the biosynthesis of specific glycopeptides. That GDP–mannose can be metabolized to GDP–fucose in mammalian tissues was shown by Foster and Ginsburg (1961). One could speculate that vitamin A functions as a carrier of the carbohydrate moieties in the biosynthesis of glycoproteins within membranes, in a manner analogous to the polyisoprenol carrier of carbohydrates in bacterial system (Dankert *et al.*, 1966). However, any such speculations with respect to the involvement of the vitamin in control of differentiation are still premature.

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Vitamin E. Regulation of the Biosynthesis of Porphyrins and Heme

Padmanabhan P. Nair,* Hari S. Murty, Priscilla I. Caasi, Sherry K. Brooks, and Jeffrey Quartner

Vitamin E deficiency in the rat leads to a decrease in the activity of hepatic δ-aminolevulinic acid dehydratase, the second enzyme in the biosynthetic pathway to heme. Since this was accompanied by lower concentrations of microsomal cytochrome P_{450} and b_5, as well as by lowered activities for hepatic catalase and tryptophan pyrrolase, we postulated the existence of a defect in heme synthesis in these animals. This was confirmed by following the incorporation of labeled δ-aminolevulinic-4-^{14}C and porphobilinogen-^{14}C into microsomal protoheme. Vitamin E was also found to have a second locus of action in blocking the induction of hepatic δ-aminolevulinate synthase and dehydratase by phenobarbital and allylisopropylacetamide. Structure–activity studies using several other substituted tocols and a group of synthetic antioxidants revealed that the action of vitamin E (α-tocopherol) in this system is not mediated by a mechanism similar to that of the antioxidants.

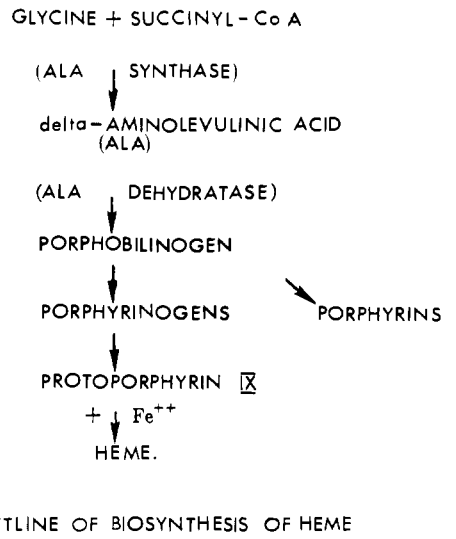
Vitamin E or α-tocopherol has been known as a nutritional factor for over five decades. Among the several fat-soluble vitamins discovered during that era, vitamin E is one that has remained an enigma.

Vitamin E deficiency has been associated with a wide variety of syndromes in several animal species (Table I). This pleomorphism in the manifestations of the deficiency of a single nutritional agent has prevented the enunciation of a satisfactory generalized hypothesis for its mode of action at the cellular level.

The several theories that have been proposed to explain the mechanism of action of this vitamin could be classified into two groups: those that are specific and those that are nonspecific (Table II). Although a considerable amount of literature exists supporting each of these hypotheses, none of these by themselves have provided a satisfactory unified concept. The idea that the deficiency of a single nutritional agent can give rise to several different apparently unrelated disease states in various animal species led us to believe that this vitamin may have a function fundamental to all living cells. In our search for such a function, our attention was drawn to the original observations of Dinning and Day (1957) on a nutritional anemia in primates fed a tocopherol deficient diet, indicating a possible involvement in the synthesis of heme or heme proteins. This led us to initiate studies on aspects of the regulation of heme synthesis and its relationship to vitamin E nutrition (Murty and Nair, 1969; Murty *et al.*, 1969; Nair *et al.*, 1970; Murty *et al.*, 1970a,b).

The synthesis of heme is an attribute common to all aerobic cells and is initiated by the condensation of glycine and succinyl-CoA, intramitochondrially by the enzyme δ-aminolevulinate synthase (Figure 1). The next step in this pathway is carried out extramitochondrially and involves the condensation of two molecules of ALA to give porphobilinogen (PBG). The other intermediates that follow PBG are the porphyrinogens, colorless tetrapyrroles, which upon oxidation yield the

Biochemistry Research Division, Department of Medicine, Sinai Hospital of Baltimore, Inc., Baltimore, Maryland 21215.



