, relating to flux rates of metabolites into cells, have fixed limits which must be of importance in defining the limits of adaptation.

The factors that determine the rates at which certain nutrients will become available, the rates at which they will be transported, and the rates at which they will be metabolized, need more quantitative investigation.

Potter: I am particularly interested in the data by Dr. Niemeyer as it relates to our own work. Dr. Waterlow earlier this morning made

reference to the small amount of fuel reserves represented by the glycogen store. I suggest that the young growing animal does not have very much margin in his enzyme capacity or in his fat reserves. We have studied the size of the fat pads in the young growing animal, and we have studied what happens when you change the feeding pattern from daily food intake to the 8+40 regimen. What happens to these young growing animals that have never fasted before? The fact is that they burn themselves out. They kill themselves running if they have access to an exercise cage. They die with a total depletion of all of their fat pads because they simply do not have the capacity to store enough fat to get them through a 40-hour period of fasting. As they get just a little bit older, however, they can develop that capacity.

Now what is very important in the ability to develop the capacity to store fat? It is glucose-6-phosphate dehydrogenase, among other things, because this is an enzyme that generates reduced triphosphopyridine nucleotide (TPN) which is needed for fat synthesis.

We have shown that the animals adapted to the experience of fasting function with about six times as much glucose-6-phosphate dehydrogenase as the non-adapted animal, so that, in addition to the enzyme patterns that Dr. Niemeyer sees with changing ratios of protein, carbohydrate, and fat, we see tremendous variations in the levels of enzymes simply with the same diet, but under situations where carbohydrate storage doesn't solve the problem. They have to have fat storage.

OPENING STATEMENT—Afternoon

John C. Waterlow, Chairman

Dietary and hematological surveys are a necessary part of any public health program. Their aim is to detect groups or individuals who may be classified as "deficient" or "anemic." Today, more and more reliance is being placed on biochemical and hematological measurements to provide evidence of marginal or subclinical states of deficiency. The afternoon meeting is concerned with the general problem of how such measurements should be interpreted. Does the concept of subclinical deficiency have any meaning? If many biochemical functions show adaptive changes, how can one define the range of adaptation compatible with normal function? Are there any logical criteria for distinguishing between normal and abnormal? These are questions of fundamental importance for preventive medicine, where we are dealing not with the sick, but with the potentially sick. The papers in the afternoon session will be directed towards this general problem, discussing it from different aspects, and with examples from different fields of study.

THE CONCEPT OF "NORMAL" IN NUTRITION

John C. Waterlow

The word "adaptation" presupposes that people may be exposed to different situations, stresses, or environmental factors. Obviously there would be no question of adaptation if the conditions of life were identical for everyone and never varied. Adaptation, then, means the preservation of normal bodily function in the face of these differences. Although this definition tells us nothing that was not said long ago by Claude Bernard, Walter Cannon, or L. J. Henderson, it serves to bring out the first point I wish to make: *the concept of adaptation and the concept of normal levels are two aspects of the same thing.* The problem, then, is to define the range for any parameter within which adaptation has occurred and beyond which it has broken down. This is not a new problem, but it becomes increasingly important as medicine ceases to be preoccupied mainly with the cure of disease and is concerned more and more with the maintenance of health. We study the normal ranges of various systems in order to detect impending disease. It is a classical approach in hematology and in nutrition to try to identify by chemical measurements individuals or groups who are risks because they fall outside the so-called "range" of normal.

There seem to be four ways of approaching this problem of defining normal ranges or ranges of adaptation.

The statistical approach

This is the method commonly used in clinical chemistry. Measurements are made on a large group of apparently healthy people, and the normal range is taken as the mean ± 2 standard deviations (*6*). The solution, however, is not as simple as this. Figure 1 shows the distribution of hematocrit in healthy women. Garby and Killander (*3*), from whose paper this figure is taken, comment as follows on this distribution curve: "The data can answer the question of the probability of observing a hematocrit value of a given magnitude, provided that we have a healthy female individual. . . . It is important

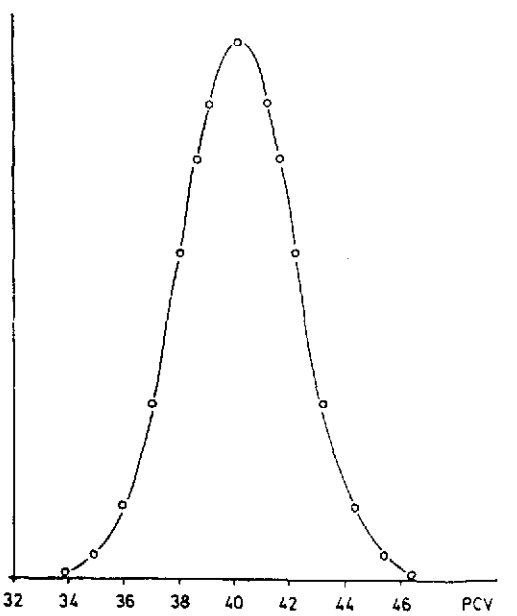

Figure 1. Distribution of packed cell volume (PCV) in healthy women from the combined data of Wintrobe, Scott, and Pritchard. From Garby and Killander (3) by permission of Almqvist and Wiksells, Uppsala.

to note, however, that this is *not* the question that we want to ask. The question that we want to ask is completely different: namely, What is the probability that a given hematocrit value is optimal given a randomly chosen female individual? This information is not contained in the data." For instance, a woman whose normal hematocrit was 43 per cent might reasonably be regarded as anemic if it fell to 37 per cent, yet this value is well within the range of 95 per cent of the observations.

This figure was chosen for another reason—its symmetry. It represents a statistically "normal" distribution. Here we are using the same word in two quite different senses—statistically normal and biologically normal. This causes great confusion, which can be avoided by using the proper term "Gaussian" for a distribution of the kind shown in Figure 1. There is a tendency to think that in a healthy group of people all parameters should have a "normal" distribution. This is quite fallacious. An excellent example of a highly skewed distribution in a clinically normal group is given by Cook, Layrisse, and Finch (*1*) in their paper on iron absorption (Figure 2).

This kind of situation greatly increases the difficulty of interpreting a group of results, since a mathematical transformation as proposed by Cook and co-workers may not always meet the case. A practical example of such a difficulty is shown in Figure 3. This represents the distribution of values for serum alkaline phosphatase in children in day nurseries in London (*8*). At first sight this looks like a Gaussian distribution with a tail of "abnormal" values to the right. In fact, however, one cannot say on statistical grounds alone that these results do not all come from the same population. If one were to accept the commonly used standard that a value for serum alkaline phosphatase of more than 25 units is biochemical evidence of rickets, clearly a substantial proportion of children would be at risk. In fact, however, they were all receiving supplementary vitamin D. The only way in which one can find out the meaning of the high

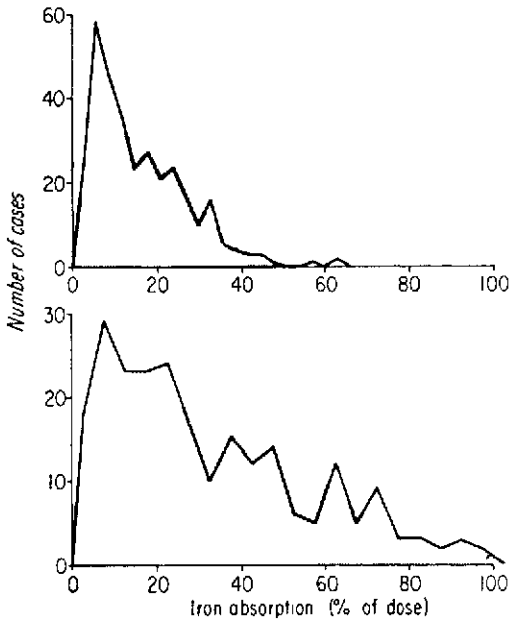

Figure 2. Frequency distribution of iron absorption, expressed as per cent of administered dose. The upper figure relates to Swedish subjects, the lower to subjects in Caracas, Venezuela. From Cook, Layrisse, and Finch (*1*) by permission of the Editors of *Blood*.

values is by further biological measurements, for example, by determining whether or not they represent bone phosphatase.

Another example of the pitfalls in interpreting distribution curves is shown in Figure 4, taken

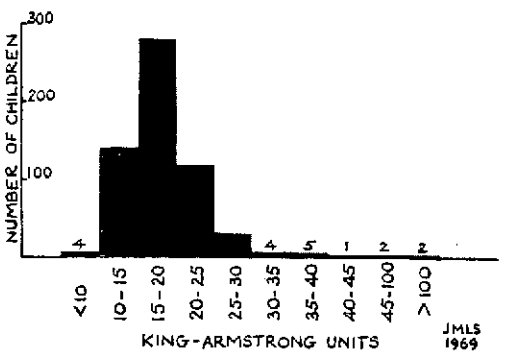

Figure 3. Distribution of plasma alkaline phosphatase in 583 children in day nurseries in Britain. From Stephen and Stephenson (*8*).

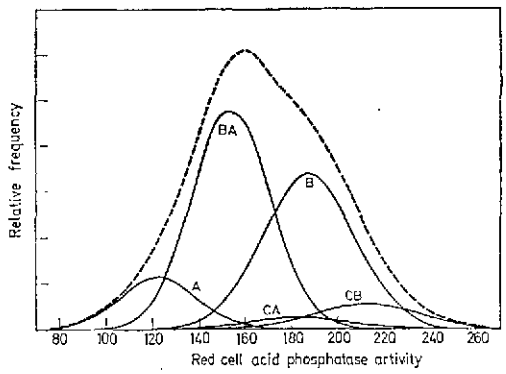

Figure 4. Distribution of red cell acid phosphatase activities in the general population (top line) and in the separate phenotypes. Phosphatase activity expressed in μmoles p-nitrophenol liberated per gm hemoglobin in 30 minutes at 37°C. From Harris *et al.* (4), by permission of Grune and Stratton.

from Harris *et al.* (*4*). Here an apparently Gaussian distribution of red cell acid phosphatase is in fact the sum of the distributions of five different phenotypes.

Statistical methods, therefore, describe the range of variation between individuals, and the probability that groups belong or do not belong to the same population, but they cannot provide value judgments of what is normal or abnormal. This is an elementary fact, which I think is often overlooked for the simple reason that we are often biased in our approach to these matters. As L. J. Henderson emphasized, adaptation is *relative*. It is natural to take the position that I am normal, and that you, if you are different, are at best "adapted," at worst "abnormal."

Associations and long-term consequences

This is essentially the approach of the clinician, who knows by long experience that a certain level of blood sugar indicates impending diabetes, or a certain level of blood pressure, impending hypertensive disease. When in nutrition we use a biochemical measurement, for example, of serum vitamin A or alkaline phosphatase, to indicate so-called subclinical disease, two things may be implied. The first is that, if the same conditions continue, the subjects will become clinically ill. This can only be proved by long-term studies, such as those made by the British Medical Research Council during World War II on volunteers receiving very low intakes of vitamins A or C. I am not aware of any studies of this kind on people under natural conditions. I do not know whether it has ever been shown that a person whose serum vitamin A concentration puts him in the category of deficient by accepted standards will, in fact, if untreated, develop signs of avitaminosis A. Dr. Arroyave will deal with this topic in more detail.

The other implication is that a person who is graded biochemically as being deficient may be in a steady state in which he is not obviously ill but is functioning in a suboptimal way. He may be so near the margin that the slightest stress will precipitate him into illness. These, it seems to me, are the only possible meanings of the unfortunate term "subclinical deficiency."

Measurements of function and performance

If an adaptation means a response which maintains normal function, the best test of adaptation is through appropriate measurements of function or performance.

Since anemia is considered to be one of the most widespread deficiency diseases, I have chosen an example from this field, which will be covered in more detail by Dr. Waters. The example comes from the work of Elwood and his colleagues (*2*), who have surveyed the hemoglobin concentrations of a large population of women in South Wales. In a recent paper, Elwood and co-workers report that women with Hb concentrations of less than 10.5 gm/100 ml had significantly lower blood cholesterol levels than those with Hb of more than 10.5 gm/100 ml. They go on to say: "It is therefore difficult to escape the conclusion that mild anaemia has a beneficial effect. . . . Furthermore, to the possible benefit of a lower serum-cholesterol in anaemia should be added the protective effect of the greatly increased number of anastomoses in the coronary circulation which has been demonstrated in anaemic subjects and may have devel-

oped because of anaemia. On the other hand there is remarkably little evidence that levels of circulating Hb down to about 8 gm/100 ml are associated with any harmful effects, or that treatment with iron has any beneficial effect on morbidity or on function."

It may well be, therefore, that a reduction in Hb concentration is a beneficent adaptation to the sedentary conditions of modern existence, in which there is no great demand on the oxygen transport mechanism. Could one speculate further and suppose that women are less liable than men to cardiovascular disease because they have on the average lower hemoglobin concentrations than men?

Analysis of the physiological and biochemical mechanisms of adaptation

I believe that in the long run the best way to define the limits of an adaptive process is through a better understanding of its mechanism. It is this approach that brings together the two parts of our symposium.

The classical example of the practical value of a physiological analysis is provided by disturbances of acid-base and electrolyte balance. Our ideas of what is "normal" rest on an understanding of the homeostatic mechanisms derived from the work of L. J. Henderson, Gamble, Darrow, and others. These ideas are put to the practical test in the treatment of patients.

In going back to the teachings of Claude Bernard, it must be recognized that in an adaptive process, there are some functions that are fixed within narrow limits and others that are allowed to vary rather widely. It is important for understanding the mechanisms of adaptation to identify and distinguish functions according to their range of variation. Figure 5 is an example from a paper published several years ago by Dr. Hoffenberg and his colleagues (5). It shows a linear relation between the serum albumin level and the catabolic rate of serum albumin in healthy subjects fed low-protein diets. The point it brings out is that albumin concentration is relatively fixed; when it falls

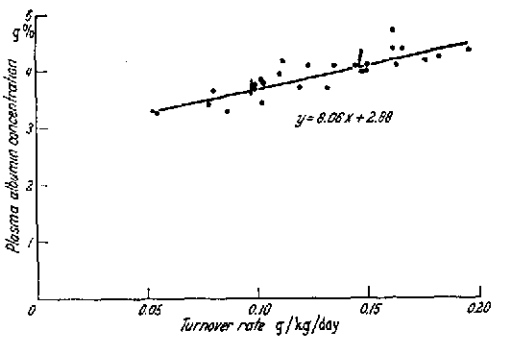

Figure 5. Albumin turnover rate plotted against plasma albumin concentration in healthy adults on diets of varying protein content. From Hoffenberg *et al.* (5), by permission of Springer Verlag, Berlin.

by 25 per cent, the catabolic rate is reduced by 75 per cent. It is reasonable to conclude, as Hoffenberg and all others who work on this subject have done, that a change in catabolic rate is an adaptation to low-protein intakes which helps to maintain a constant albumin concentration.

A priori, it seems likely that a function that is closely fixed under a variety of conditions must be physiologically important. Table 1 shows another example from the field of protein metabolism—the total rate of protein synthesis in infants at different levels of protein intake (7). It would be reasonable to conclude that the overall rate of protein synthesis is a function that must operate at a certain level, and that adaptive mechanisms come into play to maintain this level. The next step in considering the body's reaction to changes in protein intake is

Table 1

Rates of protein synthesis and catabolism in infants at different levels of protein intake, measured by intragastric infusion of ^{15}N-glycine [a]

	High protein	Low protein
No. of infants	6	5
Taken in (gm protein/kg/day)	5.2	1.2
Synthesized (gm protein/kg/day)	6.5	6.2
Catabolized (gm protein/kg/day)	4.2	5.5
Fraction of ^{15}N excreted	24.3%	3.4%

[a] From Picou and Taylor-Roberts (7).

to identify the mechanisms of homeostasis. I have considered this problem in more detail elsewhere (*9*).

To summarize, the concept of adaptation and the concept of a normal range of variation are two aspects of the same thing. A statistical approach is essential for describing and summarizing data and for establishing differences between groups, but "normal" and "abnormal" can only be distinguished by biological methods. These, I suggest, are of three kinds: long-term experience of consequences; functional tests; and analysis of the basic mechanisms of any adaptive process.

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DISCUSSION

Hilleboe: Dr. Waterlow, you said the statistical approach leaves much to be desired, yet you used it throughout your discussion. Is this not inconsistent? I should like to point out, and I speak as an epidemiologist, that in making a statistical comparison, two critical points must always be considered—one, time, and the other, place. Therefore, the fact that there are differences in a frequency distribution in May as compared to November, does not decrease the significance of the variations at a particular time. Furthermore, the fact that the cholesterol levels in the United States are quite different from those in the Netherlands does not mean that statistically they are not sound within the context of each place. Consequently, unless a better substitute for statistical analysis than statistical analysis itself is found, I cannot accept the premise that it is not a good approach. Indeed, statistics are indispensable for describing frequency distributions, for describing variations, and, particularly, for describing the role of chance in some of the various relationships in human physiology and medicine. If one is to develop valid statistical associations, one needs to use the statistical method as epidemiologists do in their critical evaluations of quantitative measurements. If one could not use quantitative methods in applying molecular biology to nutrition and other factors in human ecology, we would have to stop doing research.

From the standpoint of administration, I must always consider what I am going to do—in terms of application—with the facts or new information that researchers give me. Unless I can see some use for the output, I do not see any benefit toward the improvement or maintenance of the health of the people. Standards must be used, no matter how arbitrary or crude they may be. It is a little bit like our procedure for planning, programming, and budgeting. We use statistical standards to give administrators some data upon which to base a decision or solve a problem. Both descriptive and analytical statistics are essential in planning and executing health programs. Give us indices of health with varying ranges, under varying conditions, especially time and place, and we then will determine levels of plausibility in the use of these indices in health programs. Health administrators are interpreters as well as implementers.

Chairman Waterlow: I certainly do not deny the value of statistics for many reasons, and especially the two you mentioned. First, they describe a population, and this description we need. Secondly, they help us to establish associations. However, the point I was trying to make was that a description of populations does not help to identify one or another as being "normal" or "abnormal." When you said you would give the statistics to the administrator and let him make of them what he may, I would give you my example of the alkaline phosphatase measurements. No administrator can answer the question, "What really does this mean?" Only the biologist can answer that question. We had to have these figures, but figures alone do not answer the question. I hope I get my message across. I do not deny at all what you say.

Potter: In listing your four approaches, I wondered why you did not mention a fifth approach, which would be a linear time record of the individual. By correlating that record with functional tests, and thereby knowing what the individual's record shows when he is healthy, one would have more information when he is ill. This linear record of the individual would contribute to functional tests and physiological mechanisms as well. This would be particularly helpful, not only for measuring a single factor,

such as alkaline phosphatase, but several factors, to see which parameters were correlated.

Chairman Waterlow: I really meant to include those factors under the heading "associations and long-term consequences." I entirely agree that it should be combined with functional measurements.

Arroyave: I would like to support Dr. Hilleboe's words. When a statement about the characteristics of a population is made—on nutritional status, for example—a statement of probability is being made. That is statistical. We cannot get away from it. Moreover, when a physician, who is more apt to look at the individual case, makes a statement about the characteristics of a patient, he is also making a statement of probability on the basis of knowledge acquired through examination, no matter what or how many parameters he uses. Presumably, he does this with a certain degree of uncertainty, or probability of being wrong. We must remember, furthermore, that even the subject that we are characterizing is in itself a population—a population of cells.

Neel: In the unaccustomed role of peacemaker, I would take a position somewhat intermediate between the two that have been expressed.

Yes, statistics are useful in providing descriptions of populations, but the slide which Dr. Potter showed is extremely important. I believe the slide had reference to dissecting a population curve of acid phosphate levels into subcurves, based on the different genetic types. With one genotype for this system, one will be in the low end of that descriptive curve based on the population no matter what one eats or how one sleeps, but with another set of alleles, one will be in the upper end and nothing can change that.

Statistics improperly used can conceal the heterogeneity, which it is important to understand, if we are going to understand populations.

PHYSIOLOGICAL AND SOCIAL ADAPTATION IN FAMINE

Jean Mayer

There are at least four different categories relating to physiological and behavioral adaptation to famine, and there has been a risk of confusing them though they are, in fact, quite different. The first category is individual variation, the second, seasonal variation. Both of these parameters lend themselves to description by classical statistical methods, and although they have to be accommodated in any elaboration of requirements for a population on a yearly basis, they do not, properly speaking, fall within the concept of adaptation.

The third category in question is adaptation without loss of function, and there are, of course, examples in nutrition of this. People can function quite well on a great variety of diets if these provide adequate amounts of the required nutrients. It has been my good fortune to be the nutritionist for the United States Olympic team, and as such I have observed young people with the most extraordinary variety of diets either self-prescribed, or prescribed by coaches who are more enthusiatic than literate in the field of nutrition. As long as the diets are not deficient, it does not matter very much (at least in the short run) what curious combinations of foods they eat. If they have enough water, calories, and nutrients, they can eat yeast, molasses, or wheat germ oil *à gogo*. If they train hard enough and are determined enough, they will do very well.

It is not always known what the loss of function may be if an odd diet is adhered to for a very long time. Those of us who have been involved, however tangentially, with the nutritional aspects of the space programs have always recommended making the range of adaptation or the need for adaptation as small as possible, at least for trips of a month or more. If the trip is further prolonged, the range of adaptation will certainly be very small as regards oxygen and water and may not be too great as regards food.

There may be situations, one may add, where a loss of function in one respect and an improvement in another may be observed. In any situation where calories are inadequate, for instance, one usually finds a decrease in cardiovascular catastrophies, but an increase in the prevalence of tuberculosis, particularly among adolescents.

Finally, we come to the fourth category—adaptation with loss of function. The loss of function may be less obvious, but eventually equally grave when it is delayed. This may be called adaptation with a long-range cost. Examples abound in developed countries. For hundreds of thousands of years man has spent most of his time roaming around the forest or the desert clubbing deer or running away from tigers. Only for the past ten or fifteen thousand years has he been engaged in agriculture, and it is only in the past generation that most people have become sedentary. The present generation is really the first that spends most of its time sitting down.

Man has inherited an organism which is not adapted to such a life. Two examples of long-range costs from living under conditions to

which the organism is not adapted by natural selection are obesity and atherosclerosis. Inasmuch as most of the conditions that follow from this inactivity occur after the reproducing age, it is quite possible that basically there is no way to adapt through classical methods of evolution to conditions brought about by modern life.

There are genetically determined differences between individuals in the way they can adapt to new conditions. It has been shown, for instance, that with regard to regulation of food intake no individual is really adapted to zero activity. In this respect man is in the same position as the caged animal. Our appetite mechanism which functions quite well in adjusting intake to output at moderate activity levels does not function at zero activity. But it functions better for some individuals than for others. Those individuals who are characterized by anthropologists as ectomorphs seem to be better adapted to very low levels of activity in terms of not being likely to become obese. They also appear to have a lower expectancy of coronaries than mesomorphs or endomorphs. Thus, one can say that ectomorphs are better adapted to new conditions that in this case are brought about by social changes.

