tion and massive ecological disturbance whereas others remain untouched. Long-term existence of reefal enclaves with stable community structure intermingled with others subject to disequilibrium and repeating patterns of ecological succession should have important evolutionary consequences. Induction of a biotic province with an enhanced capacity for speciation and the generation of unusually high diversity levels should result.

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Nutritional Importance of Pyrroloquinoline Quinone

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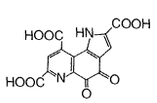
Mice fed a chemically defined diet devoid of pyrroloquinoline quinone (PQQ) grew poorly, failed to reproduce, and became osteolathyritic. Moreover, severely affected mice had friable skin, skin collagen that was readily extractable into neutral salt solutions, and decreased lysyl oxidase. The identification of functional defects in connective tissue and the growth retardation associated with PQQ deprivation suggest that PQQ plays a fundamental role as a growth factor or vitamin.

THE QUINOPROTEINS WERE RECOGnized in the late 1970s as a novel class of bacterial oxidoreductases that utilize PQQ (or Methoxatin) as a cofactor (*1*). PQQ also functions as a cofactor for important plant and animal enzymes (*2*). It is present in a number of common foods and food components (*3*), and it is a product of fermentations (*4*). Such observations raise questions regarding its metabolism and possible role as an essential dietary factor or vitamin.



The inhibition of lysyl oxidase, which in mammals requires PQQ, leads to osteolathyrism characterized by decreased cross-linking of collagen and elastin (*5*). We show that PQQ deprivation causes decreased levels of lysyl oxidase in mice, which results in mice that are lathyritic, grow poorly, and fail to reproduce. Although severe measures are required to render animals deficient in PQQ, deprivation can be achieved with an experimental protocol (*6*) similar to that used to produce deficiencies of essential ultratrace minerals or vitamins.

When PQQ was omitted from a chemically defined diet containing antibiotics (*6*), growth impairment was observed (Fig. 1). A normal growth response was observed when PQQ was added to the deficient diet, or whenever PQQ was detectable in the fecal samples or as a diet or water contaminant. The latter often occurred when resistance was developed to the succinyl sulfathiozole that was added to inhibit growth of intestinal microflora.

About 20 to 30% of mice, for which no PQQ was detectable in food or excreta, had clear external signs from the PQQ deprivation. These signs included friable skin, mild alopecia, and a hunched posture. The signs were occasionally quite severe (Fig. 2). Of a total of 40 mice assigned to PQQ-deficient diets (maternal dams plus the offspring in four separate litters), eight died by week 8 of deprivation. Three of these deaths were due to an aortic aneurysm or abdominal hemorrhages. Diverticuli were also common in the deficient animals. In contrast, only one of the mice in PQQ-supplemented groups (33 total) died during the course of the experiments.

A striking response was the friability of skin in those mice that appeared most affected by dietary PQQ deprivation. The response suggested that decreased maturation or deposition of skin collagen was a component of the lesion. Indeed, collagen extractability (*7*), a measure of collagen maturation and cross-linking, was increased abnormally in skin from mice fed deficient diets. The total amount of collagen extracted from skin of PQQ-deprived mice was about twice that of supplemented mice (*7*). Although it was not possible to confirm unequivocally that the increase in collagen extractability was due entirely to reduced amounts of cross-linking amino acids, a significant reduction occurred in the tissue levels of lysyl oxidase (*8*). PQQ deprivation caused a net decrease in lysyl oxidase to 10 to 30% of the normal values for skin, as estimated by an enzyme-linked immunosorption assay (*8*).

In addition, attempts to breed young female mice, which were fed the PQQ-deficient diet for 8 to 9 weeks, resulted in either no litters or in pups that were immediately cannibalized at birth. Such responses or behaviors were not observed for female mice fed the supplemented diet; greater than

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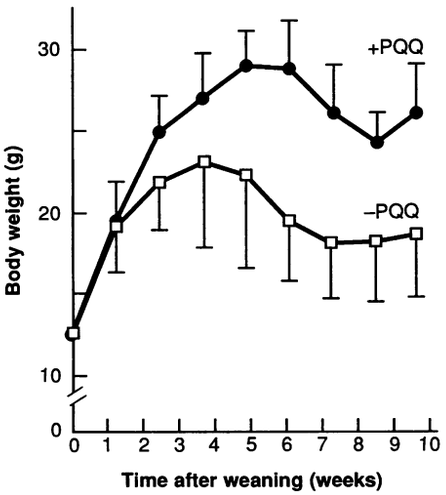