OUTLINE OF BIOSYNTHESIS OF HEME

Figure 1. Outline of the biosynthesis of heme

red protoporphyrin IX. The terminal step in the pathway is completed intramitochondrially by the introduction of Fe^{2+} into protoporphyrin IX by the enzyme ferrochelatase.

In this biosynthetic pathway, the two enzymes ALA synthase and dehydratase, either individually or in tandem, are rate-determining for heme synthesis.

Our studies have proceeded along two main lines–one involving the study of the effects of vitamin E deficiency on the rate-determining enzymes in the pathway to heme and on the overall synthesis of protoheme and several known heme proteins, and the other involving studies on the effects of the vitamin on chemical porphyria, which is characterized by an excessive induction of hepatic ALA synthase under the influence of chemicals and drugs such as allylisopropylacetamide and phenobarbital.

EXPERIMENTAL

Male Wistar strain rats were reared on a vitamin E-deficient diet for 15–16 weeks, as described earlier (Weglicki *et al.*, 1968). The control animals were fed the basal vitamin E-deficient diet supplemented with vitamin E, 200 mg per kg of diet.

For intraperitoneal administration, vitamin E was dissolved in 0.3 ml of ethanol and made up to 10 ml with propylene glycol.

The methods used for the assay of various hepatic enzymes are referred to in earlier publications from this laboratory (Nair *et al.*, 1970; Murty *et al.*, 1970a). Hepatic δ-aminolevulinate synthase (ALA synthase) and δ-aminolevulinate dehydratase (ALA dehydratase) were induced either by making the animals porphyric by repeated subcutaneous injections of allylisopropylacetamide (AIA) (Nair *et al.*, 1970) or in short-term experiments by a single subcutaneous injection of AIA, 150 mg/kg body weight. For the induction of these enzymes with phenobarbital, male Sprague–Dawley rats weighing 100–120 g were given two intraperitoneal injections of the inducer in physiological saline at a dose of 60 mg/kg body weight 24 hr apart. Vitamin E, when administered, was given intraperitoneally at doses shown in each experiment.

RESULTS AND DISCUSSION

Our attention was first focused on the activities of the hepatic enzymes, ALA synthase, and ALA dehydratase in livers of animals on a vitamin E-deficient diet. Hepatic ALA

Table I. Syndromes of Vitamin E Deficiency

Animal species affected	Syndrome
Rat (male)	Sterility
Rat (female)	Fetal resorption
Rat (both sexes)	Liver necrosis (selenium)
Rabbit	Muscular dystrophy
	Myocardial degeneration
Dog and guinea pig	Myocardial degeneration
Chicken	Encephalomalacia
	Exudative diathesis
Primate	Macrocytic anemia
	Muscular dystrophy

Table II. Vitamin E: Postulated Mechanisms of Action

Specific	Nonspecific
Cofactor in electron transport	Biological antioxidant
Regulator of TCA cycle	Protects peroxidizable lipids and and cofactors (PUFA, Vitamin A etc.)
Regulator of nucleic acid metabolism	

Table III. Hepatic Catalase and Tryptophan Pyrrolase in Vitamin E Deficiency[a] (Murty *et al.*, 1970a)

Groups	Catalase, units/g of tissue	Tryptophan pyrrolase, μmol Kynurenine/hr/g tissue
Control	1721 ± 32	11.6 ± 0.9
Vitamin E-deficient	1033 ± 63	4.2 ± 0.7

[a] Experimental animals were maintained on a vitamin E-deficient diet for 18 weeks. Results are expressed as means ± SE of five animals in each group.