This last point leads me to the social dimension of nutrition. If the human race has not yet progressed to a real consideration of the implications of being a family—and a crowded family at that—traveling in a small spaceship in an extremely empty and hostile universe, at least it is becoming more conscious of the sociological matrix in which nutrition takes place.

Social factors determine first of all how many people there are. Man is a social animal, he lives in communities, and nutrition is a social and economic activity as much as an individual one. Hence, at some point, social factors in adaptation should be discussed if we wish to be realistic and constructive.

It is sometimes very difficult to differentiate in practice between individual physiological and behavioral adaptation and the reaction of society as a whole to a particular nutritional situation. Famine provides a very good illustration of the interaction between individual and social phenomena in what is, after all, the most extreme and the most disastrous of all nutritional situations.

I have had the very doubtful fortune of seeing several famines in my life, and further, of being involved in relief in a number of them. The last was in Biafra in the spring of 1969. I have been fairly continuously involved in relief work in that country through the slow recovery period of the past few months.

One all too striking fact about famine is how reproducible the situations are in terms of what happens, not only to individuals, but to society. It is very saddening to see how little the experience of one famine is applied to the next. Everything has to be reinvented every time when, in fact, what happens is depressingly similar.

Famines occur by and large in the same areas periodically. They are caused, in large measure, by widespread crop failures because of lack of water. Droughts are followed by dust storms and loss of seed. Some of the causes of recurrent famines have been made worse over the years, and in the areas of periodic crop failure erosion has made famines more likely.

Other famines are due to destruction of crops by various diseases or by pests (fungus and locusts, for example), by disruption of farming operations caused by wars and civil disturbances, or to a combination of factors affecting both crops and farmers as a result of great natural disturbances such as floods and earthquakes.

Many authors, Ancel Keys in particular, have counted the known famines over the centuries, and observed that there has been at least one serious famine somewhere every year throughout recorded history.

Social inequality, which is a characteristic of underdeveloped countries even more so than it is of developed countries, makes the social aspects of famine considerably worse. By and large, the ruling groups tend to escape the catastrophe, and therefore, they are slow to react to the problems.

Concretely, what physiological changes in the individual occur in a famine? First, there is a

wasting of fat deposits starting with the subcutaneous adipose tissue, with the deeper fat pads being affected as well. Then the abdominal and thoracic organs are affected; liver size, for example, drastically diminishes. Reduction of organ size is in part a reflection of the body burning what is available. This process has an adaptive value in that, to a certain extent, it permits lower maintenance at that particular level.

Following these changes, the intestinal mucosa becomes thin and smooth, hence it loses some of its absorptive capacity and diarrhea results. Gastric achlorhydria too is generally present. These changes mean that what little food is available is poorly utilized. Far from being adaptation, these occurrences represent a vicious circle which accelerates further decay: this involves brown atrophy of the heart so characteristic of starvation, the fall in blood pressure—with the systolic pressure in some cases becoming difficult to estimate, and the diastolic pressure dropping perhaps as low as 75 millimeters of mercury—and finally, a drop in the pulse rate. Edema then occurs. This is due to possible disruption of osmotic tension, lack of elasticity of the skin, renal and endocrine disturbances, and the like.

Allow me to stress that none of the above has any perceptible adaptive value. They represent distinct pathology which eventually may become irreversible. Certainly, other symptoms —growth of lanugo hair and finally cancrum oris—are straight pathology.

The behavioral aspects of malnutrition are ambivalent. Though they may have some adaptive effects or benefits for the individual, in many cases they are very dangerous to society as a group. The individual becomes obsessed with food, mentally restless, physically apathetic. This means that his energy expenditure is reduced. In turn, there is a depressed requirement for food because of the decreased body weight, decreased metabolic rate, and decreased activity. These factors eventually cause a plateauing of body weight if the famine is not too acute.

If this is adaptation, then one could say that most adaptation to starvation is behavioral rather than physiological, and one could also say that this form of adaptation, although it may protect the individual, may, in many cases, be dangerous to the survival of society and therefore, in the long run, not particularly helpful to the individual.

The disorganization of society which takes place in such situations means that infection is much more likely to become rampant, families become disorganized, children get lost, and adolescents classically form gangs and are driven to bandit-like practices.

Unless society as a group organizes itself, or is organized from the outside, by the creation of small famine hospitals, by spreading its medical personnel, by keeping good records so that whatever supplies are available can be directed into the areas which are most in need, famine, as a social problem, is not going to be dealt with.

In summary, there are situations, as exemplified by famine, where society itself has to adapt to cope with problems. This is especially the case where individual adaptation may not necessarily function in accordance with the best interests of social adaptation.

Although famine is the most dramatic example of failure to adapt, others come to mind. When one considers the enormous mortality rate from cardiovascular diseases, especially atherosclerosis, it becomes obvious that social adaptation to the new conditions in which man is living is a *sine qua non.* In our own society, this means planning cities, which are pleasant to walk in rather than simply convenient to park in and, in general, creating a mode of life for our children different from the one the present generation has drifted into.

DISCUSSION

Hoffenberg: Could Dr. Mayer say anything about possible long-term effects of famine? One knows, for example, that there are reported effects on brain size and intellectual capacity after infantile protein malnutrition, and I wonder whether there are any known long-term effects on survivors from famines of the sort you describe?

Mayer: When you are in the middle of a famine as acute as the recent one in Eastern Nigeria, it is difficult to believe that those children who have survived, have survived unscathed. I have been in villages where basically all the children under six were dead. Incidentally, it is only when you start doing epidemiological studies that you realize how bad the situation is. The casual observer walking in the village may think that everything is in reasonably good shape, and it is only when you start doing age group distributions that you fully realize the problem. Those few children who survived would seem to be candidates for the usual sequelae of acute starvation. To my knowledge, no one has every really undertaken a study of the sequelae of famine. As a matter of fact, the amount of epidemiological information that we do *not* have is enormous. In the Biafra famine, the mortality from measles, for instance, was between 25 and 50 per cent. I have no data for comparison, however, on the number of irreversible complications from measles following starvation compared to those in normal children. There have been a number of theories. One concerns liver cancer and the possibility that periodic starvation may be one of the factors in predisposition to primary cancer of the liver. I think none of these have received any verification. Perhaps our Chairman would know more about it than I do. Nonetheless, I believe the information is very, very poor.

Zubirán: Those of us who have closely viewed malnourished populations have seen the consequences of which Dr. Mayer spoke a moment ago. Our children in many of the rural villages in Latin America, and I refer specifically to those of Mexico, live under conditions of food shortage similar to those of acute famines, but they live it daily throughout the year. Many of these children reach adulthood with chronic aftereffects, which still retain most of the characteristics of the acute state. Those children who continue to live on an inadequate diet are shorter in stature, lower in weight than normal, have a lower mental capacity, and consequently, throughout their lives, have a reduced ability to adapt to environmental stress.

Tourists invariably take pictures of Mexicans dozing with crossed arms under a big sombrero. This posture takes less effort socially, and is, in fact, an adaptation of a malnourished people. These consequences from malnutrition are translated into a reduced work capacity, an inability to maintain sustained effort, and also, no doubt, give rise to social retardation. Such people do not share in the development of their country because of their physical incapacity and because of their need to adapt to an inadequate diet.

Another result also pointed out by Dr. Mayer is an insufficient defense against disease. One finds a higher mortality, irrespective of the disease, but particularly from measles. Here the human has undergone adaptation, both clinical and social, in order to survive under centuries of insufficient dietary conditions.

Chairman Waterlow: Dr. Mayer, would you like to reply to these remarks? You asked me whether I could give any information about the long-term effects of famine. The answer is I do not have the information any more than you.

Mayer: It is very likely that acute starvation, or continuous malnutrition, as Dr. Zubirán dealt with, is responsible for many of the phenomena that we observe—decreased stature, greater susceptibility to this or that disease, greater prevalence of congenital malformations, and greater prevalence of children with low IQ's. One can say in a few cases that there is a clear cause and effect relationship between malnutrition and the above deficiencies. In many cases, however, this relationship can only be inferred through fairly constant epidemiological association. All of this means that we have to consider the cost of adaptation. It is not enough to say that some individuals seem to function very well with almost no detectable vitamin A level in their blood. First, we do not know whether all of them would. Secondly, to get back to Dr. Hilleboe's question, if you are cast in the position of an administrator, there are chances you are willing to take and chances you are not willing to take.

Although adaptation is an obvious fact, it may not apply to all nutrients. In terms of the first law of thermodynamics, adaptation to low calories has to be at the cost of size, or at the cost of activity. Size may not by itself be a great benefit. One may say that in a crowded world maybe we should strive to make people smaller and smaller so that we could accommodate the population explosion for one more generation. But whatever the theoretical arguments, there is not very much we can do about the fact that people want larger children, particularly larger boys. If activity is decreased by social factors, as it is in this country, it is at a certain cost as, for example, obesity, increased prevalence of heart disease, and perhaps diabetes. This decrease in activity has a number of undesirable effects on human physiology and perhaps on mental health. Certainly, none of us would advocate that physical activity be further reduced by malnutrition, as is the case in the populations that Dr. Zubirán described.

I do think, Mr. Chairman, that we should have the session address itself to only two questions: What are the limits of adaptation without long-term or short-term physiological cost? What is the cost of adaptation beyond that particular level, and is this an acceptable cost? You raised the example earlier today of the pH and electrolyte concentrations of the blood as examples of regulations where the limits of normal are well-known in view of the disastrous consequences of going beyond them. Another analogy is the regulation of body temperature. There is a zone of thermal neutrality in which vasoconstriction, vasodilation, or in the case of man, the putting on of a coat, or not putting it on permits effective control of body temperature without any additional thermal energetic cost. This is one zone of "adaptation," the "thermoneutral" range. There is also another range on both sides where we can adapt, but at some cost. We have to eat more in the cold and, on the other hand, we have to reduce drastically our physical expenditure in the heat, particularly in humid heat. But there are the extreme zones where, in effect, we cannot adapt. Both extreme cold and extreme heat lead to death. It seems to me we may have an analogous situation regarding nutrition. Moreover, we must consider social adaptation as well as individual adaptation.

STANDARDS FOR THE DIAGNOSIS OF VITAMIN DEFICIENCY IN MAN

Guillermo Arroyave

Introduction

The spectrum of vitamin deficiency states, as any other nutritional deficiency, extends between two theoretical limits: that of optimal nutrition, when the metabolic needs for vitamins are amply satisfied, and that of extreme deficiency, when the lack results in such severe metabolic and organic alterations as to interfere with the life process. In between, however, there is a continuum approaching either extreme. This concept is suggestive of the difficulties involved in the definition of standards, since the implication is that they must be quantitative and express in measurable terms the distance between the particular situation of an individual or a group from either or both of these two extremes.

It is evident, then, that the first task in the elaboration of standards is to define as clearly as possible the characteristics of the subjects that exist at the two extremes. The second would be to develop means to express, quantitatively, deviations from them. To define these points, several criteria have been used that, at the risk of oversimplification, can be classified as follows: dietary, biochemical, clinical, and functional. I have separated functional criteria from the rest, despite the fact that good function is dependent on the normality of the biochemical aspects of the organism, but the expression of these functions is dynamic and may be best referred to as "performance," whether this be biochemical, physiological, physical, mental, or otherwise. Actually, functional tests should provide the ultimate criteria, but unfortunately, they are also the most difficult to evaluate.

In view of the latter difficulty, efforts have been made to develop *indices,* particularly dietary and biochemical. In general, these are numerical expressions of measurable characteristics which ideally are, or may be, related to a state of functional alteration, but which are practical to apply to human subjects. They express arbitrary estimates of the position occupied by the subjects in the spectrum delimited by the two extremes mentioned above. Unfortunately, this relationship of the indices with function is as yet in many instances poorly defined.

Clinical criteria

Clinical criteria are of limited use as standards for various reasons: (a) they are often unspecific and hence seldom pathognomonic of a particular deficiency. Other factors in the physical and microbiological environment may result in the same or similar alterations; (b) the concomitant presence of several deficiency states obscures the appearance of clear-cut clinical signs, thus making their interpretation doubtful. Unfortunately, this is often the case with human populations; and (c) in general, their presence in the individual reflects the already extreme state of deficiency and, therefore, they do not

lend themselves to the evaluation of the gradient-type of situation characteristic of vitamin deficiency.

There is, however, one situation in which clinical signs may lend themselves to mathematical, or rather to statistical treatment. When dealing with population groups, and provided that their nutritional origin can be demonstrated, prevalence figures could conceivably be set up as standards. The bases for defining these limiting acceptable prevalences are still empirical, but they have value in comparative studies of population groups. An example of such an effort by Jolliffe is given in Table 1 (*12*). I will not comment on the soundness of the specific prevalences proposed. They are several

Table 1

Clinical criteria of nutritional status

Nutrient	Clinical sign	Abnormal
Calories:		
Obesity	*Skin thickness:*	
	Scapulae	Over 30 mm.
	Lower Axillae	Over 25 mm.
	Height-weight tables	Over 10 percent.
Leanness	*Skin thickness:*	
	Scapulae	Under 8 mm.
	Lower Axillae	Under 8 mm.
	Height-weight tables	
Protein	*Dependent edema*	Over 0 percent [a] under age 50. In absence of beriberi starvation, and pregnancy.
Vitamin A	*Follicular Keratosis* of arms	Over 5 percent[a] in adults and pre-adolescents. Not reliable in starvation and in adolescents.
Vitamin D	Under six months *Craniotabes*	Over 0 percent.[a]
	Six mo. to two years *Beading of the ribs.*	Over 0 percent.[a]
	Over two years ( ) *and X deformities of legs.*	Over 0 percent.[a]
Thiamine	Absent Achilles tendon reflexes.	Over 1 or 2 percent.[a]
Niacin	*Tongue lesion more advanced than hypertrophy of papillae at tip.*	Over 5 percent.[a]
	Reddened tongue	Over 1 or 2 percent.[a]
	Pellagrous dermatitis	Over 0 percent.[a]
Riboflavin	*Angular stomatitis*	Over 5 percent [a] in non-denture-wearing population.
	Conjunctival hyperemia (circum corneal infection).	Over 5 percent.[a]
	Magenta tongue	Over 0 percent.[a]
	Red hyperemic gums	Over 1 or 2 percent [a] in children.
Ascorbic acid		Over 5 to 10 percent [a] in adults.
	Perifolliculosis	Over 0 percent.[a]

[a] An educated guess of frequency that these signs might occur in a well-nourished population group.

From: *Methods of Evaluation of Nutritional Adequacy and Status.* National Research Council (U.S.), 1954.

years old, and perhaps the time is due for a revision of these standards by clinical nutritionists.

Dietary and biochemical criteria

I deem it impossible to discuss these two approaches separately because in most instances the criteria for establishing the dietary standards have been based on biochemical alteration.

Efforts have been directed towards the determination of some biochemical characteristics of subjects who are at the lower limit of the spectrum of deficiency, and there is a great deal of information on this point for many vitamins. The determination of the other extreme, the extreme of sufficiency, is somewhat more vague. Here we either have a level of intake of a vitamin which will be sufficient to satisfy all metabolic needs and no more, and which will determine certain biochemical characteristics of the subjects, or, on the other hand, we can have an excess of the vitamins which produce no harm but no further benefits either, and that will, nevertheless, change some biochemical measurements. The danger here lies in not being able to discriminate between sufficient and excessive and thereby running the risk of setting unduly high standards. I have partaken in the efforts of at least two scientific groups that have proposed standards to assess vitamin deficiency or adequacy, and I guarantee that, to the extent of our present knowledge, this danger has been carefully considered.

The next question is how to establish standards to judge situations in between these two limits. In reviewing the literature, I have come to the conclusion that the prevailing criterion has been based on the following: (a) values occurring in individuals who are in a definite deficiency state. These have been produced experimentally both in animals and humans. Conditions of practically "complete depletion" have been obtained in which histological and physiological alterations are clearly evident; (b) values occurring in individuals who present no evidence whatsoever of abnormality, whether physical or physiological, and who furthermore possess a certain amount in concentration of the vitamin in question in their body or specific tissues which acts as a reasonable insurance against rapid depletion under conditions of deprivation; and (c) the intermediate values between the upper range of values under (a), and the lower range of values under (b) may be and in general are considered indicative of undesirable situations at "risk" to a smaller or larger extent. Subjects in these intermediate stages are in situations that can be changed from a doubtful status by increasing the supply of the nutrient to the body.

It would be impossible in the allotted time to review the approaches which have been used by groups of experts or individual investigators to set up dietary standards and corresponding biochemical indices for all the vitamins concerned. Depending on the extent of our knowledge, these proposed standards range from what could be considered "educated guesses" to relatively well-documented figures.

Derivation of standards

Only three cases will be discussed. These can be taken as models of what has been done to elaborate standards, perhaps under the best circumstances, that is, when a relatively adequate amount of knowledge has been available. There are less fortunate cases, but emphasis is made on one point: whatever information is available now with regard to standards cannot be improved by discussion or criticism; it has to be done through research.

Ascorbic acid

There is still much controversy regarding the amount of ascorbic acid to be recommended as an adequate intake for humans. I believe that this controversy is due more to differences of opinion than to contradictory data. If we take two standards, those of the National Research Council of the United States (NRC) (*17*) and those of the Canadian Council on Nutrition

Table 2

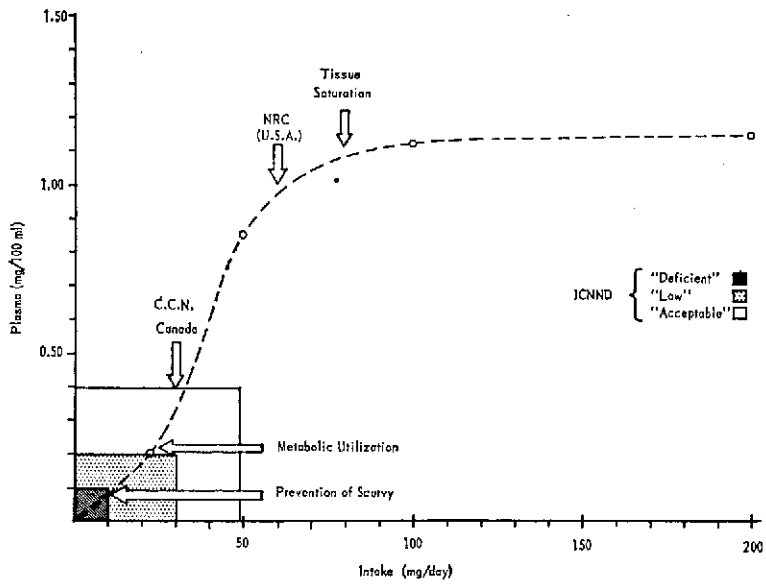
Guides for the interpretation of ascorbic acid intakes and plasma levels (young adult male)

	Deficient	Low	Acceptable	High
Dietary intake (mg/day)	<10	10–29	30–40	≥50
Plasma concentration (mg/100 ml)	<0.10	0.10–0.19	0.20–0.39	≥40

From United States Interdepartmental Committee on Nutrition for National Defense of the United States of America (ICNND). *Manual for Nutrition Surveys*, 2nd ed., Bethesda, Md., 1963.

(CCN) (*5*), it can readily be realized that their traditionally divergent standards are both derived from the same data. While NRC recommends a daily intake of 60 mg for the adult man, the Canadian standard recommends exactly half. Another United States group, the Interdepartmental Committee on Nutrition for National Defense (ICNND) (*11*) went somewhat further, proposing categories of levels of intake and corresponding expected plasma ascorbic acid concentrations which could be used as indices. These are shown in Table 2.

What are the data on which these standards are based? In Figure 1 are represented some of the pertinent associated phenomena in relation to levels of dietary intake. The data are mainly those from Lowry *et al.* (*15*) and Rally *et al.* (*18*). The intake which results in maximum plasma levels with minimum urinary loss is approximately 80 to 100 mg/day. Levels of 8 to 10 mg are sufficient to prevent scurvy. Information regarding intakes below that level is scarce and doubtful, but there is some evidence that four mg/day is not enough for adequate reversal of some clinical alterations (*7*). Studies with ^{14}C ascorbic acid indicate, further-



Based on data from Lowry et al. J. Biol. Chem., 166: 111, 1946, and Rally et al. Proc. Soc. Exp. Biol. Med., 40: 604, 1939.

Figure 1. Relation of intake of ascorbic acid and blood plasma concentration.

more, that the actual metabolic utilization by healthy men is around 22 mg/day (*2, 3, 19*).

In support of the recommendation of the NRC for the higher standard, two main arguments have been brought forward: (a) that animals that can synthesize ascorbic acid maintain a tissue concentration at saturation levels; and (b) that the higher amount provides a margin of safety in cases of increased need and also protects a proportion of individuals who, by the mere fact of individual variability, may have a higher requirement. In favor of the lower standards are the following arguments: (a) that 30 mg is at least three times the intake needed to prevent scurvy; (b) that it provides an amount somewhat above the ascorbic acid actually metabolized daily by a normal man; and (c) that it has not been possible to demonstrate satisfactorily any further benefits from amounts in excess of this standard.

Thiamine

Several criteria have been considered in the determination of standards for thiamine nutrition. Among the most informative we may list: (a) the attainment of a point of minimum urinary excretion of the vitamin. It is based on the observation that as the thiamine intake is gradually lowered from a high level, the urinary excretion decreases proportionally to a point after which further lowering of the intake results in only minor changes in urinary output. This point is assumed to represent the minimal intake of thiamine that will maintain the tissues with a physiologically adequate thiamine concentration. Intakes higher than this critical point result in spilling and wasting of the vitamin; (b) closely related to the above criterion are the so-called dose-retention tests. They are conducted by the administration of a dose of thiamine either orally or parenterally, and the determination of the percentage of the dose that is excreted in the urine during the subsequent four hours. The values obtained are also supposed to represent the extent of saturation of the tissues with thiamine; (c) appearance of abnormalities in carbohydrate metabolism. These abnormalities are judged mainly by alterations in the concentrations of pyruvate and lactate in the blood and are particularly useful when the metabolic paths involved are subjected to a metabolic stress, such as a load of glucose, plus physical exercise. Recently, transketolase activity in erythrocytes has been shown to depend on a minimum intake of thiamine, but so far no direct application to the estimation of minimum requirement has been reported; and (d) appearance of early clinical signs such as impaired appetite, lowered blood pressure, slight edema, diminution of deep reflex activity, and dulling of vibration sense. The estimation of plasma and red blood cell thiamine has also been used, but they appear to be too insensitive.