Fig. 1. Growth in response to PQQ addition to a chemically defined diet (*6*). Virgin female mice were adapted to the basal or PQQ-supplemented diet (800 ng of diet per gram) for 2 to 3 weeks before mating. The basal or supplemented diet was continued throughout lactation. The offspring were then transferred to the maternal diet (usually after 21 days). Each data point is a composite from two separate experiments and represents the means ± SD for 20 to 30 determinations. The differences at week 5 and subsequent points are significant at $P < 0.03$ to $P < 0.06$ by a Dunnett's test.

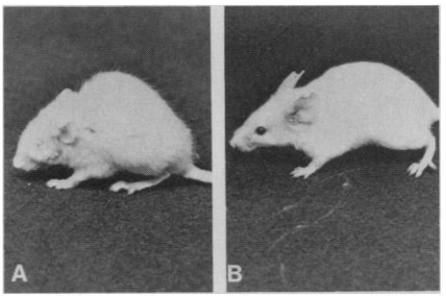

Fig. 2. Appearance of (**A**) PQQ-deprived and (**B**) PQQ-supplemented mice at week 8 after weaning. Mice with the appearance of (A) quickly recovered after PQQ addition to their diet or evidence of PQQ production by gut microflora.

90% of these offspring were successfully weaned.

The difficulty of recognizing PQQ as a potentially essential nutrient or growth factor has probably been the result of its ubiquitous presence in feedstuffs and growth media. Our experience has been that it is difficult to prepare liquid-based diets without some PQQ contamination (*3*). Even domestic water sources may contain traces of PQQ. If the requirement for PQQ is of the same order of magnitude as biotin or vitamin K, that is, 100 to 400 ng/g or 20 to 80 μg per 100 kcal (*9*), then 300 to 1200 ng per mouse per day may be a reasonable estimate of daily need. This amount of PQQ may be found easily as a contaminant in semipurified or some types of purified diets (*10*). For example, we have found that diet-grade starch preparations contain as much as 100 to 150 ng of PQQ per gram.

Moreover, primary deficiencies of many other essential nutrients are also difficult to achieve. For example, biotin, vitamin K, or folic acid deficiencies in animal models often require antibiotics or antagonists to effect clear signs of nutritional deficiency (*9, 10*). For PQQ, the natural antagonists are the aminonitriles, such as β-aminopropionitrile from the pea *Lathyrus odoratous* (*5, 11*). Further, enzymes such as lysyl oxidase are also developmentally regulated. A given deficiency sign is most obvious when the deprivation period coincides with periods of maximal expression of a targeted enzyme or other features important to developmental regulation. Consequently, we attempted to optimize the expression of a deficiency by depleting mice at the most critical stages of growth.

Whether eukaryotic cells synthesize PQQ has yet to be established, although it is known that PQQ acts as a mammalian cell growth factor when added to culture medium (*12*). If PQQ is produced in the mouse by an unusual metabolic pathway, it does not occur in amounts sufficient to maintain normal extracellular matrix maturation or growth. Although it is possible that the growth stimulation elicited by PQQ is the result of its potential probiotic effects (*12*), in most respects, PQQ seems to qualify as a vitamin as usually defined. At the very least, there appears to be a high degree of dependence on nutrition or intestinal flora for a supply of PQQ. Moreover, given the effects that PQQ deprivation has on extracellular matrix maturation, a useful line of future research could be the design of PQQ structural analogs that influence the rate of extracellular matrix deposition in control of fibrotic processes and wound healing. That the adrenal gland contains high concentrations of PQQ (*3*) suggests other important areas that should be examined.