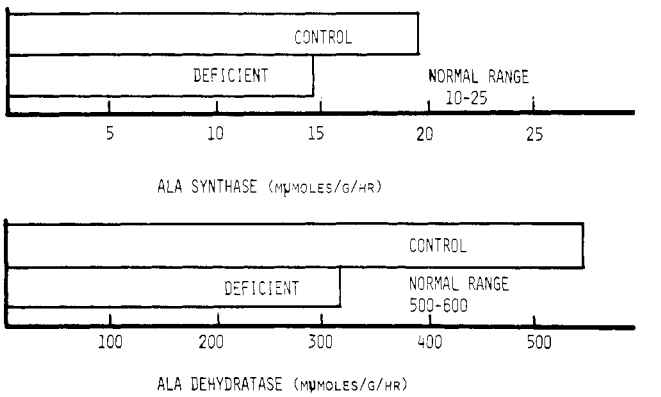


Figure 2. Hepatic ALA synthase and dehydratase in vitamin E deficiency

synthase remained within the normal range of 10–25 mμmol ALA/hr/g of tissue, while hepatic ALA dehydratase was distinctly depressed in deficient animals (Figure 2). This was the first indication of a possible metabolic aberration at the level of ALA dehydratase.

Now the question was raised whether this observation reflected a specific metabolic defect. The decision to examine other aspects of heme synthesis was dictated by three important considerations. First of all, a defect is this biosynthetic pathway would be reflected in at least some of the heme proteins, both functionally and quantitatively. Secondly, if ALA dehydratase becomes rate-limiting in vitamin E deficiency, we expected to observe a decrease in the incorporation of ALA-4-^{14}C and not of PBG-^{14}C into protoheme. Furthermore, in order to ascribe specificity to this metabolic aberration, it would be necessary to examine other hepatic enzymes unrelated to the synthesis of heme.

Table IV. Hepatic Microsomal Cytochromes b_5 and P-450[a] (Murty *et al.*, 1970a)

Groups	Cytochrome b_5, mμmol/mg protein	Cytochrome P-450, mμmol/mg protein
Control	0.72 ± 0.02	2.03 ± 0.06
Vitamin E-deficient	0.32 ± 0.09	1.06 ± 0.29

[a] Experimental animals were maintained on a vitamin E-deficient diet for 17 weeks. Results are expressed as means ±SE of three animals in each group.

Table V. Effect of Vitamin E Deficiency on Incorporation of Radioactivity from ALA-4-^{14}C and PBG-^{14}C into Microsomal Protoheme[a]

Groups	dpm/mμmol protoheme	
	ALA-4-^{14}C	PBG-^{14}C
Control	9010	83
Deficient	624	41

[a] Experimental procedures are as described in an earlier publication (Murty *et al.*, 1970a). Animals were maintained on a vitamin E-deficient diet for 17 weeks. Results are mean of three animals.

Table VI. ATPase Activities of Rat Liver Homogenate and Mitochondria[a]

Groups	ATPase activity, mg orthophosphate liberated/hr/g of tissue	
	Whole homogenate	Mitochondria
Control	34.4 ± 5.5	3.8 ± 0.5
Deficient	27.5 ± 5.9	3.8 ± 0.7

[a] Animals were maintained on a deficient diet for 18 weeks. ATPase activity was determined as described by Masoro *et al.* (1962). Results are expressed as means ±SE of observations from three animals.

Table VII. Activities of Pyridine Nucleotide Linked Oxidoreductases in Rat Liver Fractions[a]

Groups	Mitochondrial		Microsomal NADPH-cytochrome C reductase[d]	Soluble glucose-6-P dehydrogenase[e]
	Malate dehydrogenase[b]	Isocitrate dehydrogenase[c]		
Control	0.7 ± 0.1	0.05	707 ± 178	2.8 ± 0.5
Experimental	0.9 ± 0.7	0.06	1009 ± 52	4.8 ± 0.9

[a] Animals were maintained on a deficient diet for 17 weeks. Results are expressed as means ±SE of observations from three animals. [b] Expressed as μmole of NADH oxidized per minute per gram protein (Ochoa, 1955). [c] Expressed as μmole of NADP reduced per minute per gram of tissue (Ochoa, 1955). [d] Expressed as mμmol of cytochrome C reduced per mg of protein per minute (Baron and Tephly, 1969). [e] Expressed as μmol of NADP reduced per minute per gram of tissue (Glock and McLean, 1953).