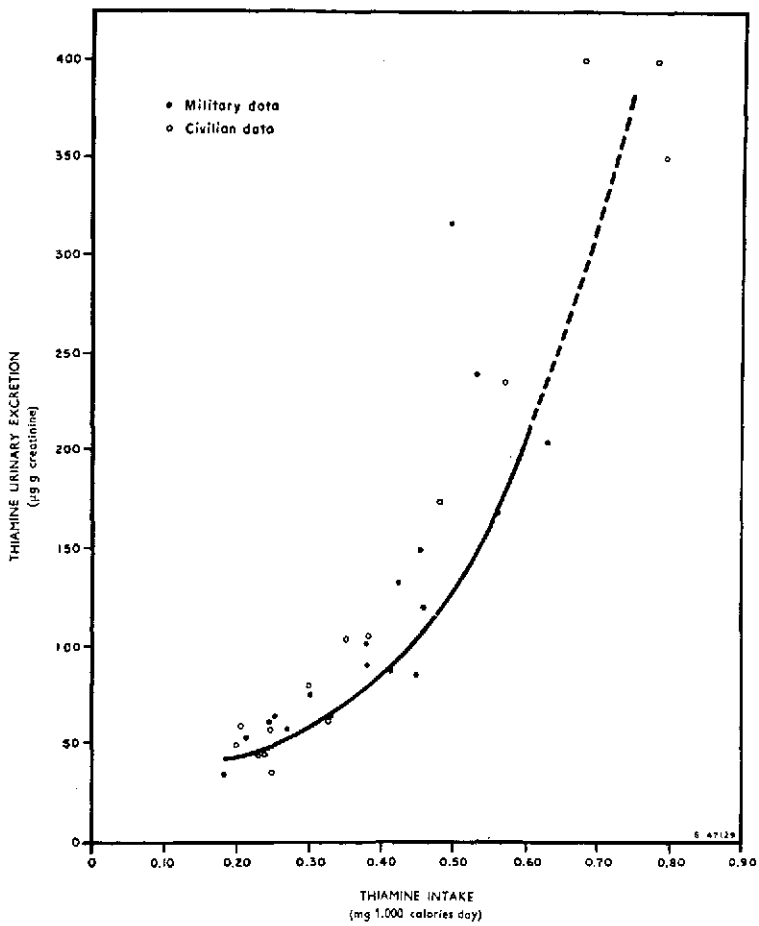
Several independent groups of investigators have searched for the minimum daily intake required by humans using either one or a combination of the above criteria in a comprehensive approach.

There is general agreement in expressing the requirements as mg daily per 1000 calories. The available evidence has been reviewed and carefully analyzed by several groups of experts. There is little question that intakes of 0.3 to 0.5 mg of thiamine/1000 calories are about the highest range in values reported as a minimum requirement. One exception is the work of Williams *et al.* (*21*), who concluded that even 0.45 mg/1000 calories was slightly less than required. Their criterion, however, is open to question, because it was based on an arbitrary set excretion level in the urine which has no definite basis and on a slight elevation of the blood pyruvate after a glucose load, which may not necessarily be abnormal. Values of 0.20 to 0.25 mg can be considered as the lowest range of the values reported as minimum intakes. Keys *et al.* (*13*), for example, conclude that "for the periods studied, no benefit of any kind was observed to be produced by an intake of more than 0.23 mg of thiamine/1000 calories." These authors state, however, that no conclusions can be drawn for more prolonged

periods of time. Their observation period lasted only from 10 to 12 weeks. Another very carefully conducted study in adult men in which levels in this low range were evaluated by comprehensive criteria was that of Horwitt and his group (*9*), who concluded that 0.40 mg of thiamine was below the minimal requirement for subjects who were consuming about 2000 calories/day (about 0.20 mg of thiamine/1000 calories).

An interesting observation is that the extrapolation to man of the formulas developed by Cowgill in 1934 for mice, rats, pigeons, and dogs indicate that the calculated thiamine requirement for a 60 kg woman and a 70 kg man would be 0.25 and 0.30 mg/1000 calories respectively (*16*).

The stimulatory effect produced by the addition of thiamine pyrophosphate (TPP) to the transketolase activity of red blood cell hemolyzate is increased under conditions of dietary thiamine restriction (*4*). Data are beginning to appear indicating that a TPP effect of more than 20 per cent correlates with "deficient"



[1] Data from civilian and military groups surveyed by the Interdepartmental Committee on Nutrition for National Defense of the United States of America.

From: FAO Nutrition Meetings Report Series No. 41.

Figure 2. Relationship between thiamine intake and thiamine urinary excretion in adults of 18 countries.

thiamine intake and excretion, and more than 25 per cent may be regarded as an index of thiamine deficiency (*6*).

Reference to other works only serves to confirm what has already been stated. This discussion leaves a situation of relative agreement, but the decision regarding a minimum requirement figure still has to be made. A figure corresponding to the lowest range mentioned, of approximately 0.20 mg/1000 calories, might be selected. This is the position adopted by the CCN (*5*), which states that the results of the available studies indicate that "for the adult person 0.2 mg of thiamine/1000 calories is at the lower limit of adequacy and that 0.3 mg/1000 calories is near the level to produce tissue saturation, and is undoubtedly adequate." On these bases the Council adopts 0.3 mg/1000 calories as the recommended amount.

It is interesting to note that in the latest edition of the *Recommended Dietary Allowances*, the National Research Council of the United States of America (NRC) (*7*) also bases the "dietary allowance" for thiamine on the evidence that the minimal requirement approximates 0.2 mg/1000 calories. The NRC, however, recommends 0.5 mg/1000 calories on the assumption that this amount maintains whole blood thiamine levels and permits relatively high urinary excretion.

The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Group on Requirements of vitamin A, thiamine, riboflavin, and niacin (*7*) proposes 0.4 mg/1000 calories. They state that on the basis of available evidence, a value of approximately 0.33 mg/1000 calories represents the requirement and "with an allowance of 20 per

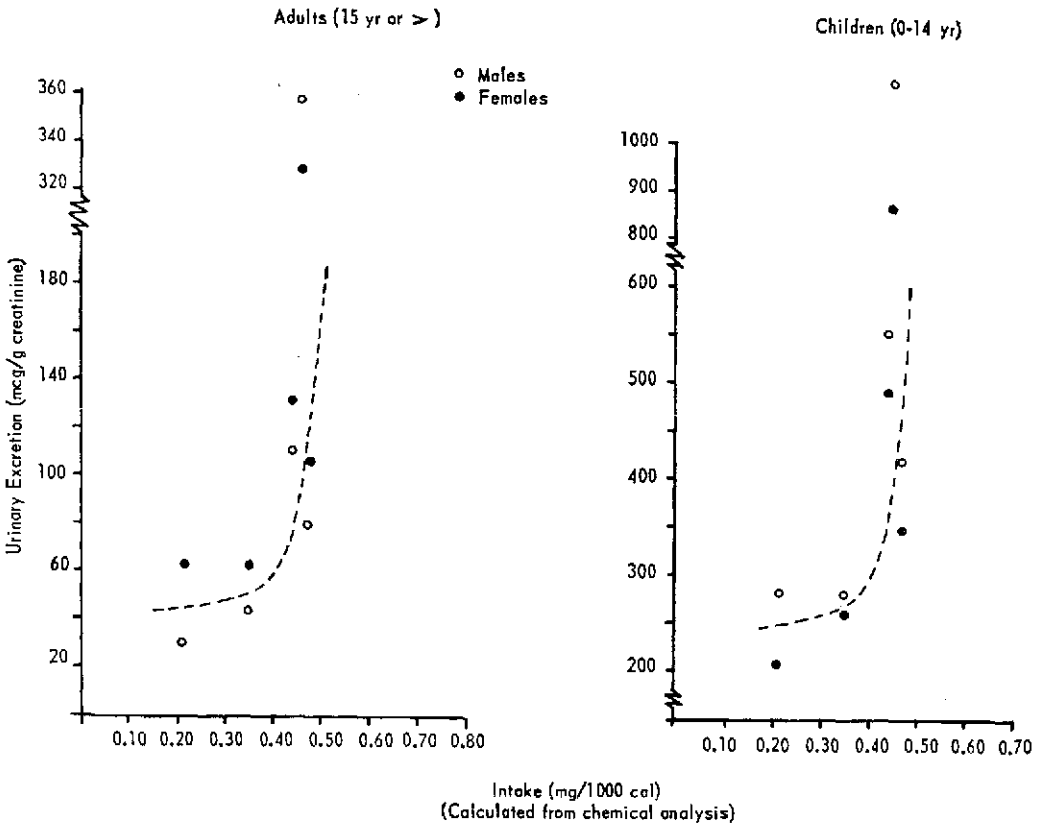


Figure 3. Relationship between intake and urinary excretion of thiamine in Central America.

cent for individual variation, the recommended intake for thiamine is 0.40 mg/1000 calories."

Data obtained from nutrition population studies seem to support this "critical" relationship between intake and urinary excretion. Figure 2 shows data collected by ICNNDUSA (*11*). These data are for adults only and were collected by different groups from 18 communities. In the Nutrition Survey of Central America and Panama, it was possible to derive similar relationships for a large number of subjects of different ages using the same biochemical and dietary techniques (*1*). The results are illustrated in Figures 3 and 4.

In fact, the "critical intake," that is, the level at which the tissues become "saturated" (the excess thiamine being excreted in the urine) is usually found to be around 0.35 mg/1000 calories, that is the mid-point between the Canadian and the FAO/WHO recommended figures. Measurements of the thiamine metabolites excreted in the urine have indicated that the actual metabolic utilization of the vitamin is approximately 0.33 mg/1000 calories/day and suggest this as the minimum daily requirement (*22*). A model curve illustrating this relationship is shown in Figure 5, which also presents the intakes recommended by the CCN, NRC, and FAO/WHO. The ICNND guide to interpretation of thiamine intake data is also included. Its scientific basis is quite evident. We must emphasize again that these figures are all based on the same set of data and that the difference between the views of the two groups is a question of balance between the degree of risk accepted as reasonable and the feasibility of attaining in practice an intake to meet a relatively high recommendation.

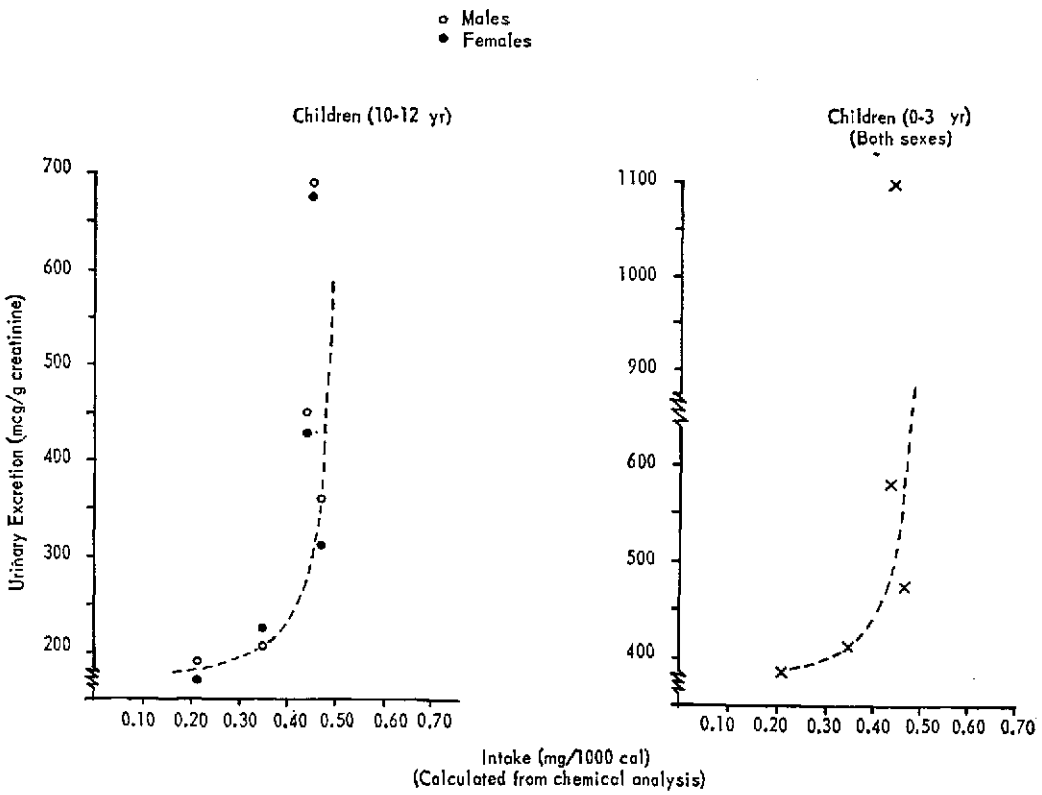


Figure 4. Relationship between intake and urinary excretion of thiamine in Central America.

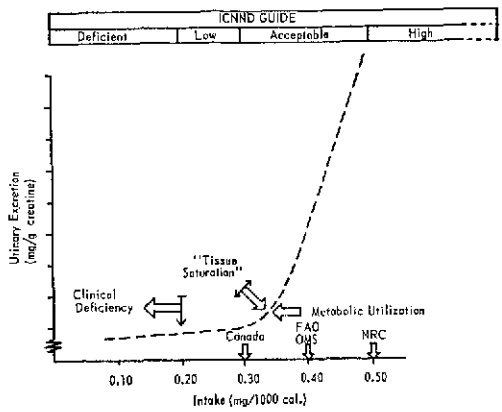


Figure 5. Relationship of thiamine intake and urinary excretion.

Vitamin A

In theory, the standards for the assessment of vitamin A deficiency should be based on the interrelationship of four types of data: dietary intake, concentration of vitamin A in liver, blood levels of the vitamin, and the appearance of clinical signs, including abnormal dark adaptation, which could be considered as a functional test.

In practice, the data available are difficult to interpret because of several factors: (a) vitamin A accumulates in liver tissue, and can be used as a reserve supply for other tissues for variable periods of time, depending on the extent of the reserves; (b) there is a need for more studies in humans to establish the quantitative relationship between vitamin A serum levels and vitamin A liver reserves, as well as to determine factors other than nutrition which affect this relationship; and (c) the scarcity of information on the biological activity of the carotenes as provitamin A compounds.

In this discussion I will follow the recommendation of the Joint FAO/WHO Expert Group that, because of the availability of crystalline vitamin A alcohol (retinol) as a reference standard, the practice of expressing vitamin A values in terms of international units (IU) should be discontinued.

Intakes of compounds with vitamin A activity must, therefore, be expressed in "retinol equivalents." The retinol equivalency of the different carotenes will not be discussed here. The reader is referred to the literature (*7*).

The determination of blood plasma concentrations of retinol is still the only feasible biochemical tool to assess the state of vitamin A nutrition. I will try to describe the situations along the spectrum of vitamin deficiency states. At the upper extreme, sufficiency is obtained with an intake which will be compatible with normal function, particularly dim light vision and with complete absence of clinical signs. Furthermore, it is accepted that at that level the liver should contain significant amounts of retinol. After reviewing thoroughly the available evidence, the FAO/WHO Expert Group (*7*) concluded that an intake of 750 μg of retinol (2500 IU) ensures such a situation in the human adult. This figure was proposed by them as the daily recommended intake. The Sheffield study (*20*) is the main source of data on which these conclusions are based. The intake of 750 μg/day in two individuals maintained the plasma retinol levels at approximately 25 to 27 μg/100 ml. Another "landmark" to be derived from these studies is the plasma level of 15 μg/100 ml (50 IU). To quote: "A fall of the average plasma level to below 50 IU (15 μg) per 100 ml in every case preceded the deterioration of dark adaptation by a few weeks, and no abnormal values for dark adaptation were noted in any subject as long as the plasma level of vitamin A remained above 50 IU/100 ml." The next lower "landmark" set by the Sheffield study is 12 μg (40 IU/100 ml). Since this was the average plasma level found at the time, the deterioration of night vision was pronounced. Furthermore, none of the deprived individuals whose dark adaptation remained normal had average plasma levels below 12 μg/100 ml.

In the literature reviewed, a few other observations in humans do little more than confirm the general magnitude of the figures given above. For example, the retinol levels of large

Table 3

Plasma vitamin A (μg/100 ml)

Deficient	Low	Acceptable	High
<10	10–19	20–49	≥50

From ICNND. *Manual for Nutrition Surveys*, 2nd ed., Bethesda, Md., 1963.

groups of urban children or adults in Central America fall mostly between 20 and 50 μg/100 ml (*10*).

As seen by the Group of Experts of the ICNND (*11*), these data would support the guide to interpretation of blood vitamin A data shown in Table 3.

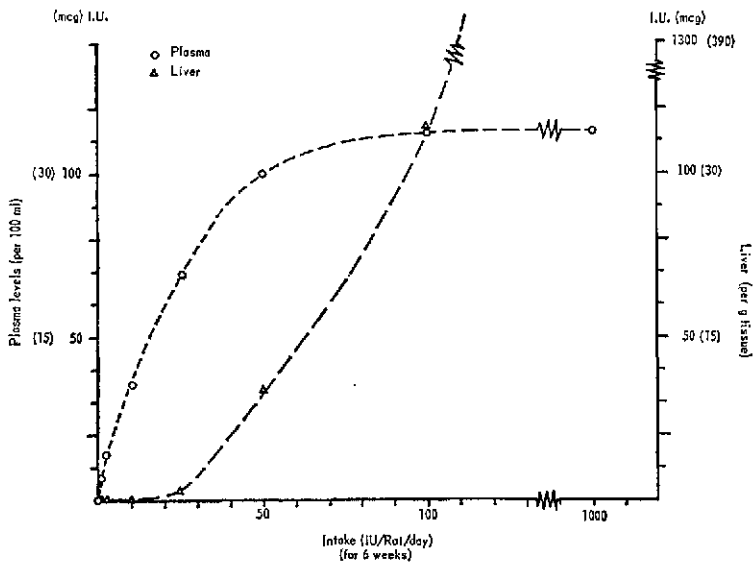
This is a reasonable approximation of great practical value. In Figure 6, data from Lewis *et al.* (*14*) indicate that intake and plasma levels relate quite linearly to each other up to plasma levels of 25 to 30 μg/100 ml. Liver retinol behaves differently, since it does not begin to accumulate until the plasma values are around 18 to 20 μg/100 ml. These data permit three conclusions: (a) that a low plasma level results from a low intake, and is undesirable because it means a low vitamin A availability to the tissues and the absence of reserves in the liver; (b) that a higher intake of vitamin A, sufficient to elevate significantly the plasma levels, does not necessarily result in appreciable liver reserves ("risk situation"). Under these conditions, the supply of retinol to the tissues will be operating, but in terms of the economy of vitamin nutrition of the population, the situation is one of risk; and (c) that with an even higher intake (upper extreme of the spectrum), both high plasma and liver retinol levels are ensured.

Table 4

Intake of retinol equivalents

Deficient	Low	Acceptable or high
<390	390–740	≥750

From the Sheffield experiments (*20*) it can be deduced that intakes of around 390 μg/day (1300 IU) of retinol would place the subjects under situation (b). One could also suppose that intakes below that would eventually lead to depletion of hepatic reserves.



Based on data from Lewis et al., J. Nutrition, 23: 351, 1942.

Figure 6. Relationship of vitamin A intake to blood plasma and liver concentration.

A reasonable guide to interpret "retinol equivalent" intakes (μg/day) would be, for the FAO reference man, that presented in Table 4.

Comments

Other standard values have been constructed on bases similar to those illustrated here. The Appendices include those which have been proposed by ICNND for some vitamins of public health significance, those for blood serum and red blood cell folates, and for serum vitamin B_{12} used by the Institute of Nutrition of Central America and Panama (INCAP).

In conclusion, I would like to state that the standards are to be recognized for what they are. They are "yardsticks" against which to assess the relative position of subjects in the spectrum of vitamin deficiencies. Furthermore, they define a goal, or the position in the spectrum which, within reason, represents the smallest probability of being deficient or at risk. They are most useful when taken as the basis for the statistical expression of the nutritional characteristics of a population.

It is not my intention to defend the specific figures proposed, but rather to emphasize the soundness of the principles involved in their development as well as the general usefulness and limitations of the standards.

Appendices

INCAP [a] suggested guides to interpretation of blood folates and vitamin B_{12} (all ages)

Constituent	Deficient	Low	Acceptable or high
Serum folates (ng/1 ml)	<3.0	3.0–4.9	≥5.0
Serum vitamin B_{12} (pg/1 ml)	<100	100–149	≥150

[a] Prepared by Drs. Fernando Viteri and Jorge Alvarado, Division of Biomedical Research, INCAP.

Suggested guide to interpretation of vitamin intake data for reference man

	Deficient	Low	Acceptable	High
Ascorbic acid: mg/day [a]	<10	10–29	30–49	≥50
Thiamine: mg/100 cal [a]	<0.20	0.20–0.29	0.30–0.40	≥0.50
Riboflavin: mg/day [a]	<0.7	0.7–1.1	1.2–1.4	≥1.5
Niacin: mg/day [a]	<5	5–9	10–14	≥15
Retinol equivalents: μg/day [b]	<390	390–740	≥750	

[a] ICNND

[b] Suggested by author of present paper.

ICNND suggested guide to interpretation of blood vitamin data [a] (young adult males)

Constituent	Deficient	Low	Acceptable	High
Plasma ascorbic acid: mg/100 ml	<0.10	0.10–0.19	0.20–0.39	≥0.40
Plasma vitamin A: μg/100 ml	<10	10–19	20–49	≥50
Red blood cell riboflavin: μg/100 ml	<10.0	10.0–14.9	15.0–19.9	≥20.0

[a] Serum levels of nutrients in children do not differ appreciably beyond infancy from those of adults. Similarly plasma levels of these blood constituents in women of child-bearing age are comparable to those of males. No guide is suggested by ICNND for RBC riboflavin during pregnancy. For ascorbic acid and vitamin A, the same values are given.

ICNND suggested guide to interpretation of urinary vitamin excretion data

	Deficient	Low	Acceptable	High
		Adults (males and non-pregnant, non-lactating females)[a]		
Thiamine:				
μg/6 hours	<10	10–24	25–49	≥50
μg/gm creatinine	<27	27–65	66–129	≥130
Riboflavin:				
μg/6 hours	<10	10–29	30–99	≥100
μg/gm creatinine	<27	27–79	80–269	≥270
N-Methylnicotinamide:				
mg/6 hours	<0.2	0.2–0.59	1.6–4.29	≥4.3
mg/gm creatinine	<0.5	0.5–1.59	0.6–1.59	≥1.6
		Provisional guide for urinary excretions in children[b]		
Thiamine: μg/gm creatinine:				
Age (years):				
1–3	<120	120–175	176–600	>600
4–6	<85	85–120	121–400	>400
7–9	<70	70–180	181–350	>350
10–12	<60	60–180	181–300	>300
13–15	<50	50–150	151–250	>250
Riboflavin: μg/gm creatinine:				
Age (years):				
1–3	<150	150–499	500–900	>900
4–6	<100	100–299	300–600	>600
7–9	<85	85–269	270–500	>500
10–15	<70	70–199	200–400	>400

[a] The urinary values indicated above for adults are based on an expected creatinine excretion of 1.5 gm daily for a reference man weighing 65 kg, for creatinine coefficient of 23.

[b] The guides offered here for children are based on considerably less extensive data than are the guides for adults.