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3. PQQ can be measured chemically with the assay described by M. Paz, P. M. Gallop, B. M. Torrelio, and R. Flückiger [*Biochem. Biophys. Res. Commun.* **154**, 1330 (1988)] and R. Flückiger, T. Woodhi, and P. M. Gallop [*ibid.* **153**, 353 (1988)]. This assay estimates PQQ even in the presence of 100 to 1000 molar excesses of other quinones or enediols, such as ascorbic acid. Our estimates of PQQ indicate that PQQ is present in egg yolk, adrenal tissue, and many citrus fruits in amounts that range from 500 to 20,000 ng/g. Many diet supplies (for example, purified caseins, starch, and soy protein) and commercial tissue culture media contain PQQ in amounts ranging from a trace to 10 to 20 ng/ml or about 100 ng/g. These estimates are in keeping with values first reported by M. A. Paz, R. Flückiger, E. Henson, and P. M. Gallop [in *Proceeding of the First International Symposium on PQQ and Quinoproteins*, J. A. Duine, Ed. (Delft Univ. Press, Delft, the Netherlands, in press)].
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6. The composition of the basal diet was modified slightly from that developed by D. A. Hirakawa, L. M. Olson, and D. Baker [*Nutr. Res.* **4**, 891 (1984)]. Purified amino acids were the nitrogen source. A 2.5:1 mixture of sucrose:starch was the carbohydrate source. The antibiotic succinyl sulfathiozole was added at 2% at the expense of the sucrose:starch mixture. Thiamin, pyridoxine, retinyl acetate, α-tocopheryl acetate, vitamin B_{12}, folic acid, vitamin K_2, and biotin were added at levels at least five times the requirements for the mouse. The vitamins and minerals were prepared first as premixes. All chemicals were reagent grade and purchased from Sigma Chemical Co., Baker Chemical Co., or Ajinomoto Chemical Co. PQQ was added to the basal diet at 800 ng/g. Only trace amounts of PQQ were present in the basal diet (<30 ng/g) as estimated by the chemical assay (*3*) and confirmed by a microbiological assay described by O. Geiger and H. Görisch [*Anal. Biochem.* **164**, 418 (1987)]. The mice were housed in a laminar flow unit. All food cups and cages were sterilized before use. Likewise, all food storage containers, the amino acid mixture, the mineral mixture, and the carbohydrate sources were autoclaved separately before formulation into the diet. The diets and distilled water were supplied ad libitum. The distilled water was filtered through a Millipore filter to reduce further the possibility of bacterial contaminations. Mice could usually be maintained 5 to 6 weeks before we saw evidence of succinyl sulfathiazole resistance, that is, PQQ production, by intestinal microflora.
7. Fresh samples of skin were serially extracted sequentially with 1*M* NaCl, 0.05*M* disodium phosphate buffer (*p*H 7.5), 0.5*M* acetic acid, and 4*M* urea in sodium phosphate buffer (*p*H 7.5), each for 24 hours. The volume of extracting solution to the initial fresh tissue weight was maintained at 10:1. Aliquots were then taken for estimation of soluble collagen as described by M. Marotto and G. Martins [*Anal. Biochem.* **150**, 86 (1985)]. Collagen α chains that are newly synthesized and not extensively cross-linked are extracted into 1*M* NaCl solutions. Amounts of collagen extracted (milligrams per gram ± SEM) for four or five samples were: with PQQ, 40 ± 8 (NaCl), 18 ± 2 (acetic acid), and 15 ± 4 (urea), for a total of 73 ± 6; without PQQ, 73 ± 4 (NaCl); 23 ± 2 (acetic acid), and 28 ± 5 (urea), for a total of 124 ± 5 ($P < 0.02$). Values for the acid-stable cross-links, hydroxylysinorleucine (HLNL) and dihydroxylysinorleucine (DHLNL) were also determined as described by K. M. Reiser and J. A. Last [*Collagen Relat. Res.* **6**, 313 (1986)]. The values (average for three determinations each) expressed as micromoles per micromole of collagen were: with PQQ, HLNL, 0.54 and DHLNL, 0.1; and without PQQ, HLNL, 0.49 and DHLNL, 0.09. These data indicate that PQQ deprivation develops slowly, since HLNL and DHLNL represent cross-links that are characteristic of longer lived or mature skin collagens.
8. We isolated lysyl oxidase from rat uterus as a