Table III presents data on the activities of hepatic catalase and tryptophan pyrrolase. As would be expected, deficient tissues exhibited significantly lower activities for both these enzymes, compared to that of the corresponding control. Similarly, two other heme proteins, microsomal cytochromes b_5 and P-450, were also depressed by vitamin E deficiency (Table IV).

The locus of the enzymatic defect in vitamin E deficiency was further studied by following the incorporation into microsomal protoheme of radioactivity from ALA-4-^{14}C and PBG-^{14}C, the two intermediates that serve as the substrate and product of ALA dehydratase. As seen in Table V, the ability to incorporate ALA-4-^{14}C into protoheme was significantly impaired in the deficient microsomes, an observation that was consistent with the concept of a defect in the step involving ALA dehydratase.

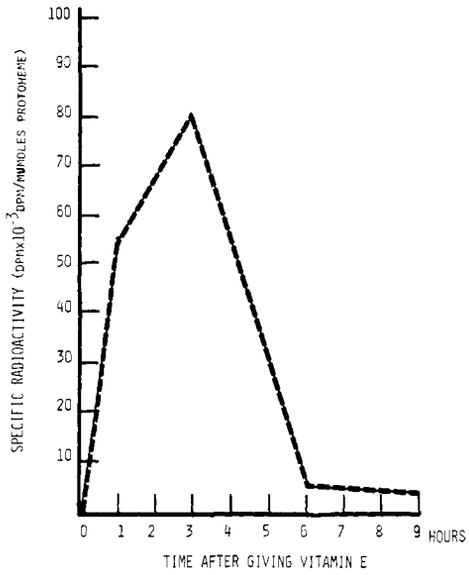


Figure 3. Time course of incorporation of ALA-4-^{14}C into microsomal protoheme following administration of vitamin E

Since hepatic and mitochondrial ATPase activities remained unchanged, it was assumed that the endogenous pool of ATP had not been depleted by hydrolytic cleavage (Table VI). A few pyridine nucleotide-linked oxidoreductases in hepatic cell fractions were also not affected by the deficiency (Table VII).

We now turned to the study of the effect of supplementing deficient animals with a single dose of vitamin E intraperitoneally. Microsomal heme synthesis was followed by pulse-labeling protoheme at various time intervals after the administration of the vitamin. In this experiment each animal received a pulse dose of 10 μCi of ALA-4-^{14}C 1 hr before being killed. Figure 3 presents a plot of the specific radioactivity of microsomal protoheme at various time intervals. Vitamin E administration resulted in a dramatic stimulation of heme synthesis, approaching a maximum value at about 3 hr, and then steadily declining 6 to 9 hr later to a level similar to that observed in nondeficient microsomes.

Based upon these observations we postulated that vitamin E deficiency in the rat leads to a defect in the synthesis of hepatic heme at a locus identical to that of ALA dehydratase, the second enzyme in the biosynthetic pathway to heme (Figure 1). While these studies were in progress, we were also preoccupied with the regulation of heme synthesis at the level of ALA synthase, an enzyme which is normally in a state of partial repression in hepatic cells but can be induced by a variety of drugs (phenobarbital, allylisopropylacetamide) and steroid hormones (Granick, 1966). Furthermore, in hepatic porphyrias as well as in experimental porphyrias, induced by chemical agents such as allylisopropylacetamide, the activities of this enzyme are significantly enhanced. In this connection earlier studies from our laboratories had already shown that vitamin E prevents the induction of experimental porphyria (Murty and Nair, 1969) and causes a remission in human hepatic porphyrias (Murty *et al.*, 1969; Nair *et al.*, 1971).