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DISCUSSION

Schaefer: Dr. Arroyave's excellent presentation has my whole-hearted support. The history of the establishment of the ICNND guidelines was precisely as you heard. The guidelines were based on the best evidence we could find regarding biochemical levels associated with clinical or functional changes related to dietary intake. They are guides for interpretation of data to identify populations at risk. I often find myself included with many other researchers who have a tendency to over-interpret the guidelines. I need not remind this group that the guidelines are not to be used as definitive diagnoses of malnutrition in individuals. Definitive diagnosis is dependent upon a response of the syndrome, be it a physical lesion or a functional alteration, to a specific therapy. They are merely a measuring mark or bench mark, and if one wishes to confirm a suspicion of a biochemical (tissue level) or dietary risk, other confirmatory data, such as specific response to therapy, or some other test, such as a "saturation" or "load test" would be required. There is an urgent need for more simplified screening tests. For example, we are extremely interested in the research that Dr. Hodges at Iowa University is conducting on longitudinal studies with prisoners on a vitamin A-free diet in which he is observing histological alteration in the epithelial cells of the buccal mucosa, intestinal, and urinary tract. It appears that these changes occur much earlier than serum vitamin A levels would indicate. It is very obvious from his longitudinal studies that by measuring total body pool or store of vitamin A, one can predict from a linear curve exactly when the serum vitamin A level will arrive at 20 micrograms or 15 micrograms per 100 ml. We are hopeful that a simplified histochemical test of changes in the epithelial cells may be a quick screening method for vitamin A nutrition. It is this kind of study that we need to encourage. Similarly, the recent work on the total protein content of hair follicles as an indicator of protein nutrition holds promise. It appears to be a much more sensitive method than serum albumin levels.

Our knowledge of the human organism's ability to adapt to stress is indeed meager as regards nutritional status. In populations consuming marginal or suboptimal levels of various nutrients such as thiamine, riboflavin, vitamin A, and even calories, does the body adjust to lower intakes? Do these individuals have a lower requirement or have they merely reduced physical output? Such problems can not be answered using current nutritional status assessment techniques. Dr. Arroyave has mentioned a few promising new approaches toward assessment of nutritional status. For many of the nutrients there are indeed sophisticated biochemical and microbiological methods that are more precise and sensitive. However, their use in large scale epidemiological surveys are limited. For example, the determination of the vitamin C of white blood cells is much more sensitive than serum or plasma vitamin C assays; also transketolase activity of serum is a better indicator of thiamine nutrition than urinary thiamine values. One always needs to appreciate the limitations and constraints in interpreting dietary or merely biochemical data. It is much more meaningful to assess nutritional status on the basis of biochemical, physical, and dietary findings. We all know the restrictions of dietary information. This is usually obtained over an extremely short period of time. One is required to obtain this information, however, since the major purpose of any diagnosis or epidemiological study is to determine the po-

tential cause and then develop remedial and preventive action. I have often been disturbed that most nutrition research—and I will have to admit most of us are guilty of the same thing—is restricted to studies of a single nutrient. In our experience, when one finds malnutrition determined or defined in a broad sense, very rarely does one find a single nutrient deficiency. There are exceptions, of course, such as iodine deficiency and goiter.

The general impression with reference to kwashiorkor is that it is a protein malnutrition problem. Our experience, in fact, has shown it to be a multiple nutrient deficiency. Most of the nutrition research studies have not been directed towards the influence of the total food supply and environment. We blithely establish guidelines and standards based on controlled studies, and these values may not be appropriate for assessing marginal, multiple nutritional states.

Cohen: Several important considerations have emerged from Dr. Arroyave's presentation which I appreciated hearing. It will be very helpful as background for later presentations. Dr. Arroyave pointed up very clearly one of the dilemmas facing administrators. If one has to supply a community with vitamin C, does one decide on a daily requirement of 30 milligrams or 60 milligrams? Decision-making thus may be economically very important. There are a number of comparable situations in dealing with the dietary needs of a population that this group has concerned itself with over the years. If we find that the dietary intake of a constituent in one population is lower than that recommended for another population, does this mean necessarily that we are dealing with a "deficiency" of that dietary constituent? If we are committed to raising the level of intake to that recommended for another population, are we certain that "readaptation" to a higher intake is really necessary or even desirable? These questions need to be answered and it would be appropriate to ask Dr. Schaefer's opinion on this. The popular press, in particular, has given wide publicity to certain of Dr. Schaefer's findings, to the effect that large segments of the population in the United States are suffering from malnutrition because the level of intake of a particular nutrient may be 20 to 30 per cent below that recommended. Dr. Schaefer, you suggested, in fact, that this position is not finally established, but rather is only an initial and tentative assessment which needs to be followed up for confirmation. I am certain that the members of this meeting would like to have you comment on this.

Schaefer: In our presentation of the National Nutrition Survey data, whether to Congress or to the press—and I beg no credit for the press interpretation—it was repeatedly stressed that we were defining a population at risk, based on tissue levels of the six nutrients we studied. We further stated that if these population groups were subjected to continued low intakes or to other stresses (parasitic, chronic disease, and such), they were candidates for the appearance of clinical lesions associated with malnutrition. We pointed out that the prevalance of clinical lesions as indicators of malnutrition were, indeed, rare in our population sample. However, there were some cases of clinical findings that we had not expected to see in the United States; for example, the finding of eight cases of kwashiorkor, 21 cases of Bitôt spots, a prevalence of rickets of two to six per cent among the 0- to 3-year-old population in some states and goiter in five to six per cent of the population in two states.

On the manner in which we selected our population—a random sample from the lower quartile of income areas—I honestly would have expected in this sophisticated society that these people would have been identified either by their parents, by their neighbors, by the school, or through our health care system. Obviously, we are dealing with a population that is unaware of problems of malnutrition. Likewise, the prevalance of goiter occurred in one state in which 40 per cent of the stores did not stock iodized salt. One storekeeper wanted to know

why he should stock iodized salt and then stated that none of his customers asked for it. These were some of the points we made. Again, we did not say that 25 per cent of our population is severely anemic; we said 25 per cent of the population studied had hemoglobin levels that placed it in a risk category. This distinction is usually not made by lay interpreters. There *is,* however, reason for concern. When we compared our findings, moreover, with the Central American studies, bearing in mind that we are studying populations living in the lower quartile of income areas, we found some states with a higher prevalence than we found in Central America. This was not hard for me to believe, but it was for others. My case rests.

Mayer: The difficulty that Dr. Schaefer described, "At what point do you make the decision that your data in nutrition show this or that situation and that a specific action is necessary?" is not very different from the mental process involved in research; for there is not such a great difference between being a scientist and being an administrator. Our Chairman quoted Claude Bernard a little earlier. I would like to quote him in a different context by way of illustration. Bernard said that all research starts on a bet and ends on a bet, but you hope that somehow you improved the odds by your research. It seems to me that at some point you have to decide that your figures concerning, for example, vitamin A levels or vitamin B_1 levels mean one thing from the point of view of knowledge and something else from the point of view of action. The fact that we cannot demonstrate that in every case people are affected by low vitamin B_1 levels is not an obstacle to knowledge; it is merely a statistical fact. By the same token it should not preclude action.

The danger, obviously, both from the viewpoint of knowledge, and from the viewpoint of action in misinterpreting the data—a practice that the press is sometimes guilty of as Dr. Cohen rightly showed—lies in the fact that if you have standards which have some individual variation and some margin of built-in safety, such as recommended dietary allowances, then a fraction of the population can be below the standard without it necessarily representing a danger.

A problem for this group at some further session should be: what is the acceptable minimum below which we must act? In the case of vitamin C, it may be less than 50 per cent because there is a large margin of uncertainty built-in. In the case of vitamin B_1, I would say that 50 per cent of the recommended dietary allowance is too little for acceptable long-term function for any large population or group.

Perhaps we need to have minimum levels below which action is indicated. Merely having recommended dietary allowances for the feeding of the population in general may not be the only guide we ought to have in terms of public health analysis of data.

Arroyave: I would like to mention a few other arguments, not to defend, but to explain the practical use of standards. Let me make a comparison with the use of standards in economics. For example, a country sets up minimum salaries, which does not mean that persons who earn slightly below the minimum are going to be necessarily suffering because of lack of money. It is merely a goal established on the basis of the fact that families earning less have a high probability of being at risk economically. Does one prefer to have just enough income to be obliged to spend the last cent at the end of the month, or does one prefer a situation that allows for some money in the bank so that in case of loss of work or unusual needs, one is not going to be in a critical financial state suddenly? One can look at vitamin A nutrition in somewhat the same way. It may be stated that persons with less than ten micrograms of serum vitamin A (no liver reserves) are doing well. But suppose there comes a period of scarcity of sources of vitamin A. The individuals under these limiting condi-

tions would definitely be at a higher risk of developing acute deficiency. One final point: standards, as I mentioned before, are to be used to make decisions about particular situations. In this context different situations may call for different standards, depending on the circumstances under which the decisions are to be made or the objective of these decisions.

ANEMIA AND NORMALITY

A. H. Waters

Introduction

The automation of diagnostic laboratory procedures has made it possible to extend the scope of clinical assessment to the community outside the hospital. Fundamental to the interpretation of each observation is a knowledge of the range of values which should be considered normal for the person concerned. "Normal" values for hemoglobin, as for other body constituents, have frequently been determined from testing only a small number of persons in a single laboratory, or by accepting and applying the published results of other workers, often out of context, especially in relation to the comparability of methods used. Widespread use of automated techniques should make it possible to reassess normal values for hemoglobin and other body constituents, making use of large numbers of observations and also of improvements and standardization of methodology.

The purpose of this paper is to review the published criteria of normality for the hemoglobin concentration. In particular, the importance of careful selection criteria will be discussed, especially in relation to the exclusion of latent deficiencies of iron, vitamin B_{12} and folate, which may reduce the Hb concentration below its optimal level in otherwise apparently healthy subjects. The functional significance of a suboptimal Hb concentration will also be discussed in relation to adaptive mechanisms which compensate for the reduced oxygen-carrying capacity of the blood.

Definition of anemia

In clinical medicine anemia is defined as a reduction of the hemoglobin concentration below normal. This is a functional definition based on the oxygen-carrying capacity of the blood. It can be argued whether the Hb concentration or the total red cell mass is the most valid index of anemia. However, the former is more directly related to tissue oxygen supply and is certainly the only practical clinical measurement. The packed cell volume (PCV or hematocrit) can be used as an indirect screening test for anemia, and in practice this measurement is usually made in conjunction with the Hb concentration, from which the mean corpuscular hemoglobin concentration (MCHC) (Hb gm per cent $\times$ 100/PCV) can be calculated.

Normal Hb concentration

The term normal is a statistical concept reflecting biological variability and refers to the range of Hb values obtained in a random sample of apparently healthy subjects. However, what we really want to know is the optimal Hb concentration, which is a physiological concept implying maximal performance for age and sex in a particular environment.

Population surveys

A precise assessment of the normal Hb concentration is of more than just academic interest because of the increasing interest in community

Table 1

Norwegian survey of hemoglobin concentrations[a]
Proposed normal values and criteria for anemia

Age (Years)	Male	Anemia (Hb below)	Female (Non-pregnant)	Anemia (Hb below)
7–9	12.7 ± 1.6[b]	11.0	12.7 ± 1.6[b]	11.0
10–13	13.2 ± 1.6	11.5	13.2 ± 1.6	11.5
14–16	15.0 ± 2.0	13.0	14.2 ± 2.0	12.0
17–20	15.5 ± 2.0	13.5	14.2 ± 2.0	12.0
>20	15.7 ± 1.8	14.0	14.3 ± 1.8	12.5

[a] gm/100 ml.
[b] Mean ± 2SD (= 95% of population).

From Natvig and Vellar (*46*); Natvig *et al.* (*47*).

health, and in particular in view of the prevalence of anemia in various population groups.

In determining the normal Hb concentration of a population group, it is essential to ensure the good health of the subjects studied, for the significance of the results obtained depends on the extent of the screening criteria used. Because of its relative frequency, it is important to exclude iron deficiency, for it is well recognized that the early stages of this deficiency may be associated with a fall in the Hb concentration of the particular subject without overt hematological changes (*2, 9*), and on the other hand, patients with overt iron-deficiency anemia may escape detection for they often have no definite symptoms (*27, 28*). One way to exclude significant iron deficiency in apparently healthy men and women is to examine the bone marrow iron stores. Using this method, Scott and Pritchard (*48*) and Hallberg *et al.* (*31*) found the lowest Hb concentration at which iron was consistently present in the bone marrow of non-pregnant women was 12.0 gm/100 ml. Recent studies in Sweden (*29*), and Norway (*46, 47*) have drawn attention to the value of therapeutic iron trials in apparently healthy individuals in determining optimal values for the various hematological indices of anemia.

Table 1 summarizes the proposed normal Hb values for males and females at different ages in the Norwegian survey. These results are in general agreement with the Hb values found in previous surveys of unselected healthy subjects in Britain (*5*) and Australia (*20, 55*). However, in these latter surveys, although the Hb values for men were essentially the same, the range of values for women[1] were lower than in the Norwegian survey, which may reflect the inclusion of some women with latent iron deficiency who were especially excluded from the Norwegian survey. Natvig and co-workers (*46, 47*) applied their criteria to determine the incidence of anemia in apparently healthy Norwegian subjects (Table 2). In children (boys and girls) anemia was most prevalent around puberty and early adolescence (10 to 16 years). In adult men subnormal Hb values (below 14 gm per cent) were most frequently found after the age of 40. In women, however, subnormal Hb values (below 12.5 gm per cent) were common in all age groups, the incidence reaching a peak between 30 and 40 years, but remaining relatively high thereafter. It is of interest to compare the results of the Norwegian survey conducted in affluent peacetime with the Medical Research Council (MRC) (U.K.) survey of Hb levels in Britain during World War II

[1] Berry *et al.* (*5*)
14.0 ± 1.25 gm% (11.5 = M — 2 SD)
Walsh *et al.* (*55*)
13.9 ± 1.16 gm% (11.6 = M — 2 SD)
Davis *et al.* (*20*)
14.3 ± 1.2 gm% (11.9 = M — 2 SD)
Natvig and Vellar (*46*)
14.3 ± 0.9 gm% (12.5 = M — 2 SD)

Table 2

Incidence of anemia in control subjects

Age (Years)	Male		Female	
	Hb below (gm%)	% Anemic	Hb below (gm%)	% Anemic
7–9	11.0	0.6	11.0	0.6
10–13	11.5	2.1	11.5	2.1
14–16	13.0	2.9	12.0	3.4
17–20	13.5	0.7	12.0	1.7
20–29	14.0	1.9	12.5	3.4
30–39	14.0	1.1	12.5	12.5
40–49	14.0	4.8	12.5	3.9
50–59	14.0	4.1	12.5	6.8
60+	14.0	6.2	12.5	7.1

From Natvig and Vellar (*46*); Natvig *et al.* (*47*).

(*41*). There was a relatively high incidence of anemia among civilian men and women (Table 3), and children (Table 4), much higher than in the Norwegian survey (Table 2). There was also a striking difference in the incidence of anemia between civilian men and Canadian soldiers stationed in Britain (Table 3). This in itself suggested that the civilian Hb values were suboptimal, and may reflect the more rigid medical criteria for selection of the soldiers and their better nutritional background. Furthermore, Berry *et al.* (*5*) obtained a higher mean Hb value of 15.7 gm/100 ml on 245 men tested in Britain some years after the war compared with the MRC survey mean value of 15.1 gm/100 ml. These differences in Hb values emphasize the importance of rigid criteria of good health and the exclusion of iron deficiency and other factors which may affect the Hb concentration when selecting a group of subjects for determining the optimal range of Hb values.

On the basis of these and similar surveys throughout the world (Table 5) (*13*), the World Health Organization (*63*) has proposed

Table 3

MRC survey of Hb levels in Great Britain in 1943 [a]
Incidence of anemia [b] in civilian men and women and Canadian soldiers stationed in Great Britian

Hb level gm% (% Haldane)	Civilians [c]			Canadian soldiers [d]
	Single Women	Married Women	Men	
Below 12.0 (80%)	5.8%	9.6%		
Below 12.5 (85%)	**14.9%**	**19.9%**		
Below 13.0 (90%)			7.4%	0.2%
Below 14.0 (95%)			**17.5%**	**0.5%**

[a] MRC Special Report Series No. 252 (*41*).

[b] Using criteria for anemia in Table 1, namely:

Men Hb $<$14.0 gm/100 ml ($\doteq$ 95% Haldane),
Women Hb $<$12.5 gm/100 ml ($\doteq$ 85% Haldane).

[c] From Table VI, p. 25, MRC Report (*41*).

[d] From Table XXXIV, p. 106, MRC Report (*41*).

Table 4

MRC survey of Hb levels in Great Britain in 1943 [a]
Incidence of anemia in children

Age (Years)	Anemia [b] (Hb less than)	Incidence (%) [c] Boys	Girls
5–10	<75%	3.9% (0.6)[d]	3.8% (0.6)[d]
11–14	<80%	4.4% (2.1)	9.0% (2.1)
15–19	<90%	8.1% (2.9)	26.5% (3.4)

[a] MRC Special Report Series No. 252 (*41*).

[b] Approximate equivalents to criteria for anemia in Table 1 expressed as Hb per cent (Haldane).

[c] From Table XIV, p. 32, MRC Special Report No. 252 (*41*).

[d] Figures in brackets indicate corresponding incidence of anemia in the Norwegian survey (see Table 2).

certain criteria for the diagnosis of anemia which are summarized in Table 6. Some workers may prefer, however, to use their own or other criteria, but whatever criteria are adopted, the results of Hb surveys should be reported not only as the mean ± SD, but also as a percentage incidence in 1 gm per cent (or smaller) Hb increments, so that the data can be analyzed by different criteria of anemia should the necessity arise.

Clinical significance of suboptimal Hb levels

While the concept of a normal range of Hb values is useful for population surveys and clinical screening, it overlooks the problem of the individual patient whose Hb concentration, although within the normal range, is below his optimal value. This applies particularly to the early stages of iron, B_{12} and folate deficiencies, which are of particular nutritional significance in population surveys.

Iron deficiency

The well-documented sequential changes in the development of iron deficiency are shown in Figure 1. From this it can be seen that the iron

Table 5

Published criteria for anemia in adult men and women [a]

Author	Country	Men	Women
Wintrobe (*64*)	USA	14.0	12.0
Bierring (*8*)	Denmark	13.0	11.5
Berry *et al.* (*5*)	Britain	13.5	11.5
Walsh *et al.* (*55*)	Australia	13.5	11.6
Kilpatrick (*39*)	Britain	12.5	12.0
Scott and Pritchard (*48*)	USA	—	12.0
Garby *et al.* (*29*)	Sweden	—	11.5
Natvig and Vellar (*46*)	Norway	14.0	12.5
Hallberg *et al.* (*31*)	Sweden	13.0	12.0
WHO (*63*)	Composite series	13.0	12.5
Dacie and Lewis (*19*)	Composite series	13.5	11.5
Davis *et al.* (*20*)	Australia	13.3	11.9
Weatherburn *et al.* (*61*)	Canada	12.5	10.8

[a] Hb gm per cent below which anemia occurs: Mean—2 SD

Table 6

WHO criteria for the diagnosis of anemia (63)

	Hb gm/100 ml
Children:	
6 months to 6 years	11 [a]
6 years to 14 years	12
Adult females:	
non-pregnant	12
pregnant	11
Adult males	13

[a] >95 per cent of normal subjects have Hb concentrations higher than the values given. However, upward correction must be made for high altitudes.

stores are depleted by the time the Hb starts to fall. At this stage, however, the Hb level, although suboptimal for the patient, may be still within the normal range and an examination of the blood film may not reveal any significant morphological changes. In a study of apparently healthy adults, Natvig and Vellar (*46*) found a higher incidence of subnormal MCHC values than of subnormal Hb values which led them to suggest that a subnormal MCHC might be a useful index of mild uncomplicated iron deficiency in cases where the Hb concentration is still within the normal range. These subjects responded to iron therapy with a rise in the MCHC to normal and an increase in the Hb concentration, even when this was initially within the normal range (Table 7). Hallberg *et al.* (*31*), using the absence of reticular iron in the bone marrow as an index of iron deficiency, showed that there was a considerable overlap of the frequency histograms of Hb levels of women with and without reticular iron in the marrow, but that the Hb levels of the latter group were shifted towards lower values (Figure 2).

The optimal Hb concentration will therefore be underestimated if subjects with latent iron deficiency are not excluded from the population sample, and, furthermore, the prevalence of iron deficiency may be underestimated if only anemic are considered. Some epidemiologists may argue that this is of little clinical significance in relation to symptoms and physical activity (*27, 28, 51*), while others suggest that even slight iron deficiency may affect work capacity (*6, 32*). In nutrition surveys, however, where it is important to know the full extent of the problem, it is essential to have sensitive tests for iron deficiency, in addition to the measurement of Hb concentration and MCHC, which can be ap-

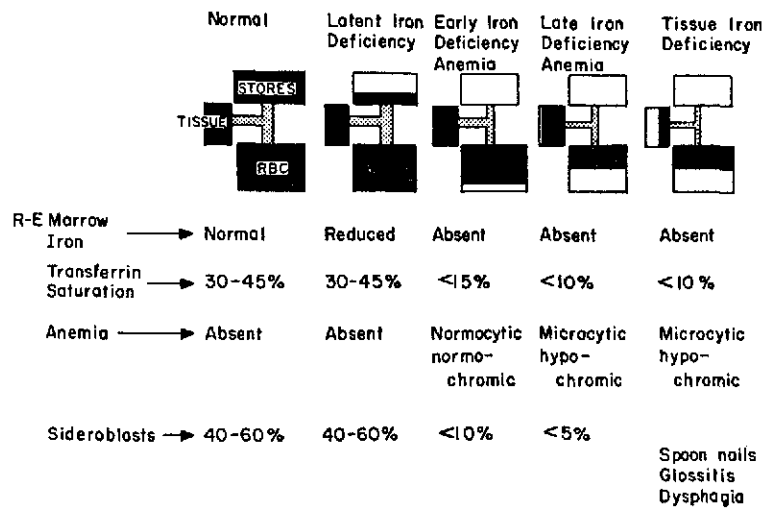

Figure 1. Sequential changes (from left to right) in the development of iron deficiency. From Simon, Giblett, and Finch (*50*). Rreproduced by permission of University of Washington Press, Seattle.

Table 7

Effect of iron therapy on the Hb and MCHC of 25 men and 23 women whose initial MCHC was subnormal (i.e. < 30.5%) [a]

Duration of iron therapy	Men [b]		Women [c]	
	Mean Hb (gm%)	Mean MCHC (%)	Mean Hb (gm%)	Mean MCHC (%)
Before iron	14.54	29.6	12.88	29.0
After 1 month	15.42	33.8	13.80	32.7
After 2 months	15.37	33.5	13.78	32.2
After 3 months	15.67	34.0	14.25	33.6
Normal	15.7	33.6	14.3	33.6

[a] From Natvig and Vellar (*46*).

[b] Only 3 of the 25 men had Hb <14 gm per cent.

[c] Only 8 of the 23 women had Hb <12.5 gm per cent.