copurified mixture of isoforms using methods adapted from S. S. Tang, P. C. Trackman, and H. M. Kagan [*J. Biol. Chem.* **258**, 4331 (1983)]. The enzyme was measured in 4*M* urea extracts of skin samples [H. M. Kagan and K. A. Sullivan, *Methods Enzymol.* **82A**, 637 (1982)] by an enzyme-linked immunoassay. The net accumulation of lysyl oxidase in the extracellular matrix of dorsal skin (in micrograms per gram ± SEM) was reduced from 500 ± 123 to 146 ± 55 by week 8 ($P < 0.01$; for two replicates, $n = 4$ to 6 each). The lysyl oxidase antibody preparation and the enzyme-linked immunoassay were done as described by H. Bode and H. Stegeman [*J. Immunol. Methods* **72**, 421 (1984)] and S. B. Carroll and B. D. Stallar [*J. Biol. Chem.* **258**, 24 (1983)]. A preliminary report on the characterization of lysyl oxidase and its quantitation by enzyme-linked immunosorbent assay (ELISA) has been published by D. Tinker, N. Romero, and R. B. Rucker [*FASEB J.* **3**, 624A (1989)].

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Translational Blockade Imposed by Cytokine-Derived UA-Rich Sequences

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The messenger RNAs specifying certain proteins involved in the inflammatory response and certain oncoproteins contain a conserved UA-rich sequence in the 3′ untranslated region. This sequence, which is composed of several interspersed repeats of the octanucleotide UUAUUUAU, has been shown to destabilize mRNA in some eukaryotes. However, this effect is not seen when mRNAs are transferred to *Xenopus* oocytes, which made it possible to separate stability from translational regulation. For interferon, granulocyte-macrophage colony-stimulating factor, and c-*fos* RNAs, the UA-rich sequence was observed to preclude mRNA translation.

THE MODULATION OF THE TRANSLATIONAL efficiency is an important post-transcriptional regulation of eukaryotic gene expression. Various characteristics of messenger RNAs are known to markedly influence translation efficiency. Several features present at the 5′ end of the molecule have been shown to be prerequisites for efficient translation of a eukaryotic mRNA: the presence of a cap structure (*1*), a favorable context surrounding the AUG initiation codon (*2*), the absence of stable secondary structures, and of an out-of-frame initiation codon (*3, 4*). At the 3′ end, the presence of polyadenylate [poly(A)] not only enhances mRNA stability (*5*) but also appears to affect the translation of some mRNAs (*1, 6*). Recently, the translational efficiency of several mRNAs has been shown to be specifically regulated by the presence of structural elements located in the 5′ or 3′ untranslated regions (UTRs) (*7, 8*). However, the mechanisms by which these post-transcriptional controls are specifically mediated are still not elucidated.

We have previously reported that the 3′ untranslated region of the human interferon-β (Hu–IFN-β) mRNA has an inhibitory effect on translation in certain systems like the reticulocyte lysate or the *Xenopus* oocytes. Furthermore, the addition of this Hu–IFN-β 3′ UTR at the 3′ end of the chicken lysozyme mRNA also leads to a large decrease of its translational capability in both systems (*9*). We have also shown that the poor translational efficiency of the Hu-IFN-β mRNA is not caused by a rapid degradation of the molecule. In contrast to what occurs in somatic cells, where this

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Fig. 1. The 3′ untranslated sequence of the different IFN-β mRNA constructs. The sequence is numbered from first nucleotide of the stop codon. The first 103 nucleotides of the sequence [bold letters symbolized by soIF (1–103)] are common to all constructs. In each modified construct, the natural last 99 nucleotides have been replaced by an oligonucleotide containing the UA sequence either in variable number (underlined) or modified (dotted underlined). All the SP6 plasmids encoding the Hu–IFN-β were constructed with the basic psoIF vector previously described (*11*). The in vitro transcribed IFN-β mRNA from this psoIF plasmid contains as 5′ UTR a short synthetic 19-nucleotide-long sequence. In DNA constructs psoIF $(UA)_1$, psoIF $(UA)_2$, psoIF $(UA)_3$, psoIF $(UA)_{AU}$, and psoIF $(UA)_{GC}$, the natural translation inhibitory sequence (62 nucleotides) was replaced by double-stranded oligodeoxynucleotides corresponding, respectively, to one, two, and three copies of the octanucleotide, or to one copy of a consensus sequence that is either modified or enriched in G and C residues. These oligodeoxynucleotides were inserted in the psoIF plasmid previously digested by Nde I and Xba I to remove the natural translation inhibitory sequence.