The object of our experimental approach was to determine whether the vitamin manifests itself in porphyria by blocking the excessive synthesis of hepatic ALA synthase. In several groups of animals, the effect of prior administration of vitamin E on the induction of hepatic ALA synthase by either phenobarbital or allylisopropylacetamide was studied. The results (Figure 4) showed that the vitamin prevented the in-

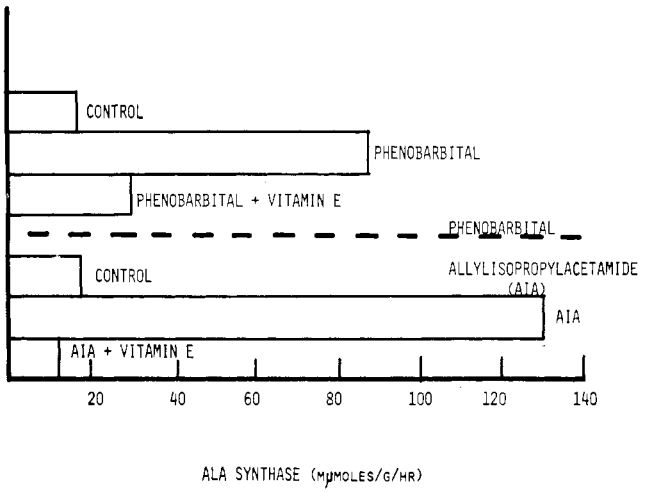


Figure 4. Effect of vitamin E on induction of hepatic ALA synthase by phenobarbital and allylisopropylacetamide

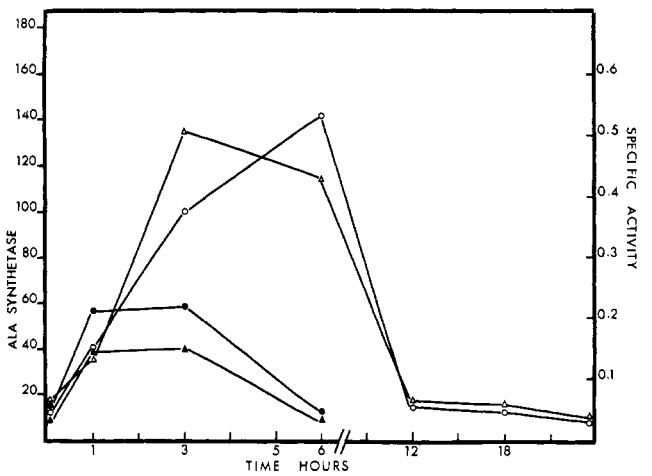


Figure 5. Effect of vitamin E on the kinetics of induction of hepatic ALA synthase by allylisopropylacetamide (AIA). △—△: AIA alone, activity/g of tissue. ○—○: AIA alone, specific activity. ▲—▲: vitamin E plus AIA activity/g of tissue. ●—●: vitamin E plus AIA specific activity

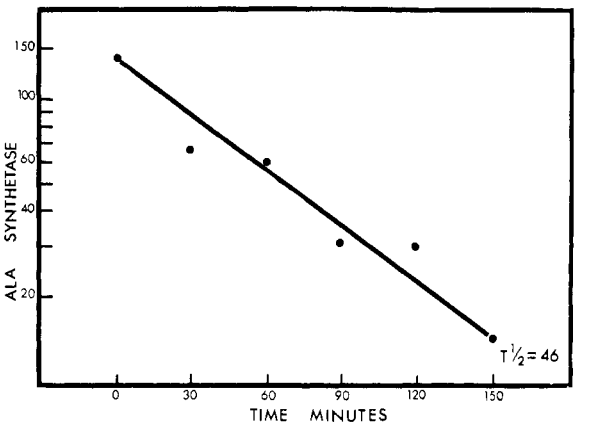


Figure 6. Kinetics of inhibition by vitamin E of hepatic ALA synthase induced by AIA