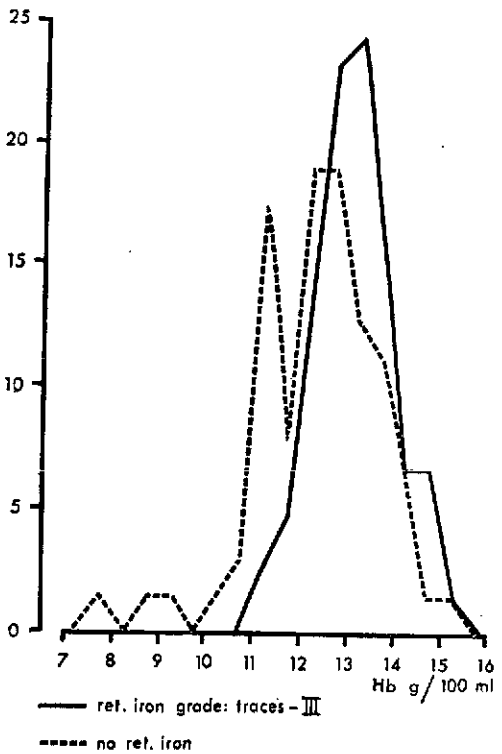

Figure 2. Comparison of the frequency histograms of hemoglobin concentrations of 193 women who had stainable iron in the bone marrow (continuous line) and 67 women who lacked such iron. From Hallberg *et al.* (*31*). Reproduced by permission of Almqvist and Wiksells, Uppsala.

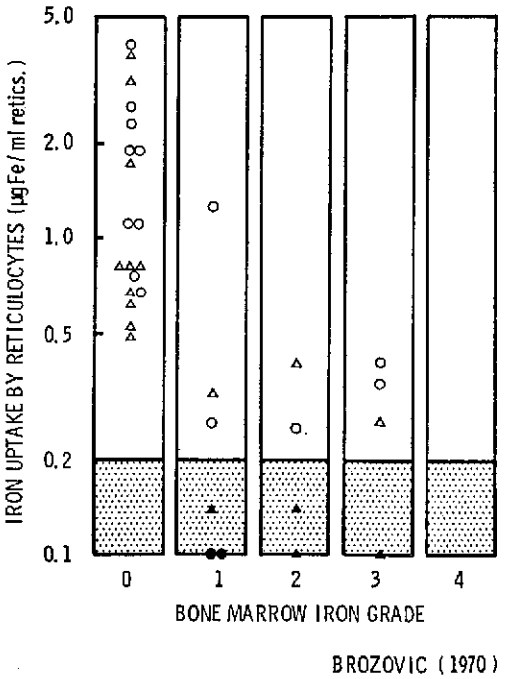

Figure 3. Relationship between the *in vitro* uptake of Fe^{59} by reticulocytes and stainable iron in the bone marrow in 33 anemic patients (Hb 5.0 to 10.5 gm per cent) suffering from conditions known to be associated with iron deficiency. The stippled area indicates the normal range of Fe^{59} uptake by reticulocytes. Normal amounts of iron in the bone marrow are indicated by Grades 2 and 3; reduced iron by Grades 0 and 1; increased iron by Grade 4. Circles represent results in males; triangles females. Closed symbols indicate normal and open symbols increased Fe^{59} uptake by reticulocytes. From Brozović (*10*).

plied to large population samples. In this respect the assessment of bone marrow iron, although probably the best index of body iron status, is not a practical proposition. On the other hand, there is a marked overlap between normal and iron-deficient subjects by the biochemical tests of iron deficiency (serum iron, TIBC, and percentage transferrin saturation) (*31*). Preliminary observations by Brozović (*10*), following up an observation by Najean *et al.* (*45*) suggest that the *in vitro* uptake of transferrin-bound Fe^{59} by reticulocytes correlates well with bone marrow iron stores in uncomplicated iron deficiency. However, in the hypochromic anemia of chronic inflammatory or neoplastic disorders, the *in vitro* reticulocyte uptake of Fe^{59} may not accurately reflect bone marrow iron stores (Figure 3), but correlates well with response to iron therapy (*10*). This is a simple and rapid *in vitro* test which may prove useful in the interpretation of equivocal serum iron and TIBC results, and may well have a useful application in nutrition surveys.

Vitamin B_{12} deficiency

The development of sensitive and specific microbiological assays for B_{12} in serum and other tissues has made it possible to diagnose deficiency at an early stage, and it is now well recognized that a person may have severe B_{12} deficiency before the onset of anemia (Figure 4). However, careful examination of the blood film and bone marrow of such patients will reveal early megaloblastic changes, which are reversible

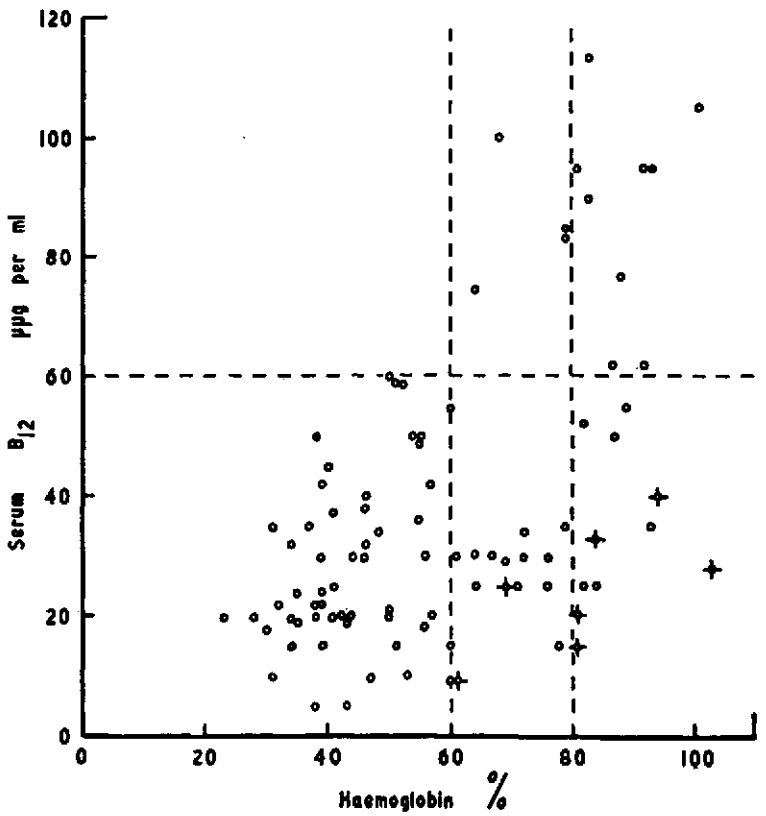

Figure 4. Relationship between the serum B_{12} and hemoglobin concentrations (100 per cent = 14.8 gm/100 ml) of 92 patients with pernicious anemia. The starred circles indicate patients with subacute combined degeneration of the cord (SACD). From Anderson (1).

by treatment with B_{12}. This treatment will also raise the Hb concentration, even when the initial Hb level is within the normal range.

Most of our knowledge of the pathophysiological effects of B_{12} deficiency in man has been derived from the study of pernicious anemia. The non-anemic stage of pernicious anemia may be asymptomatic-"latent" pernicious anemia (*7, 14, 43, 65*), but such cases are rarely detected in clinical practice except by chance or by deliberate screening for B_{12} deficiency. However, patients with severe B_{12} deficiency may suffer serious neurological damage even in the absence of anemia. As a group, patients with subacute combined degeneration of the spinal cord (SACD) tend to have higher Hb levels than patients with uncomplicated overt pernicious anemia (Figure 5), although the serum B_{12} levels are as low as in severe pernicious anemia (Figure 6). Patients may also present, during the non-anemic stage of B_{12} deficiency with a wide range of less specific symptoms including psychosis, glossitis, paresthesia, infertility, and such vague complaints as lethargy, apathy, and anorexia (*59*).

Although B_{12} deficiency may cause a wide range of symptoms before the onset of anemia, the pathogenesis of anemia in severe B_{12} de-

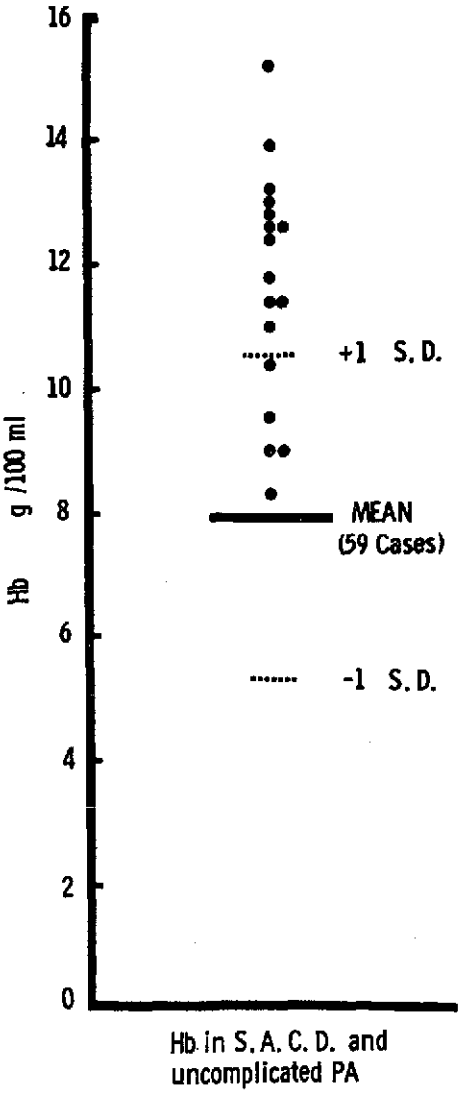

Figure 5. Hemoglobin concentrations (gm/100 ml) of 17 patients with SACD (closed circles) compared with the mean hemoglobin concentrations (± 1 SD) of 59 patients suffering from uncomplicated pernicious anemia. From Waters and Mollin (*60*). Reproduced by permission of the Wellcome Trust.

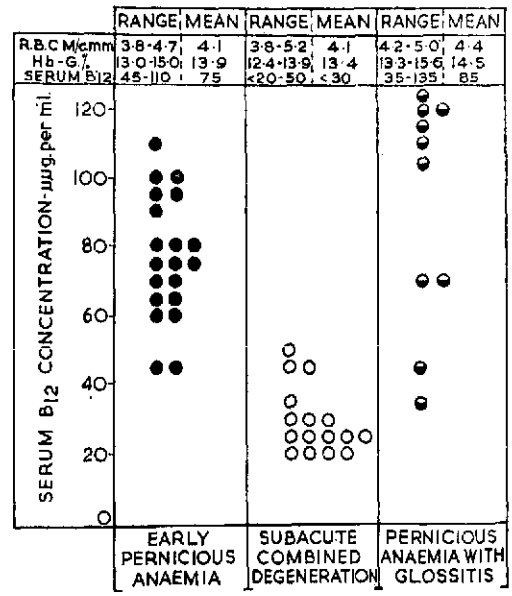

Figure 6. The serum B_{12} concentrations in: (1) a group of patients with early pernicious anemia who were not anemic and not complaining of symptoms of B_{12} deficiency; (2) a group of patients with SACD without anemia; and (3) a group of non-anemic patients with pernicious anemia whose only symptom was glossitis. From Mollin (*43*). Reproduced by permission of Athlone Press, London.

ficiency is still uncertain. As already pointed out, there is no correlation between the serum B_{12} concentration and the Hb concentration. On the other hand, there is a progressive fall in the serum folate level with increasing anemia in patients with pernicious anemia, which is independent of the serum B_{12} level (Figure 7) (*58*). It is therefore tempting to suggest that folate deficiency precipitates the onset of anemia in pernicious anemia. However, very few patients with pernicious anemia have serum folate levels in the range found in overt folate deficiency (*58*). Nevertheless, owing to the postulated "B_{12} block" in the utilization of folate in

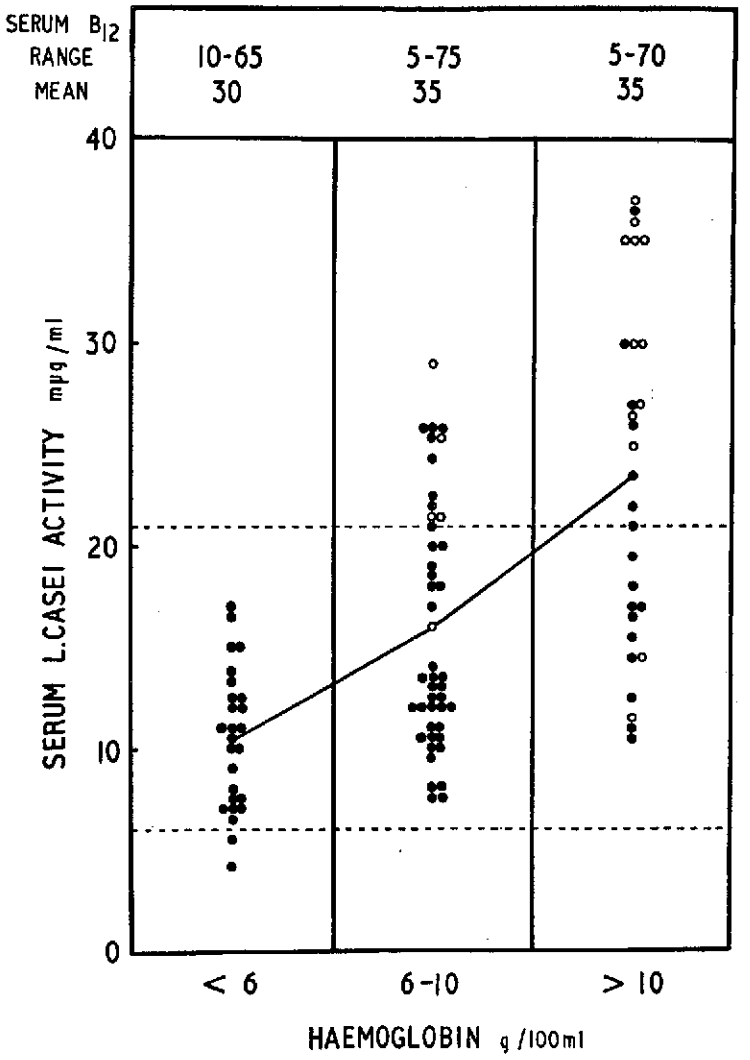

Figure 7. Serum folate (*L. casei*) concentrations of 119 patients with pernicious anemia in relation to the degree of anemia. Open circles indicate patients with SACD. The continuous oblique line joins the mean serum folate concentration in each group; the differences between the means were highly significant ($P < 0.001$). The serum B_{12} concentration (range and mean) for each group is indicated at the top of each column. The broken horizontal lines at 6 and 18 mμgm/ml (ngm/ml) indicate the range of serum folate concentrations found in 95 per cent of normal subjects. From Waters (*56*).

pernicious anemia (*12, 35, 57, 58*), it is possible that signs of folate deficiency might appear at a higher serum folate level than in the absence of B_{12} deficiency. Thus, in a particular patient, the onset of megaloblastic anemia would depend not only on the serum B_{12} level, but also on the serum folate level necessary to overcome the "B_{12} block" (*56*). Whatever the explanation, it is significant that in patients with severe B_{12} deficiency, a surfeit of folate in the diet, whether naturally as in vegans (*56, 66, 67*) or by prophylactic supplementation with folic acid (*3, 15, 17, 18, 23, 26, 54*) will tend to delay the hematological manifestations of B_{12} deficiency while allowing neurological and other complications to develop. In fact among patients with pernicious anemia, those with SACD tend to have the highest Hb and serum folate concentrations and the lowest serum B_{12} concentrations (*43, 58, 60*).

Folate deficiency

As in iron and B_{12} deficiency, significant folate deficiency may occur while the Hb concentration is still within the normal range. Furthermore, at this early stage of deficiency, morphological changes in the blood and bone marrow are minimal and may be overlooked, especially when large numbers of films have to be examined, as in a busy diagnostic laboratory or in population surveys. Consequently, the presence of early folate deficiency may be missed unless specific diagnostic tests are carried out.

Herbert (*34*) has documented the sequence of biochemical and hematological changes in the development of folate deficiency in a healthy adult whose dietary folate intake was reduced to approximately 5 μg per day of *Lactobacillus casei*-active folate (Figure 8). A fall in the serum folate concentration (after only three weeks) was the first indication of developing folate deficiency, followed much later (after 17 weeks) by a fall in the red cell folate concentration. The first hematological abnormality to appear was nuclear hypersegmentation of the neutrophil leukocytes as indicated by an increase

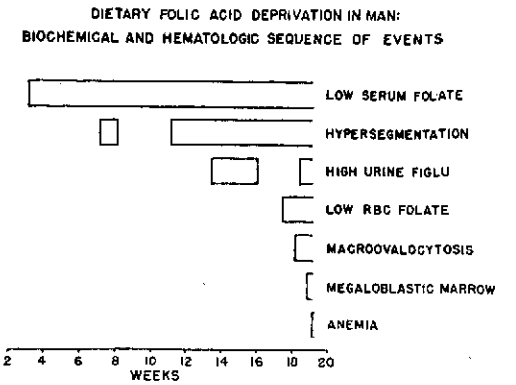

Figure 8. Sequence of biochemical and hematological changes in the development of folate deficiency due to sudden reduction of dietary folate in a previously healthy adult male. From Herbert (34). Reproduced by permission of the Association of American Physicians.

in the mean lobe count (after seven weeks). However, definite macrocytosis appeared much later (after 18 weeks) followed by megaloblastic change in the bone marrow (after 19 weeks) and last of all by anemia. Similar observations have been reported by Mollin and Hoffbrand (*44*), who compared these various parameters of folate deficiency in a number of patients suffering from a wide variety of hematological and other conditions.

The red cell folate concentration is particularly useful in assessing the severity of folate deficiency in the absence of megaloblastic anemia in a patient who nevertheless has a subnormal serum folate concentration. In this respect, it is probably the best single index of significant folate deficiency when assessing the incidence of deficiency of this vitamin in population surveys.

Table 8 summarizes some recent findings in relation to the foregoing from a recent survey of health of old people (65 years and over) organized by the United Kingdom Ministry of Health and Social Security (to be published). These results show that there was a significant deficiency of iron, B_{12} and folate in subjects whose Hb levels were within the accepted normal range, as well as in the anemic subjects. On the other hand, there was no biochemical evidence of deficiency of these nutrients in a large

Table 8

Incidence of iron, B_{12} and folate deficiency in healthy geriatric subjects (≥ 65 years) in relation to Hb concentration

Hb concentration	No. studied	Iron deficiency (Transferrin Saturation < 16%)	B_{12} deficiency (Serum B_{12} <100 pg/ml)	Folate deficiency (RCF <100 ng/ml)
Normal [a]	616	17.4%	0.8%	2.6%
Subnormal	52	53.0%	2.0%	4.0%
Total	667	20.0%	0.9%	2.7%

[a] Male Hb > 13 gm per cent; female Hb > 12 gm per cent.

proportion of subjects with subnormal Hb levels. This raises the possibility that the arbitrary lower normal Hb level may have been set too high for old people (males 13 gm per cent, females 12 gm per cent), and similar observations have been reported by Walsh *et al.* (*55*), Vellar (*53*), Kaufman *et al.* (*38*), and Weatherburn *et al.* (*61*). The explanation for these lower Hb levels in old people is uncertain, but the final report of the recent Ministry of Health survey may help to elucidate this. Apart from possible nutritional differences, other factors peculiar to this age group may be important in suppressing hemopoiesis, and in this respect, altered hormonal balance and diminished physical activity may be significant and require investigation.

In Table 8, iron deficiency was assessed in terms of a transferrin saturation less than 16 per cent, which probably overestimates the extent of true iron deficiency. Using a combination of parameters (serum iron <65 μgm per cent and total iron-binding capacity (TIBC)> 410 μgm per cent), the incidence of iron deficiency in the same population was approximately 9 per cent (Brozović, unpublished data) compared to 20 per cent, based on transferrin saturation alone. There was a surprisingly low incidence (2.7 per cent) of significant folate deficiency, as determined by a red cell folate concentration less than 100 ngm/ml, but a much higher incidence (14 per cent) of mild or incipient folate deficiency as indicated by a low serum folate concentration (<3 ngm/ml). The incidence of approximately 1 per cent of serum B_{12} concentrations less than 100 pgm/ml (that is, in the range found in overt pernicious anemia) is ten times that of "clinical" pernicious anemia in Britain reported by Scott (*49*). The survey also revealed a 10 per cent incidence of serum B_{12} concentrations in the borderline range (100–200 pgm/ml). The clinical significance of this borderline range is uncertain, but such levels occur in early pernicious anemia (*14, 43*), atrophic gastritis (*62*), and in many patients following partial gastrectomy (*22, 36*), which probably indicates mild B_{12} deficiency. The survey has therefore revealed a much higher incidence of potential B_{12} deficiency in old people than previously suspected.

Physiological adaptation to suboptimal Hb levels

Although deficiencies of B_{12} and folate (and possibly iron) may produce definite symptoms in their own right, it is still an open question whether mild anemia *per se* results in any functional impairment or lack of well-being owing to the existence of adaptive mechanisms which compensate for the decrease in arterial blood O_2 content due to anemia.

Perhaps the most important of these mechanisms is the decreased affinity of Hb for O_2 in anemia (Figure 9), which is mediated by an increase in red cell 2, 3-diphosphoglycerate (2, 3-DPG), an intermediate metabolite in the red cell glycolytic (Emden-Meyerhoff) pathway (*4, 16*). *In vivo* studies have shown that there is an increase in red cell 2, 3-DPG at high altitude (*25, 40, 52*), in cardiac failure (*68*), and in ane-

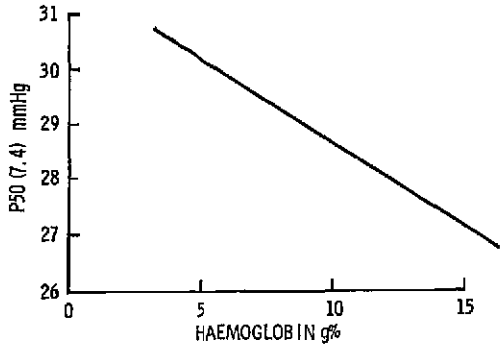

Figure 9. Average change of P_{50} (pH 7.4) (i.e., PO_2 in mm Hg required for 50 per cent saturation of blood with oxygen at pH 7.4) as a function of hemoglobin concentration. From Lenfant *et al.* (*40*). Reproduced by permission of Plenum Press, New York.

mia (*52*). The linear relationship between 2, 3-DPG and Hb concentration demonstrated in anemia and at high altitudes also exists in healthy subjects with normal Hb levels (Figure 10) (*24, 52*). The increase in 2, 3-DPG is also associated with an increase in adenosine triphosphate (ATP), but the overall effect of 2, 3-DPG on Hb-O_2 dissociation is about nine times that of ATP (*52*).

Changes in pH may also affect the Hb-O_2 dissociation curve (the Bohr effect)—acidosis leading to a right shift and alkalosis to a left shift. In a recent study of anemic patients, Torrance *et al.* (*52*) showed that acidosis was associated with a smaller increment in 2, 3-DPG

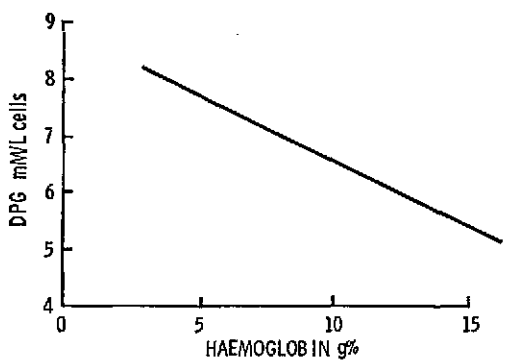

Figure 10. Average change of intraerythrocyte DPG (2, 3-diphosphoglycerate) as a function of hemoglobin concentration. From Lenfant *et al.* (*40*). Reproduced by permission of Plenum Press, New York.

than expected from the Hb concentration, and *vice versa* for alkalosis (Figure 11). The relative effect of the pH and DPG adaptive mechanisms was studied in subjects exposed to sudden hypoxia of altitude (*40*). These workers showed that when normal subjects arrived at high altitude there was a rapid increase in 2, 3-DPG ($t_{1/2}$ approximately 6 hours), which reached a maximum by 24 hours. At the same time there was a marked alkalosis due to hyperventilation, which shifts the *in vivo* Hb-O_2 dissociation curve to the left, thus aggravating the anoxia already present, and possibly enhancing the rapid formation of DPG, which then shifts the curve back to the right.

The DPG mechanism is therefore of fundamental importance in the rapid metabolic adaptation to tissue anoxia, whether this is due to anemia or to cardiopulmonary causes. In mild to moderate anemia, this mechanism may maintain adequate tissue oxygenation without affecting cardiopulmonary function, and thus account for the minimal symptomatology of such patients. The precise relationship between this mechanism and erythropoietin stimulation is uncertain, but probably the erythropoietin mechanism is stimulated when the DPG mechanism is unable to keep pace with tissue O_2 demand.

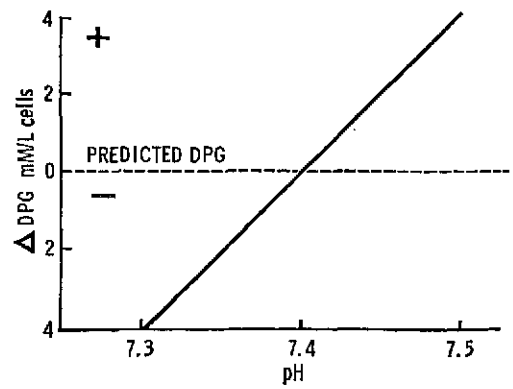

Figure 11. Average deviation of measured DPG from calculated DPG (using the regression line DPG = — 0.194 Hb + 8.89) as a function of arterial pH. These results from Lenfant *et al.* (*40*) were based on data from a group of anemic patients at Medellín, Colombia. Reproduced by permission of Plenum Press, New York.

Summary and conclusions

The differentiation between anemia and normality based on an arbitrary Hb concentration remains a statistical concept. The results of random surveys of Hb concentrations, although representative of the group under study, may not represent optimal levels. This shortcoming may be largely of academic interest when the main objective is the detection and treatment of overt anemia in underdeveloped countries where nutritional anemia is a serious public health problem. On the other hand, in clinical medicine, the individual patient's optimal Hb concentration is the significant parameter and the acceptance as normal of an Hb concentration within the statistical normal range may result in delayed diagnosis of latent deficiencies of iron, B_{12} or folate, which may reduce the Hb concentration below its optimal level in apparently healthy subjects. However, careful screening of the blood film and specific tests for iron, B_{12} and folate deficiencies, especially in patients and population groups at risk, will greatly help in the early detection of these deficiencies before the onset of overt anemia or other complications.

To make this a feasible proposition for clinical screening and nutritional surveys, it will be necessary to automate the measurement of as many nutritional parameters as possible. Progress has been made in the automation of methods for the measurement of serum iron and TIBC using auto-analyzers (*11, 30, 69, 70*). The microbiological assay of folate in serum and whole blood has also been successfully automated (*42*). This was made possible by the use of a chloramphenicol-resistant strain of the assay organism, *Lactobacillus casei* (*21*), which obviated the need for aseptic conditions. This method is a simplification of the standard manual methods (*33, 57*) and has the capacity for dealing with 80 to 100 specimens in duplicate per hour compared with approximately 200 specimens per week by the manual methods. This tremendous work capacity makes it especially suitable for providing a regional diagnostic service and nutritional surveys of large population groups. The same principles are now being applied to the assay of B_{12} in serum (Millbank *et al.* To be published).

The establishment of normal values for Hb concentration and various nutrients implies the comparability of results between different laboratories and necessitates standardization of the methods used. The International Committee for Standardization in Haematology (*37*) has made recommendations for the measurement of Hb using a cyanmethemoglobin solution as a standard, and WHO has established central reference laboratories to standardize the assays of iron, vitamin B_{12} and folate (*63*). The function of these laboratories is to distribute standards, to correlate the results of the participating laboratories, and to advise on the possible cause of discrepancies where these arise.

Correlation between Hb concentration and functional capacity is still an open question, although evidence is available that physiological adaptation to suboptimal Hb levels can maintain tissue oxygen supply without significant cardiopulmonary embarrassment. Furthermore, with the Hb concentration, as with other physiological parameters, conditions other than nutrition may affect the level of normality at different ages and under different environmental conditions.

From these observations it is apparent that, while it is still essential to define anemia as a parameter of health, the Hb concentration cannot be regarded as a sensitive index of nutritional status. This is better determined by direct measurements of the nutrients concerned and where possible by careful examination of the blood film, especially in patients and population groups at risk.

Acknowledgments

I am most grateful to Dr. B. Brozović for his help and advice on the sections dealing with iron deficiency and in particular for permission to reproduce Figure 3. I also wish to thank Dr. J. Torrance for generously allowing me to quote his paper (*52*) which is still in press.

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ENDOCRINE MECHANISM IN NUTRITIONAL ADAPTATION

Fernando Mönckeberg B.

The literature on endocrine studies of malnourished children is presently in a state of controversy and confusion.

This results from many considerations, primarily the tendency to oversimplify the etiology of malnutrition, which, in fact, is a complex process influenced by many different factors that in each geographical area may act in varying ways resulting in diverse clinical syndromes. In consequence of these variables, a conflicting terminology has developed; the same term has a particular meaning for each author. Again, some of the controversial results can be explained because the response to endocrine studies in malnutrition varies. Patient response differs: (a) in accordance with the duration and degree of malnutrition; (b) in accordance with the existence of concomitant diseases; (c) the age of the patient; (d) and finally the stage at which tests are performed—before or during recovery. With respect to the last, from the biochemical standpoint, recovery does not always coincide in time with the initiation of weight increase. For example, during treatment of marasmic infants, an increase in oxygen consumption has been observed (*25*), which precedes increase in weight by several days.

Further confusion results from the fact that few authors provide a detailed description of the patients that they are studying, and hence, again, patients with different clinical characteristics are grouped under the same syndrome. In view of the above, it is important to define the clinical characteristics of the patients that have been studied before reporting an experience.

Two types of malnutrition are generally recognized in children: marasmus and kwashiorkor. Marasmus is due to low calorie and protein intake, whereas kwashiorkor is due mainly to protein deficiency (*23*). In typical cases the symptoms are, of course, quite characteristic; however, typical cases are exceptional, most cases are of mixed etiology, and consequently present a mixed symptomatology.

In order to clarify the two syndromes, typical kwashiorkor cases are considered to be those whose etiology corresponds to a protein deficiency alone. In this type of malnutrition the disease is acute, and the whole symptomatology appears after a short period of protein deficiency. The child's growth has been normal or near normal during the first year of life, then suddenly a protein restriction—due to many socioeconomic factors—unchains the whole symptomatology. Though this type of patient is very infrequent, they have been particularly selected in order to study more clearly the effects of protein restriction upon the different biochemical parameters.

Selected marasmic patients correspond to those with malnutrition from their first months of life, and at one year of age they have almost the same weight and height as at birth. These patients had been breast-fed for a very short period of time; they then all had a very serious hypoalimentation with very diluted milk, with or without the addition of carbohydrate. No evidence of edema was found, and the changes of skin and hair, typical in kwashiorkor, were minimal

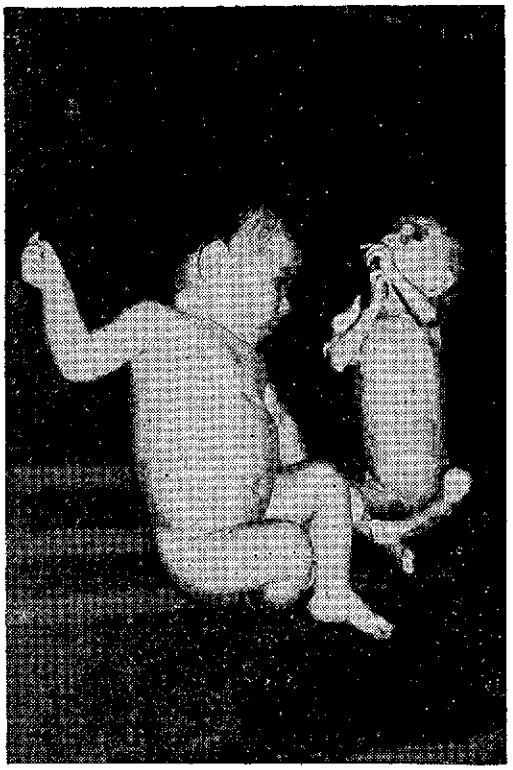

Figure 1. On the right in the photograph is a typical seven-month-old marasmic patient.

or not present. The photograph shows a typical seven-month-old patient (Figure 1).

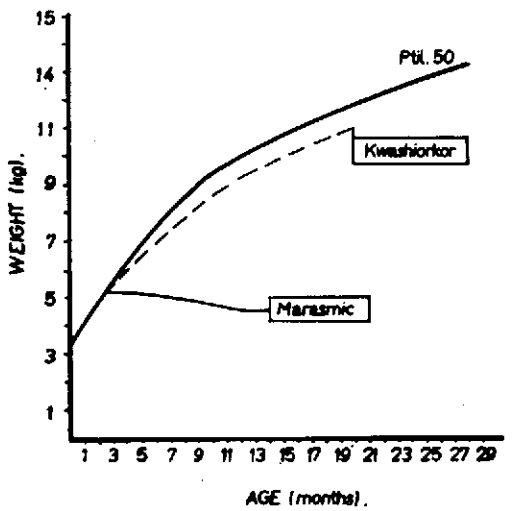

Figure 2. A typical weight curve of a marasmic and a kwashiorkor patient.

Both the marasmic and kwashiorkor cases selected were free of other concomitant diseases, and studies were initiated before treatment was started. Figure 2 shows a typical weight curve of a marasmic and a kwashiorkor patient.

In the case of marasmic patients, when hypoalimentation has been of long duration and has brought on growth retardation of 50 per cent or more, the recuperation process takes a long time to begin even when there are no signs of infection or of parasitic or other concurrent diseases (*21*). It is, in fact, a frequent experience that for four months or more, neither clinical improvement nor an increase in weight can be observed, in spite of a diet providing the proper calorie requirements for a normal child (120 calories per kilogram per day). During this

Table 1

Balance data on severe marasmic malnutrition before recovery start

Case number	Ingestion	Urine	Fecal	% Retention
	Phosphorus data (mg/kg/day)			
1	169	76	79	8
2	140	61	54	17
3	210	94	99	5
4	231	110	111	4
5	167	92	74	0
6	169	87	81	0
Average	181	86	83	5
	Potassium data (mg/kg/day)			
1	390	351	31	2
2	410	312	39	12
3	429	390	27	2
4	545	390	35	22
5	429	390	19	4
6	390	351	19	5
Average	430	364	28	8
	Nitrogen data (mg/kg/day)			
1	750	650	100	0
2	718	490	65	22
3	884	720	79	9
4	840	710	90	4
5	800	710	103	0
6	830	530	100	24
Average	803	635	89	9

period, the nitrogen balance may be negative or only slightly positive, even though the absorption of nitrogen is normal (*26*) (Table 1). Furthermore, the basal oxygen consumption is below normal, and it does not increase until recovery starts (*26*) (Figure 3).

In contrast, in the case of typical kwashiorkor, without other concomitant disease, the oxygen consumption remains normal or near normal values and the recovery starts promptly with the treatment (Figure 4).

Adrenal function

In marasmic children there are many clinical manifestations that conform to a diagnosis of adrenal hypofunction. Recovery is slow and difficult. Clinically they are characterized by weakness, apathy, skin pigmentation, electrolyte imbalance, severe diarrhea, and infections.

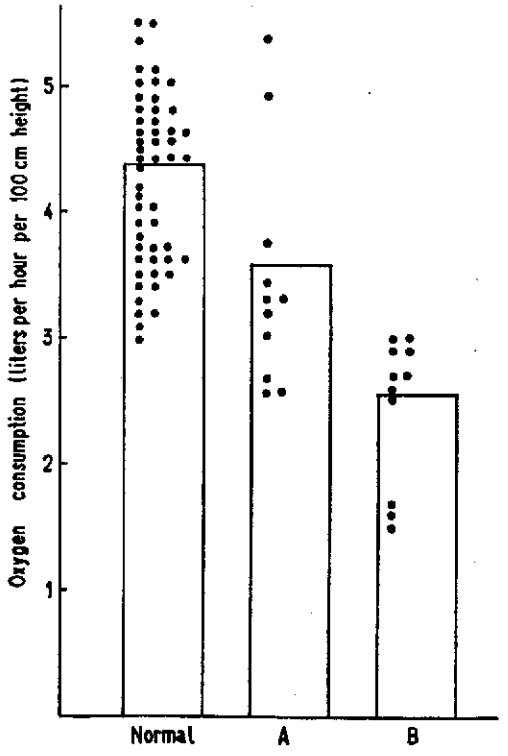

Figure 3. Basal oxygen consumption in marasmic patient is below normal and does not increase until recovery begins. A = Marasmic patients during recovery; B = Marasmic patients before improvement began.

Experimentally, observations of these patients can be summarized as follows: (a) marasmic infants have a low sodium-retaining capacity (Figure 5). Normal infants at the same age

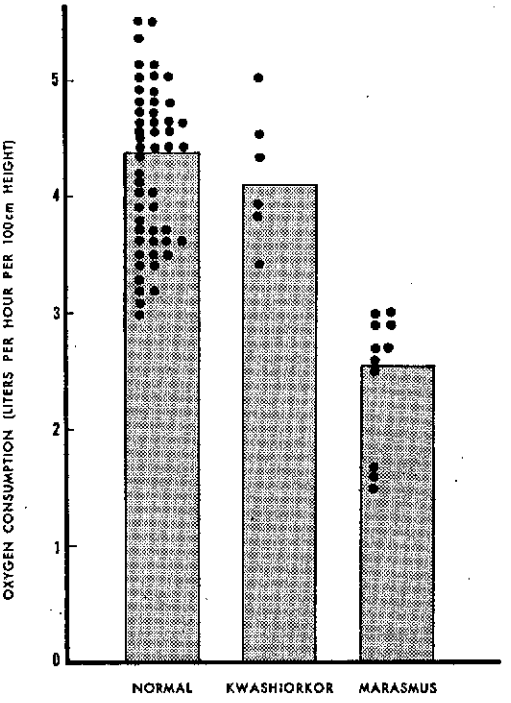

Figure 4. In typical cases of kwashiorkor, oxygen consumption remains normal or near normal values.

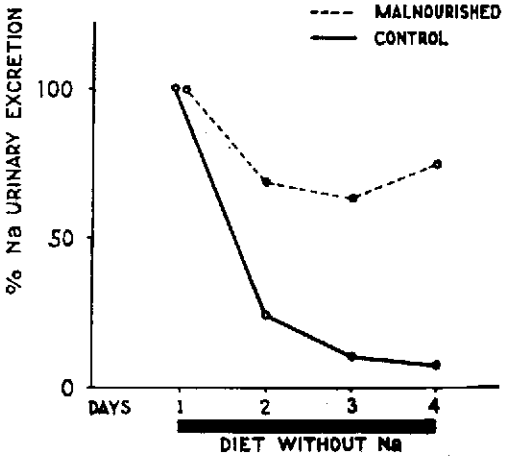

Figure 5. Sodium urinary excretion before and after a diet without sodium in 10 malnourished infants and six normal infants.

under a low sodium diet reduce the urinary Na practically to zero mEq/24 hours, whereas the marasmic, under the same test, continues to eliminate this electrolyte in considerable amount, in spite of the fact that no Na has been administered in the diet (*24*), which suggests a deficit in the aldosterone secretion; (b) when large amounts of water are administered, normal infants show a very similar diuresis of 58 per cent before and 60 per cent after the administration of cortisone. On the contrary, marasmic infants excrete only 50 per cent of the ingested water before receiving cortisone, and this elimination increases significantly after cortisone to 78 per cent (*24*) (Figure 6); (c) after stimulation with adrenocorticotrophic hormone (ACTH), infants with severe marasmus show a lower urinary excretion of 11-oxysteroids, 17-ketogenic, and 17-ketosteroids (*24*) (Figures 7, 8). Although it has been shown that the urinary excretion of 11-oxysteroids and 17-ketogenic steroids are unreliable indices of adrenal function (*12*), it is interesting to observe that the response of the marasmic child is similar to normal children during the first day, but the response decreases after two or three days of stimulation. The same happens with the sodium-conserving capacity. It is possible to assume that the same poor hormonal secretion is produced when a marasmic child experiences infection, dehydration, and other stresses.

The plasma cortisol level in marasmic patients has been determined by Rav *et al.* (*29*) and also by Alleyne and Young (*2*). Both papers give very few details about the clinical characteristics of their patients, although both describe a high level of plasma cortisol which fell with recovery. This high level could be the result of impaired breakdown and removal, or of over-production of cortisol. According to Alleyne and Young (*2*), it seems to be due to impaired metabolism of cortisol, since a significant increase of exogenous cortisol was observed in marasmic patients. The same authors describe a good response from the adrenal gland after corticotrophin administration. Nevertheless, the

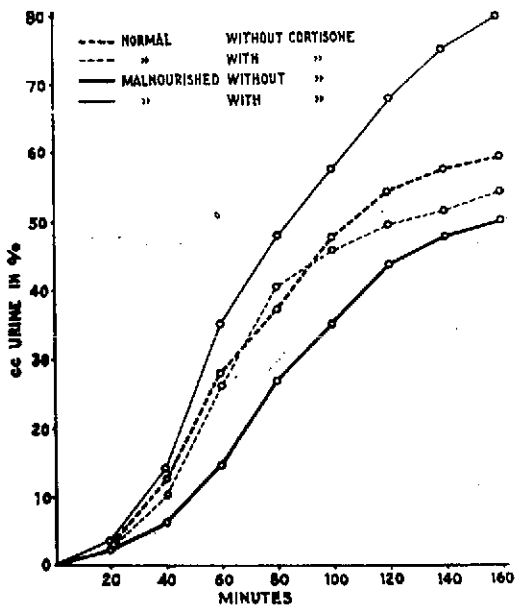

Figure 6. After intake of large amounts of water, normal infants showed similar diuresis before (58%) and after administration of cortisone (60%), whereas marasmic infants showed less diuresis before cortisone (50%) and a significant increase after administration of cortisone (78%).

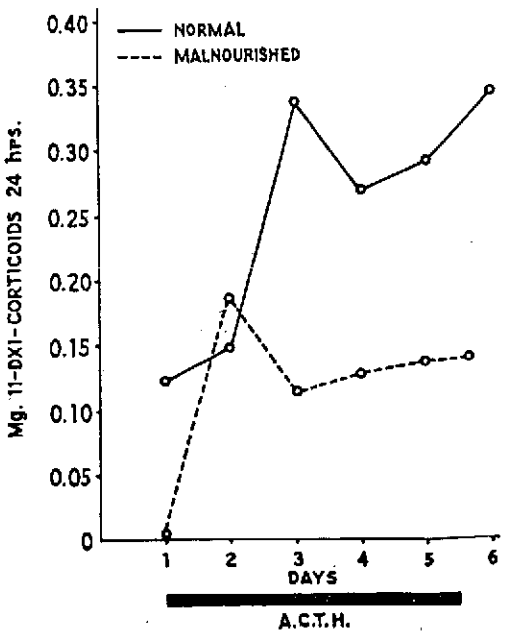

Figure 7. Urinary 11-oxycorticoids (mg/24 hrs.) in 10 malnourished infants and six normal infants.

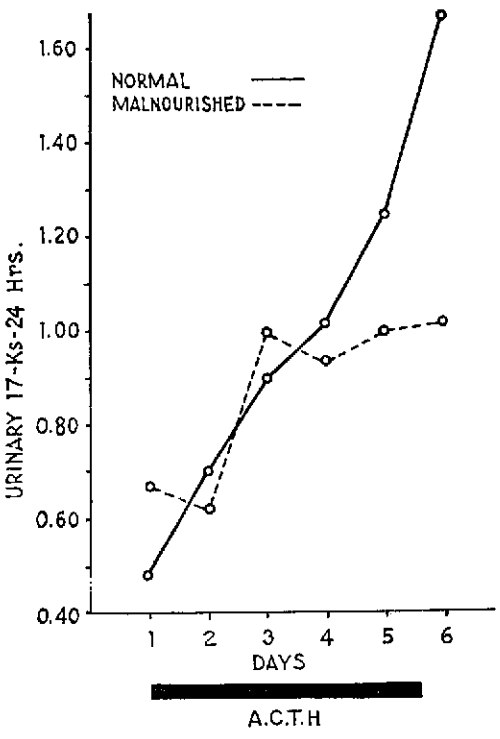

Figure 8. Urinary 17-ketosteroids in 10 malnourished infants and six normal infants during the administration of ACTH (30 mg per day).

response was studied only during a short period of time and after a single dose of corticotrophin.

Gillman and Gillman (*17*) suggest that in malnutrition there might initially be a phase of adrenal hypofunction followed by adrenal exhaustion and atrophy. Castellano and Arroyave (*11*) claimed that the marasmic infants have high, while the kwashiorkor patients have low glucocorticoid hormones. Lurie and Jackson (*20*) showed a significant reduction in excretion of 17-hydroxysteroids and 17-ketosteroids in malnourished children. As was pointed out in the beginning, these contradictory results may be due either to differences in the type of patient or to differences in terminology.

Thyroid function

Before recovery starts, the basal oxygen consumption of marasmic patients is significantly lower than that of normal children (*25*). The thyroid radioiodine uptake is also lower, as is the butanol-extractable iodine (BEI) (Table 2) (*5*).

After the administration of thyroid stimulating hormone (TSH), the marasmic patients corrected the low values of radioiodine uptake from an average of 28 per cent to 50 per cent, which is very similar to the control group (Table 2). Therefore, it is probable that the fundamental cause of the failure in the iodine uptake was the absence of TSH.

The marasmic patients, as well as the controls, showed increased BEI after TSH administration. Nevertheless, the average values attained by the marasmic infants were significantly lower than those of the control group. This result

Table 2

I^{131} uptake, BEI, and oxygen consumption in marasmic and normal infants (before and after TSH administration)

Infants	I^{131} uptake %		BEI (μg/100 ml)		O_2 consumption (liters/hours/100 cm height)	
	Before TSH	After TSH	Before TSH	After TSH	Before TSH	After TSH
Normal	39 ± 3.2 SD	60 ± 3.6 SD	5.4 ± 1.3 SD	7.9 ± 1.7 SD	4.2 ± 0.3 SD	4.2 ± 0.4 SD
	$p < 0.001$		$p < 0.01$			
Marasmic	28 ± 5.0 SD	50 ± 10.5 SD	3.4 ± 0.9 SD	5.9 ± 0.5 SD	2.8 ± 0.4 SD	3.5 ± 0.4 SD
	$p < 0.001$		$p < 0.001$		$p < 0.01$	
[a] $p <$	0.001	0.01	0.001	0.001	0.001	0.001

[a] p represents the significance between normal and marasmic infants in each period of study.

suggests that although the low values of BEI are due in part to a decrease of hypophyseal stimulation, there may also be a deficit of the thyroid function *per se*. It is probable that the mechanism of hormonal synthesis, which is more complex than the iodine uptake, is involved to a greater extent in marasmic malnutrition.

The oxygen consumption values in the normal group do not suffer important modifications after TSH administration, results which were obtained in spite of a significant increase of BEI (Figure 9). It has been suggested that, under normal conditions, the response of oxygen consumption to TSH takes several days longer (*30, 34*).

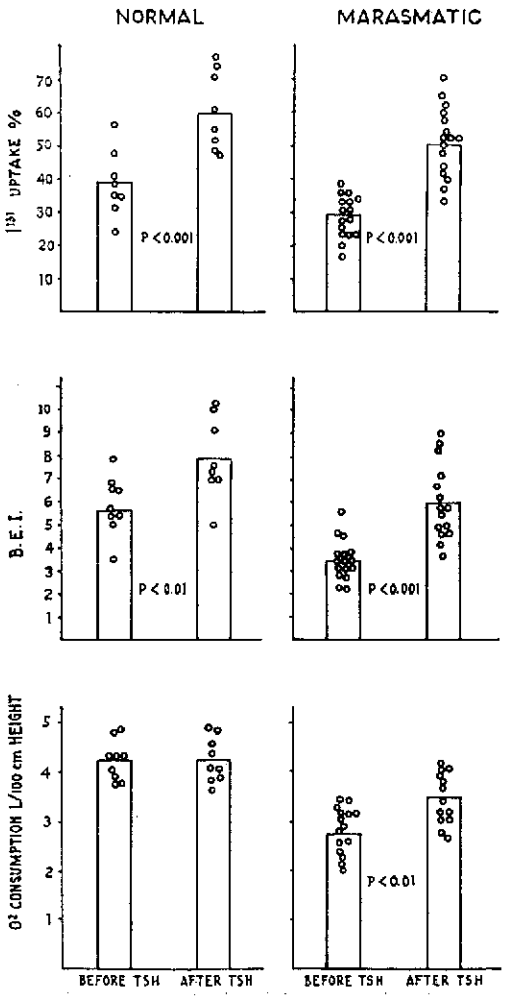

Figure 9. Marasmics and control group showed increased BEI after TSH administration, but average attained by marasmic infants were lower than control group. Oxygen consumption response to TSH administration in marasmic group was significant, but did not reach normal values.

The oxygen consumption response to TSH administration in the marasmic group was significant, but it did not reach normal values. Probably it is necessary to administer more than a single dose of TSH in order to obtain a satisfactory response. On the other hand, the lack of response to TSH stimulation may be a consequence of changes in the peripheral tissues secondary to malnutrition. It should be remembered that marasmic malnutrition, unlike kwashiorkor, is not accompanied by hypoproteinemia. The result, therefore, cannot be an artefact related to increase of protein binding of thyroid hormone (*13*).

These results allow us to conclude that in chronic marasmic infants, there is a real deficiency of the thyroid function that in some degree involves the gland, but is mainly due to a decrease in TSH stimulation. In the case of typical kwashiorkor, the oxygen consumption is normal (Figure 4), as is the iodine uptake (*3*), as if the thyroid gland were not seriously involved.

Somatotrophin studies

Chronic marasmic patients often present very difficult problems in therapy. Recovery, if it occurs at all, takes a long time. In our experience, around 30 per cent of the severe marasmic children (*21*) require more than four months of treatment before showing a consistent rate of weight gain. The mortality rate is high during this period. Nitrogen, potassium, and phosphorus balance is negative or only slightly positive. Administration of human growth hormone (HGH) during the period of stationary weight, however, produces a definite weight gain and an increase in nitrogen, potassium, and phosphorus retention (*26*). Discontinuation of HGH results again in weight stability. This fact suggests that a decrease in HGH production

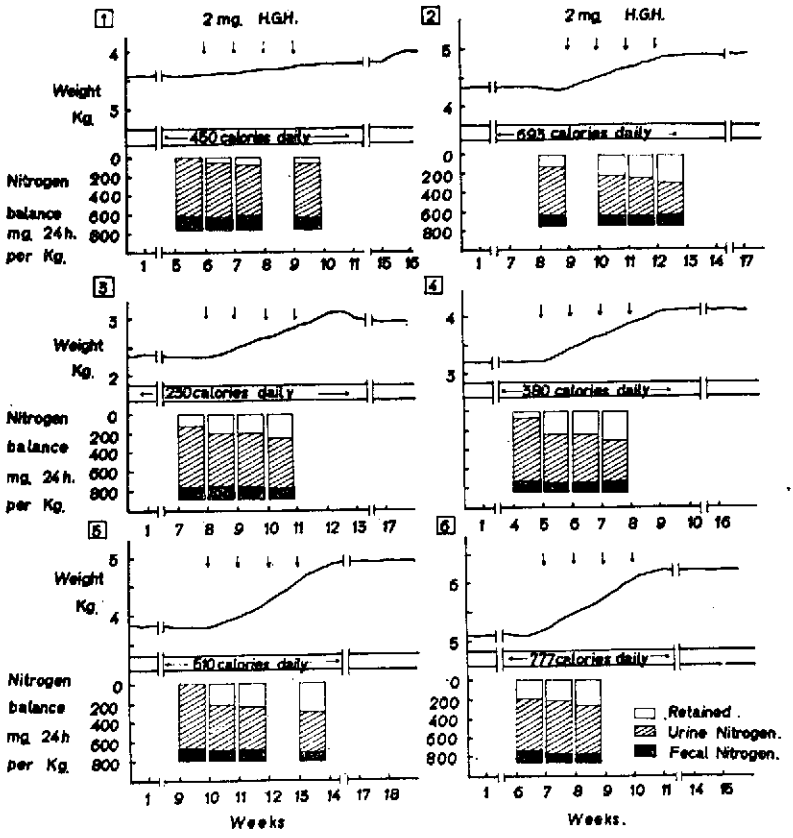

Figure 10. After the administration of HGH, marasmic patients showed a weight gain and increase in nitrogen, potassium, and phosphorus retention.

results from chronic marasmic malnutrition (Figure 10).

Beas *et al.* (*4*) have shown that basal values of HGH in this type of patient are significantly reduced (Table 3). The same result with the same kind of patient has been reported by

Table 3

Basal values of plasma growth hormone during fasting and after arginine infusion in undernourished infants (marasmus and kwashiorkor) and in normal controls

Groups	Number of patients	HGH plasma (mu/ml)		
		Fasting	After 45 min.	After 60 min.
Marasmus	6	4.1 ± 0.9	5.1 ± 1.7	5.8 ± 2.0
Kwashiorkor	6	25.6 ± 13.3	25.0 ± 17.6	14.2 ± 5.3
Control	5	7.8 ± 2.6	16.4 ± 3.7	14.6 ± 2.6

Difference between control and marasmus $p < 0.001$.
Difference between control and kwashiorkor $p < 0.001$.
Difference between marasmus and kwashiorkor $p < 0.001$.

Godard and co-author in Bolivian infants (*18*). Beas *et al.* (*4*) also describe in typical kwashiorkor a high basal level of plasma growth hormone (GH).

As in other hormonal studies, the GH determination has been the subject of controversial results. There is agreement that kwashiorkor patients present higher than normal levels of plasma GH (*1, 27, 28*). However, some authors report low values in marasmic children, and Hansen *et al.* (*27, 28*) report high values, although not as high as in kwashiorkor patients. Again we think that the confusion is due to the different kinds of patients studied. For instance, Hansen *et al.* (*27*) report that the level of GH in malnourished children is related to the level of plasma albumin—the lower the plasma albumin, the higher the plasma GH. The marasmic patients studied by these authors also have low plasma albumin. From our own point of view, the marasmic patients have normal plasma albumin, and consequently the patients that they studied would be at least a mixed form between marasmus and kwashiorkor (*27*).

It is well established that a single determination of GH, during the fasting state, is very often of limited value, because many variable levels may occur in subjects with normal pituitary functions (*14*). It has also been reported that an intravenous dose of arginine is a satisfactory test for the evaluation of human GH reserve (*22*). Based on these observations, the plasma GH level was determined in marasmic, kwashiorkor, and normal children, before and after the arginine administration (Table 3). A significant increase in the serum levels of GH was observed in normal infants after stimulation with arginine. No significant response was observed, however, after the administration of arginine in marasmic infants. However, patients with kwashiorkor presented variable GH values —the basal level was high, but there was no increase after arginine administration, and sixty minutes afterwards there was even a tendency to decrease. Godard and co-author (*18*) also have studied the pituitary reserve of GH in marasmic children after hypoglycemia provoked by insulin. Their results confirm the low basal values of GH with no significant increase after the hypoglycemia.

These results seem to indicate that in marasmic infants, chronic low calorie and protein intake produce low plasma GH levels and absence of response to different stimuli. It can also be assumed that protein deprivation, as in kwashiorkor, produces an increase in plasma GH. The full meaning, however, is difficult to interpret until further studies can be made. The increase of GH could be the consequence of an increase of GH release, a decrease in peripheral utilization, or impaired breakdown as a consequence of acute protein deficiency.

When all the accumulated data is considered, it is clear that endocrine response is different in kwashiorkor patients as compared to marasmic patients. A decrease of hypophyseal trophic hormones in the case of chronic marasmic patients is evident, whereas in kwashiorkor, facts have failed to demonstrate such a response. Kwashiorkor is the consequence of a low-protein intake with a sufficiently good calorie intake, whereas marasmus is due to a low-calorie and low-protein intake. In other words, the main difference is the calorie restriction, since both have a low-protein and probably a low-vitamin intake.

It can be postulated that, as a consequence of calorie restriction, there is a decrease of hypophyseal trophic hormone. The apparently reduced activity of the gland may, therefore, be nothing else than an indication of a reduced demand for its products, or perhaps may be a real adaptation triggered by the chronically diminished calorie intake. If it is an adaptation, it might then permit survival, in spite of a greatly lowered calorie intake, by resulting in a decrease in body activity, a lowering of the basal metabolic rate, and a diminishing or arresting of the growth rate.

Experimental results show that hypophyseal hormones, and also the availability of energy regulate the rate of cell division. Cell multiplication requires an adequate amount of energy, and depends upon glycogen concentration and

Table 4

Mitotic index in the crypts of Lieberkühn of fasted rats and rats fed on diets with different protein content

	Standard diet (15 animals)	Fasted (8 animals)	Diet 0% protein calories (8 animals)	Diet 4% protein calories (8 animals)	Diet 8% protein calories (8 animals)	Diet 20% protein calories (8 animals)
Mitotic index	6.6 ± 1.3	3.1 ± 1.2	5.0 ± 1.6	5.9 ± 0.9	5.9 ± 0.8	6.0 ± 1.1
Number of cells per mitosis	16 ± 3	36 ± 12	22 ± 7	17 ± 3	17 ± 2	17 ± 2
Significance of difference from normal; $p <$		0.001	0.05	0.10	0.10	0.10

The number of epithelial cells per mitosis is given as the reciprocal value of the mitotic index. Mean values ± SD.

carbohydrate metabolism. In rats, calorie restriction immediately decreases mitotic rate. Fasting rats have a fall in the rate of cell division, but when the calorie intake is increased, no change in the mitotic rate is observed, even with a restricted protein intake (Table 4) (*6*). Through study of the rate of cell division in the crypts of Lieberkühn by duodenal biopsy (*7*), similar results have been obtained in a group of marasmic patients in our laboratory. Marasmic infants present a significantly low mitotic index that returns to normal values with treatment. Stekel and Smith (*31, 32, 33*) have obtained similar results in hematopoietic tissue. The response in typical, acute kwashiorkor is different, and the mitotic index remains near normal values (Table 5). These results suggest that the calorie intake is of great importance in maintaining the rate of cell renewal.

Experiments on animals show that thyroid hormone and GH have a positive influence on the rate of cell proliferation (*16, 19*). The hypophysectomized animals present a lower rate of cell division in the Lieberkühn crypts (*16, 19*). These experimental studies lead to a general hypothesis: chronic decrease of calorie intake results in a decrease in hypophyseal trophic hormones, and, consequently, a decrease in cell division, growth, and metabolic rate results. The decrease is proportional to the decrease and duration of caloric restriction. On the other hand, the protein restriction alone causes a decrease in protein synthesis and many other adaptation

Table 5

Mitotic index and number of epithelial cells per mitosis in the crypts of Lieberkühn of jejunal biopsies of normal infants and infants with marasmus and kwashiorkor

	Normal (8)	Kwashiorkor (10)	Marasmus all cases	Marasmus before recovery	Marasmus during recovery
Mitotic index	3.9 ± 0.2	3.0 ± 0.6	1.9 ± 0.8	1.3 ± 0.5	2.4 ± 0.8
Range	2.6–4.5	1.8–3.7	0.7–3.6	0.7–2.1	1.4–3.6
Number of crypt cells per mitosis	26 ± 5	35 ± 9	65 ± 32	87 ±34	46 ± 16
$p <$		0.02	0.01	0.001	0.01

Numbers in parentheses are numbers of infants. The number of crypt epithelial cells per mitosis is the reciprocal value of the mitotic index.

mechanisms in protein metabolism (these have been studied and reviewed by Waterlow). Prolongation of protein restriction produces the typical protein deficiency syndrome, kwashiorkor. However, the clear separation of the two syndromes obtained under experimental conditions is very unclear in actual cases. A frequent confusion in the clinical picture is due to the fact that a deficit of proteins and calories, varying from patient to patient, is always found. In the final analysis, then, this hypothesis needs a much broader investigation.

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HOMEOSTATIC MECHANISMS IN THE REGULATION OF SERUM ALBUMIN LEVELS

R. Hoffenberg

The relative constancy of the plasma albumin concentration in health suggests the existence of some sort of regulating mechanism. Control could be effected through variations in synthesis or catabolism of the protein, or through alterations in its distribution between intra- and extra-vascular pools. Although there is evidence of increased transfer of extravascular albumin to plasma in states of protein malnutrition (*4, 12*), such measurements are usually based on isotopic tracer data which are difficult to interpret in the unsteady states that might be expected to exist in disease or after acute experimental procedures. In any event, this type of exchange must be regarded as a temporary expedient to meet acute changes; alterations in synthesis or catabolism would be anticipated in more chronic disturbances. These adaptive changes have been studied *in vivo* in human subjects suffering from malnutrition and in animals experimentally deprived of dietary protein, and attempts have been made to correlate the findings with the *in vitro* behavior of the isolated perfused liver and of cell-free systems.

Catabolism of albumin and protein malnutrition

In healthy adult men the amount of albumin catabolized per day is variously reported to be between 150-250 mg kilogram^{-1} day^{-1} (*12, 27, 29, 36*). This is equivalent to a fractional rate of 8 to 12 per cent of the intravascular pool catabolized per day. Both figures (absolute amounts and fractional rates)are slightly higher in healthy children (*29*). A limited study of apparently healthy male Africans (*5*) revealed a smaller intravascular albumin pool and a lower absolute rate of catabolism, a hint that the nutritional status of the individual might affect the turnover of albumin.

When dietary protein is limited, the plasma albumin level falls and its catabolism is slowed down. This has been demonstrated in kwashiorkor and in experimentally-induced protein depletion (*4, 8, 12, 15, 19, 20, 26*). Initially the fractional catabolic rate is unchanged and the absolute rate falls in parallel with the plasma albumin concentration and pool size. By adaptation of the catabolic rate to the synthesis rate, a simple but effective method of compensating for reduced synthesis is thus provided (Figure 1) and a new equilibrium is reached.

It should be stressed that this fall in the rate of albumin catabolism follows reduction of the intravascular pool and is not primarily determined by the reduction in dietary protein (*12, 15, 20*). Further, it is not specific to the hypoalbuminemia of protein malnutrition, but is seen in cirrhosis of the liver (*7, 30, 37*) and after plasmapheresis (*1, 11, 23*). Exceptions are seen in the nephrotic syndrome and protein-losing enteropathy where local hypercatabolism may play a part (*11, 17, 42*). In kwashiorkor (*4, 15*) and in experimental protein depletion (*12*), a fall in the fractional rate of albumin catabolism may also be observed.

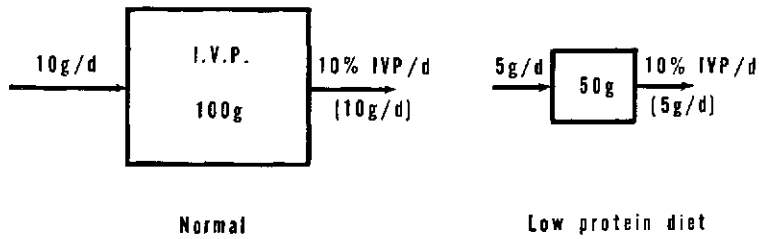

Figure 1. Diagrammatic representation of effects of diminished synthesis on albumin pool size. Maintenance of a constant fractional breakdown rate establishes a new equilibrium.

On refeeding, a reverse sequence of events is found. A return to a normal or supranormal (see below) synthesis rate rapidly restores the plasma albumin pool, and catabolism gradually returns to its normal rate, adapting once again to the altered rate of synthesis to reach a new equilibrium (*20*).

The site of albumin catabolism in the body is still not known and no specific organ has been shown to play a predominant role. Studies using the isolated perfused rat liver have led to the belief that this organ normally accounts for 10 to 15 per cent of the total amount of albumin broken down *in vivo* (*3, 10*). There is good justification for the use of this system as a model for studying adaptive changes in catabolism under experimental conditions and for the assumption that its behavior is representative of that of the intact live animal (*13*). Figure 2 shows the response of the perfused rat liver to a drastic increase in perfusate albumin concentration. A constant fractional rate of breakdown is maintained resulting in increased absolute degradation. If a liver taken from a rat which has been fed a non-protein diet for 15 to 20 days is perfused with blood taken from similarly deprived animals, a slower fractional catabolic rate is found, analagous to the observations in human subjects and experimental animals referred to earlier. It is interesting that this slow rate of catabolism can be accelerated by the addition of albumin to the perfusate pool or by perfusion with blood taken from rats fed a normal diet (Figure 3).

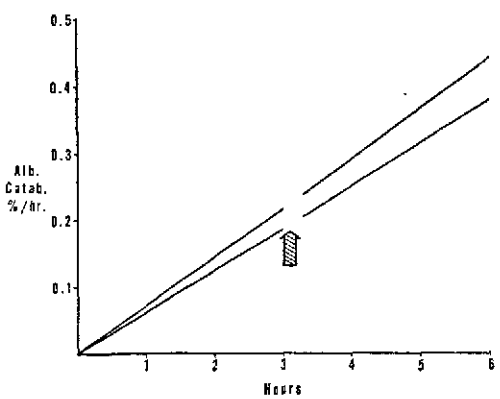

Figure 2. Effect of increased concentration on the fractional breakdown rate of albumin by the perfused rat liver. Additional albumin raised the plasma concentration of the perfusate from 21.8 to 38.9 mg/ml and 24.6 to 37.6 mg/ml in the two experiments. Catabolic rate measured from non-protein-bound/protein-bound iodide ratios.

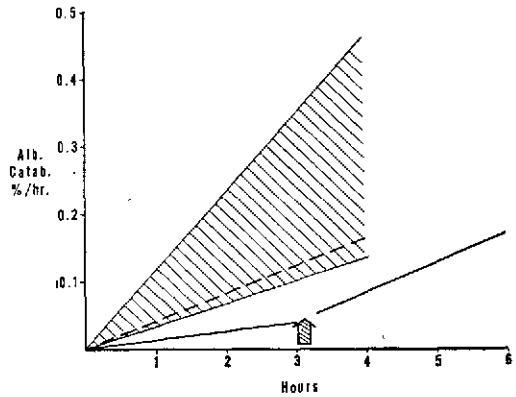

Figure 3. Albumin catabolism by livers taken from rats fed a protein-free diet. Note slow rate when perfused with blood from similarly deprived animals, and attainment of normal rate when albumin is added to increase the concentration from 18.7 to 21.6 mg/ml (solid line), or when perfused with normal blood (broken line). Normal range of catabolic rate shown by hatched area.

The observed constancy of fractional albumin breakdown rate by the perfused liver may be explained by the theory of fluid endocytosis, in terms of which hepatic cells engulf a fixed volume of ambient fluid per unit time (*14*). The number of albumin molecules introduced into the cell and therefore available for lysosomal proteolysis will be proportional to the concentration of the protein in the engulfed fluid, that is, in the extracellular milieu, provided selective adsorption of molecules at the cell membrane does not occur. This would explain the maintenance of first-order kinetics for albumin breakdown observed *in vivo* and *in vitro*. In order to interpret the lower fractional rate found with livers from protein-deprived rats, one might postulate a reduced rate of endocytosis or lysosomal activity. Protein starvation has been shown to produce structural changes in liver lysosomes as well as alterations in proteolytic enzyme activity (*25*), but the relationship to the functional changes mentioned above is not clear.

Synthesis of albumin and protein malnutrition

Amino acids for plasma protein synthesis are derived mainly from dietary sources. It is not surprising that one finds a close correlation between plasma albumin concentrations and the level of protein in the diet (*19*). With prolonged restriction of available protein, albumin synthesis is progressively reduced leading to gradual diminution in the size of the body albumin stores (*12, 15, 20*). Refeeding with a diet of normal protein composition causes a dramatic increase in albumin synthesis rate, which is apparent within 24 hours and which appears to be increased above the normal range. This leads to rapid restoration of body albumin.

Perfusion of the isolated liver provides a better model for studying albumin synthesis than it does for catabolism, since production of this protein is confined to the liver (*24*). Yet, under ordinary conditions, livers taken from normal rats appear to synthesize only half the amount of albumin produced by the intact animal. Despite this, the system has proved most valuable for comparative studies.

Rothschild *et al.* (*28*) have perfused rabbit livers with blood diluted with a solution containing glucose and amino acids. They found reduced rates of albumin synthesis when the donor rabbit had been fasted for 18 to 36 hours prior to extirpation of the liver. In a study of protein deprivation as opposed to starvation Hoffenberg, Gordon, and Black (unpublished observations) found a similar lowering of albumin synthesis. Because of difficulties in interpretation when synthesis rates of urea and protein are low, we abandoned the $^{14}CO_2$ method of McFarlane (*21*) in favor of direct measurement of rat albumin production by an immunodiffusion technique (*22*), using a system of heterologous (rabbit) plasma with homologous red blood cells. Livers taken from rats previously fed non-protein diets for 15 to 20 days showed significant depression of albumin synthesis (Table 1). Yet, these differences were abolished if liver size was taken into account and, weight for weight, livers of protein-deprived rats seemed competent to manufacture albumin. The extent to which total albumin production is impaired in these livers is comparable to that seen *in vivo* after exposure to a non-protein diet (*19*), although, as mentioned, the performance of the isolated liver does not match that found in the intact animal. With livers taken from refed animals, the findings are again similar to those in the intact rat. If the donor animal is fed a normal diet for 48 hours after 15 to 20 days of protein-deprivation, high albumin synthesis rates are found (Table 1). These are within the normal range in absolute terms, but are considerably raised when related to liver weight.

These responses of the isolated liver must be considered in the light of structural changes known to affect the hepatic albumin-synthesizing apparatus in protein-deprivation. Starvation and amino acid or protein restriction are attended by prompt alterations in polysome profile with a breakdown of membrane-bound polysomes and the appearance of more free monosomes and disomes (*2, 9, 16, 33, 34, 38*).

The shift of polysome particles from heavy to light is generally assumed to reflect a reduced

Table 1

Albumin synthesis rates. Livers taken from rats fed diets of different protein composition [a]

	mg/h		mg h^{-1} 100 gm rat^{-1}	
	$^{14}CO_2$	Imm-diff.	$^{14}CO_2$	Imm-diff.
NL	5.0	4.0	1.50	1.13
(n = 6)	(2.7–8.3)	(2.6–4.6)	(0.68–2.85)	(0.76–1.28)
OPL		1.4		0.92
(n = 5)	—	(0.9–1.7)	—	(0.55–1.13)
Refed L.	3.4	4.4	1.77	2.32
(n = 3)	(2.2–4.1)	(3.5–5.0)	(1.24–2.16)	(1.73–2.65)

[a] Albumin synthesis rates (mean and range) by isolated perfused rat livers. NL = livers from rats fed normal diet; OPL = livers from rats fed protein-free diet for 15 to 20 days; refed L = rats refed normal diet for 48 hours after protein-free diet for 15 to 20 days.

capacity for protein synthesis. Reaggregation and a restoration of ability to incorporate amino acids into protein rapidly follow the introduction of amino acids into the system. Tryptophan, in particular, seems to exert a unique and powerful influence on polysome structure and function.

The interpretation of polysome profiles in relation to dietary protein intake and amino acid incorporating ability must be adopted with caution, as aggregation may be restored when animals are refed a non-protein diet after a period of fasting, that is, exogenous protein is not mandatory for their restoration (*39, 41*). Wilson and Hoagland (*40*) have shown that 36 per cent of liver polysomes are stable after fasting, that is, not subject to these structural changes, and a majority of these can synthesize albumin. Selective disaggregation of polysomes not committed to albumin synthesis could confuse the picture outlined above.

A final word to try to relate these findings to changes in liver enzymes that have been described. In classical studies, Schimke (*31, 32*) and, more recently, Deosthale and Tulpule (*6*) have shown reduction in all hepatic urea cycle enzymes after protein deprivation. Reduced urea synthesis is reflected in low blood urea and urine excretion levels in protein malnutrition, and this was encountered in our perfusion studies on livers from protein-deprived rats. It is interesting that amino acid activating enzymes have been shown to be increased in depletion studies (*35*), suggesting that amino acids would preferentially be diverted into protein synthesis rather than into urea formation. The liver might be thought of as "primed" to synthesize protein as soon as adequate amino acids are supplied. This concept would fit very well with the prompt, even exaggerated, response to refeeding, which has been found *in vivo* and with the perfused liver system.

In conclusion, one should emphasize that body responses cannot be contemplated *in vacuo*. In particular, the role of hormones needs to be considered, as John and Miller (*18*) have recently demonstrated. In their studies, maximum albumin synthesis by the perfused liver required insulin and cortisol, as well as amino acid supplementation. Any attempt to ignore these factors must lead to an oversimplified view.

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DISCUSSION

Neel: I should like to return to Dr. Mönckeberg's paper to ask a question. We are all very much aware of the possible lasting effect of protein malnutrition on mental development. In this regard, Doctor, did your study include IQ follow-ups? When you have saved one of these children, do you have a functional citizen or not?

Mönckeberg: This is a difficult question since adequate studies do not exist. In cases in which malnutrition is severe and affects the child during the first stages of his life, a considerable retardation in psychomotor development can be observed. Our experience, and that of several other investigators indicates these damages to be persistent, leaving behind serious sequelae, even when the nutritional condition improves in a later period. We have been able to make a follow-up study of a group of 15 patients, presently nine-to-ten-years-old, who experienced severe malnutrition during the first months of life and later improved their nutritional condition. The IQ as an average is 74, compared to normal values of 94 to 110. From the physical point of view they present an anthropometrical deficit—their height is 24 centimeters below normal for their age. We also have a study on the frequency of mental deficiency in preschool children. Where malnutrition is prevalent in a slum area, almost 40 per cent of the children have an IQ lower than 80. It is very difficult to arrive at a definite conclusion about this, however, because we know that a low IQ in a slum area will be the consequence of at least two factors, malnutrition and deprivation. Unfortunately, those factors are always together—we cannot separate them. In any event, we can demonstrate a very close correlation between the degree of growth retardation and the degree of mental retardation.

Neel: A third factor may be involved, namely, the possibility that people of low IQ gravitate to the slums so that you have a constitutional factor interacting with the two factors you mentioned.

Chairman Waterlow: We are just now following up our malnourished patients after five to nine years. They seem presently to be some ten IQ points below their own brothers who were not treated for malnutrition. This only partially answers the question.

Neel: But where do their brothers stand with reference to the population norm?

Chairman Waterlow: I do not know that we can answer that, but I should not conceal from you that brothers do not always have the same father in our society.

Harper: I would like to return briefly to Dr. Hoffenberg's paper and ask if he has measured proteolytic enzymes in the liver under some of the conditions in which changes in the catabolic rate have been observed.

Hoffenberg: No, we have not. This has been done by other workers, but we have not done so. Changes have been observed, of course, in certain enzymes.

SUMMARY

J. C. Waterlow

The purpose of these symposia, which the Advisory Committee organizes every year is to bring together different ideas and disciplines in order to help PAHO in its objective of improving health. I think I speak for the Committee as a whole in thanking the contributors who have come to present these papers to us.

In conclusion I should like to comment briefly on some general ideas put forth by various speakers, particularly as they touch on my own interests and research.

Very generally, what has emerged from the sessions is that a main problem for the future is the recognition of marginal states, which are halfway along what Dr. Arroyave calls "the spectrum of deficiency."

Dr. Mayer introduced the idea of adaptation at a cost. The problem is to determine where the cost begins, and at what point on the spectrum is the division between *all right* and *not all right.* As Drs. Hilleboe and Mayer have pointed out, administrative decisions have to be taken on all these matters. It is our job to feed in the information on which those decisions are based. We had some discussion about statistics. I am glad to have the last word because I can restate my view that the collection of facts about populations is indeed very important and provides part of the data that have to be fed in—facts about individual variation, facts about differences between groups. My contention was that these facts in themselves do not provide the basis for value judgments. They tell us what the situation is. They don't tell us whether it is good or bad. But our problem is to make value judgments. To repeat what I said earlier, I think these are made possible by a study of associations between different things, such as Dr. Arroyave described in the work on vitamin requirements and vitamin standards, by studies of what happens in time, by functional tests, and, lastly, by an understanding of the basic mechanisms of adaptation. It would be quite impertinent of me to try to summarize the information given this morning and this afternoon, at various levels, on different kinds of adaptive mechanisms. I can only say that in my own work, which is concerned to a considerable extent with practical problems, such as the protein requirements of infants, how to diagnose protein deficiency and so on, I have found the work of Drs. Potter and Schimke extremely valuable and helpful. It would be unnecessary to trace the intellectual connection between their work and our problems, but it exists and I am sure the same is true for many other people working in this field.

I would like to end by recalling a remark thrown out casually by Dr. Waters at the very end of his presentation in which he said that we have to search for *sensitive indices.* That is the heart of the matter, and in my view, the way in which we are going to identify sensitive indices is through a better understanding of the biochemical mechanisms.

SUMMARY

Philip P. Cohen

What we have heard today exemplifies the spectrum of research input which is necessary in order that we can understand the adaptive factors in so complex an organism as man, both as an individual, and as a member of a group. The original question that was posed —namely, the question of adaptation, particularly in relation to diet, was explored perhaps without defining what is meant by adaptation. Adaptation, as was pointed out, is basically an individual problem. The intrinsic biochemical or molecular aspects of the cell, the organ, and the hormonal interplay operating in the whole organism have been emphasized, but as was brought out by Dr. Mayer, there is also the need to recognize that, as a member of a community, the individual has certain ecological or environmental relations which come into play, of which diet is only one consideration. The biochemical sophistication that has been developed together with a new way of looking at problems, which a molecular view permits, encourages me to believe that a concept of adaptation will eventually emerge that will be more meaningful and useful. Although the problem is complex, a reasonable and realistic position as to what standards in nutrition in terms of specific numbers are intended to mean, has been presented. If we recognize the limitations that are inherent in these numbers, and if we make certain that these numbers do not become so fixed that any deviation is considered to represent malnutrition, we can consider a reasonable beginning has been made to bring the areas of cell biology, biochemistry, genetics, and related areas, into some more meaningful frame of nutritional reference for help to the dietician and clinician in dealing with problems of diet and health. With these goals in mind, I feel that this has been a very rewarding session.

APPENDIX

THE ROLE OF MOLECULAR BIOLOGY IN HEALTH AND MEDICINE

Philip P. Cohen

A major current concern in the area of health relates to delivery of medical care with emphasis on the relative shortage of physicians. Because the time spent in medical education and specialty training has been increasing over the past 50 years in response to the advances in scientific knowledge and technology, to the extent that a period of 12 years (4 premedical, 4 medical, 1 interne and 3 residency), is usually spent before practice is undertaken, the medical profession and some medical educators are talking about a shorter curriculum with less emphasis on the natural sciences. The currently popular concept of "relevance" has led to the assignment by some of "less relevance" to the natural sciences, and "more relevance" to the social sciences (including psychiatry) as related to medicine.

Historically, it is worth noting that the major thrust and impact of the Flexner Report in 1910 was that of stressing the scientific basis of medicine, and the necessity for structuring medical education and practice on the scientific principles of the natural sciences of chemistry, physics, and biology. The great advances which had been made in the period of 1875–1910, particularly in the areas of bacteriology and the role of bacteria in disease, and in the discipline of pathology as developed particularly by Virchow in terms of a cellular basis of disease, were compelling reasons for introducing these new sciences into the medical curriculum. Indeed, the microscope became a symbol of medicine along with the stethoscope, and reflected the commitment of medicine to a scientific basis.

The exciting and very rapid advances in the natural sciences in the past 25 years have served to pose a dilemma for medicine. On the one hand, interesting new discoveries were being made in the areas of biochemistry, genetics, virology, and pharmacology, which required a more sophisticated level of scientific comprehension than was possible for most physicians. At the same time, traditional and empirical practices of medicine were being challenged by the newly emerging concepts, and laboratory techniques applicable to clinical problems.

A prominent and most promising feature of the emerging new areas of the biological sciences was that of a molecular basis for understanding complex biological events. In essence, the newly emerging concepts of molecular biology were extending, if not replacing, the cellular basis of biology to a more fundamental level, namely, the molecular. While biochemistry is in its very nature oriented to a molecular view of cellular functions, it had been concerned, particularly for the past 30 years or so, with applying techniques for the main purposes

of isolation, identification, and characterization of molecules such as vitamins, lipids, carbohydrates, and with the energetics of living systems. With the development of new techniques in the physical sciences, rapid advances began to be made by the biochemist in the isolation and characterization of macromolecules such as proteins and nucleic acids. As the biochemist became more adept at understanding the primary, secondary, and in some cases even the tertiary structure of macromolecules, such as proteins and nucleic acids (including genes and viruses), events of the greatest scientific importance occurred, particularly in the area of biochemical genetics, namely, the establishment of deoxyribonucleic acids (DNA) as the primary genetic material, the chemical and physical nature of DNA, and above all else the "cracking" of the genetic code inherent in the DNA molecule. This intellectual *tour de force* opened up a vista of scientific possibilities yet to be fully appreciated. At this point one might say that molecular biology emerged—but it should be noted, and even emphasized, that molecular biology is not in fact a discipline, but rather a way of thinking and developing concepts about biological events. The underlying basic discipline for molecular biology is in fact, today, chiefly biochemistry.

With the development of new concepts about genetic transcription and translation, regulation of cellular processes, and differentiation and development, it was to be expected that new concepts in molecular terms would emerge, particularly in pathology, to replace—or at least to extend—the cellular basis of disease developed by Virchow. As a matter of fact, the molecular view of biological processes has led to a breakdown of the traditional boundaries which earlier distinguished anatomy, microbiology, physiology, pathology, and pharmacology from one another. New journals have even emerged with such titles as *Experimental and Molecular Pathology, Journal of Molecular Pharmacology,* and *Molecular and General Genetics.* The molecular concepts of biological processes aligned with biochemical techniques have thus begun to emerge as a feature shared, to an increasing extent, by all the basic sciences of medicine. The significance of this trend is not so much that distinctions are disappearing, but rather that new concepts are emerging. This development is not simply one of substituting one set of words or interpretations for another, but rather is one which will permit, for the first time, an experimental approach to problems heretofore considered too complex to yield to experimental approach. Thus, investigators are now designing experiments to yield information on such complex systems of the brain as memory, mentation, and related functions. The advances in biochemical techniques and the new concepts of molecular biology have led to major new advances in subcellular anatomy, microbiology (in particular virology), physiology, immunology, pathology, pharmacology, and genetics. The full impact of these new concepts are yet to be fully appreciated and exploited in the fields of medicine dependent on these basic disciplines. While there is a gap—and always will be and should be—between the *implication* of a new concept in the basic sciences and the *application* of this new concept to clinical problems, the gap today seems to be disconcertingly large and growing larger. In my view, the primary reason for this is that the basic scientist is pursuing his research with a greater commitment than that of the clinician in comprehending and applying the results of the new research findings. If this position is valid, then it follows that the clinician cannot afford to be educated and trained with less science, but rather, to be effective, he must be trained with more science.

Because time will not permit a systematic review of the many areas of exciting new developments in molecular biology and biochemistry, which give promise of great importance when developed to the point of application in the clinic, I have decided to limit my comments to the area of molecular mechanisms involved in cell regulatory processes.

The problem of regulation of the cell to main-

tain its steady-state functions has begun to yield information of great interest to the biological scientist, as well as to the clinician. While the biochemist has been relatively successful in defining the energetics of the cell in terms of specific reactions involving the conversion of chemical bond energy of food stuffs to the energy required to do mechanical, osmotic, biosynthetic, and electrical work, mainly through the generation of adenosine triphosphate (ATP), the capacity of a specific cell and the whole organism to regulate these processes has begun to be appreciated only recently. Although energy production and effective utilization of transduced forms of energy are of fundamental importance, and basically similar in principle, if not in detail, in all cells, we now recognize that other forms of control are much more subtle and meaningful in the maintenance of functional health and survival of a cell or organism. It goes without saying that if a cell or whole organism cannot generate the energy necessary for effective function and regulation, particularly in higher animal forms, the organism will not survive. As a matter of fact, one has to accept the fact that if an organism survives at all, it does so because it can generate the energy necessary to do so. What is less clear is the nature and latitude of the regulatory processes which determine the survival of a given cell or the whole organism.

The regulatory processes which operate from the stage of the fertilized ovum and lead to the differentiation and development of the fetus and postpartum, the regulatory processes which operate through growth, maturation, and finally death are conceptually being developed and experimentally approached. While the model for much of the thinking about higher animal forms, such as man, is based on the models of microorganisms such as *E. coli,* cells of higher animals must utilize similar regulatory processes to one degree or another, in addition to the controls inherent in a cell which has its nuclear DNA in a segregated cellular organelle surrounded by a nuclear membrane, and its DNA bound to a family of proteins known as histones, plus a multiplicity of regulators at the level of hormones.

It is widely held that all, or almost all of the biological information that the adult human has potentially or actually is encoded in the DNA of the fertilized ovum from which he develops. A clear understanding of how this information is transcribed, translated, and finally regulated is necessary before one can appreciate the regulatory aspects of this process as the most basic consideration in the health and disease of a cell or organism. Although the basic model of transcription of DNA via messenger ribonucleic acid (RNA), and the translation of messenger RNA on ribosomes into specific functional proteins is still not complete, it is nevertheless adequate to explain the specific macromolecular defect in many cases of hereditary disorders involving a genetic defect or mutation. A considerable number of hereditary disorders have now been described in which lack of a specific enzyme, or production of an abnormal protein (for example, phenylalanine hydroxylase deficiency in phenylketonuria), or substitution of a specific amino acid (for example, hemoglobin S in sickle cell anemia in which a valine residue replaces the glutamyl residue in position 6 of the β-chain of normal hemoglobin A), can be explained on the basis of a mutant gene unable to code specifically for the necessary amino acid sequence required for a functional enzyme or protein. Although the processes involved in transcription of DNA and the translation of messenger RNA are far from being completely understood, meaningful questions can now be asked on the basis of the available concepts which can be experimentally tested.

Once the differentiated organism has developed to the stage of an adult animal, the role of the regulatory processes in maintaining the steady-state functions of the individual cell and whole organism becomes a predominant consideration. While the DNA of muscle, liver, and kidney cells, for example, all contain the

same genetic information, it is clear that each cell of these organs is regulated to carry out specialized functions peculiar to that organ. Although all the cells are programmed to produce the enzymes necessary for energy production and utilization, the cells of each organ also have the means of expressing themselves by synthesis of the unique macromolecules which are necessary for the specialized function of that organ. In essence then, the genetic information in each cell is programmed in terms of a type of repression and derepression of specific genes, inducers, plus other types of regulators. Because the macromolecules of each cell have a finite half-life time, there must be regulatory mechanisms in the adult cell which turn the genetic information on and off by some kind of a feedback or other mechanism.

A second level of regulation relates to the translation process. There is now evidence that the translation of messenger RNA, formed by the chromatin template of nuclear DNA, either before, during, or after attachment to the ribosome, is subject to regulatory factors. Levels of transfer RNA (needed for converting amino acids into aminoacyladenylates for conversion into peptides), initiation and termination factors (needed to indicate initiation and termination of the peptide chain on the messenger RNA), and special binding factors are known to operate in regulation at this level. Many hormones are now thought to exert their regulatory effects in animal systems by affecting the transcription or translation (or both) processes.

Regulation is also known to occur at the enzyme level by way of feedback inhibition, conformational changes, specific activators and inhibitors, enzyme turnover, and the like.

An area of emerging importance is that of the role of functioning membranes as regulatory systems. The animal cell has an elaborate system of membranes involving not only the surface of the cell, but also all of the intracellular organelles such as mitochondria, nucleus, microsomes, endoplasmic reticulum, and golgi apparatus. These membranes serve to compartmentalize certain cellular functions, and thus provide a system for regulation in the way of control of transport in and out of specialized areas of the cell.

The purpose of this excursion into regulation is primarily to call attention to the fact that we are on the brink of a new set of concepts which will hopefully provide a more basic understanding of the physiology of the cell and the organism. These new concepts provide a new basis for understanding disease in all of its aspects, whether the disease is a result of genetic defect, bacterial or viral infection, toxic agents, neoplasia, or nutritional deficiency. It is safe to predict that the unifying theme in the newer pathology will be in terms of a failure in cell regulation at the molecular level, irrespective of the etiology. It follows then that concepts of diagnosis, prognosis, and therapy must be based on these concepts if a rational basis for the practice of clinical medicine is to emerge and replace the large areas of empirically based medical practice. However, we will realize this goal only if it is recognized as a primary purpose of medical education and training. This, of necessity, must mean a more profound level of science training and education, rather than the trend to less profound and more superficial science.

The limited, if not erroneous view, of the fundamental basis of disease is revealed when the clinician talks—and even writes textbooks —about so-called metabolic diseases. It must be emphasized that basically all diseases are primarily the consequence of a cellular metabolic disorder. This is as true of a bacterial or viral infection, or a coronary occlusion, as it is of diabetes mellitus. The argument that this term is a convenient basis for classifying a group of diseases does not relieve the clinician who subscribes to this of the burden of ignorance of what is the underlying basis of *all* disease.

In countries with major problems of medical care and limited scientific traditions and research experience, a serious problem will exist in the attempt to apply the newly emerging scientific principles to clinical situations. The effective application of scientific principles requires the

availability of a group of basically-trained scientists to be certain that the principle is not only understood to begin with, but also is being appropriately applied and critically evaluated. After all, the great value of a scientific principle is that if fully and critically understood, it will permit a practical application free of empirical considerations. Since the clinician may not in all instances be sufficiently well trained to comprehend, appreciate, and effectively utilize the full potential of all of the new developments in the basic sciences, it goes without saying that any commitment of a society to deliver effective medical care must at the same time accept a commitment to train and maintain a sizeable group of basically-oriented scientists.

Finally, a word should be mentioned about current nutritional research. First off, it seems very clear to me that we have today all the basic knowledge needed regarding essential nutrients, but what we lack is the effective means in the different countries for application of this knowledge in the prevention or treatment of primary nutritional disorders. In addition, we lack a meaningful definition of health and what constitutes a departure from good health. Although there is uncertainty as to the quantitative requirement for one or more nutrients in one situation or another, this search for a magic number seems not likely to yield anything more than a series of quantitative limits which may be relevant to the requirement of a given individual, but of limited value as an index of nutritional adequacy—or of good health.

The area of nutritional research which remains to be explored in any depth has to do with regulation of cell function. It is obvious that in any overall process, there will be a rate-limiting requirement for any essential ingredient of that process, and any level below that rate-limiting requirement will affect, perhaps in an all-or-none manner, the operation of that process. What we need to know are the consequences of regulatory failure or inadequacy of any of the multiplicity of regulatory processes which result from dietary restrictions. At the same time, we have to know what degree of adaptability exists and the limits of this adaptability in the regulatory process affected by dietary restriction. Dr. Waterlow has discussed this matter in a recent publication (*1*). The means of regulation of cell function are complex and finely controlled; the limits of adaptability in any given dietary situation are not well understood. The kind of information needed will result not from the perpetuation of traditional experiments in human nutrition, but rather from experiments designed on the basis of the newer concepts of molecular biology and cell regulation. It is, thus, likely that well-designed animal model systems will be needed to exploit these newer concepts before suitable experiments can be developed for use with humans.

REFERENCE

1. WATERLOW, J. C. Observations on the mechanism of adaptation to low protein intakes. *Lancet* 2: 1091–1097, 1968.