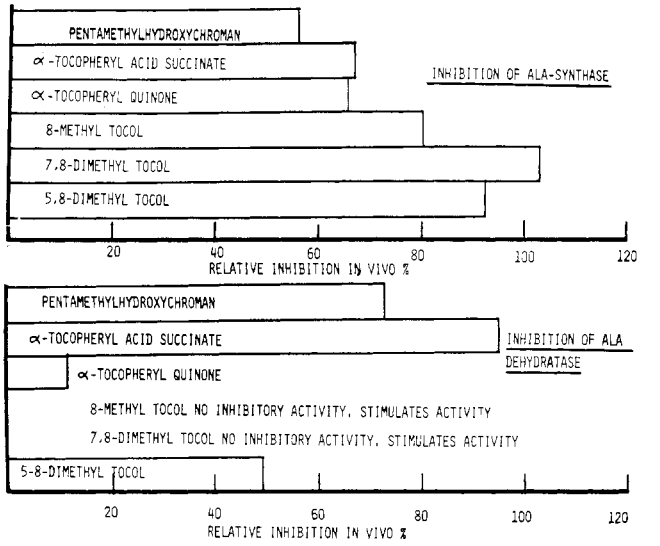


Figure 7. Relationship between structure and inhibitory activity of several substituted tocols and related compounds on induction of hepatic ALA synthase and dehydratase by AIA. Expressed as percent inhibitory activity relative to α-tocopherol (100%). All test compounds were administered intraperitoneally in propylene glycol, 5.0 mg/kg body weight

duction of the enzyme by chemical agents, thus clearing the way for a second locus of action, that of blocking the derepression of hepatic ALA synthase.

Since the activity of the constitutive enzyme in itself is very low in normal liver and remains unchanged in deficient animals, the question was posed whether the nutritional status of the animal with respect to vitamin E would modulate the response of hepatic ALA synthase to induction by chemical agents. We substantiated this assumption by showing that under identical conditions of age and dose of allylisopropylacetamide, the induction of hepatic ALA synthase in deficient animals was severalfold higher than that in normal control animals (Nair *et al.*, 1970). It should be pointed out at this stage that in all these experiments addition of vitamin E *in vitro* to homogenates of "induced livers" had no inhibitory activity on ALA synthase.

In the next step we followed the kinetics of induction of ALA synthase by allylisopropylacetamide and its inhibition by vitamin E. Several groups of male rats weighing between 100–125 g received subcutaneous injections of allylisopropylacetamide (150 mg/kg body weight). In addition, some of them received vitamin E (5 mg/kg body weight) intraperitoneally 1 hr before allylisopropylacetamide. As seen in Figure 5, the induction of ALA synthase reached a maximum at about 3 hr following the administration of allylisopropylacetamide alone. Vitamin E significantly suppressed the induction as expected.

In a parallel experiment, vitamin E was administered to several animals after maximal induction of ALA synthase, and the activity of the hepatic enzyme from livers of animals killed at various time intervals was followed. The half-life of the enzyme under these conditions was about 47 min (Figure 6). In all these experiments we had observed that the induction of ALA synthase was always followed by a similar but delayed secondary rise in the activities of ALA dehydratase, the second hepatic enzyme in the pathway leading to heme. The rise in the activity of this enzyme is also prevented by the vitamin.

We now considered it appropriate to examine the relationship between structure and activity among several analogs, positional isomers, and compounds related to the family of methyl-substituted tocols (Figure 7). When expressed in terms of percent inhibitory activity relative to α-tocopherol (100%), the dimethyl tocols were among the most active

Table VIII. Effect of Synthetic Antioxidants on Hepatic ALA Synthase and ALA Dehydratase Induced by Allylisopropylacetamide (AIA)[a]

Antioxidant	ALA synthase[b]		ALA dehydratase[c]	
	in vivo	*in vitro*	*in vivo*	*in vitro*
Control: only AIA	151	134	1306	1212
DPPD	30	8.2	1143	1029
BHT	28.7	23.3	11 9	1173
Propyl gallate	41.0	17.7	1092	1035
Methylene blue	28.8	28.6	836	1658

[a] Hepatic ALA synthase and dehydratase were induced by allylisopropylacetamide (150 mg/kg body weight). Antioxidants were either injected in a propylene glycol-based vehicle (2.0 mg per animal) or added to liver homogenates (2.0 mg per incubation). Results are means of six to eight animals in each group. The abbreviations are: DPPD—diphenyl-*p*-phenylene diamine and BHT—butylated hydroxytoluene. [b] Expressed as mμmol of ALA formed/hr/g of tissue. [c] Expressed as mμmol of PBG formed/hr/g of tissue.

against the induction of ALA synthase. This was in sharp contrast to the activity profile in the ALA dehydratase system. Here, a decrease in the number of methyl substituents on the tocol nucleus was not only accompanied by a corresponding decrease in inhibitory potency, but also a stimulatory effect with the 8-methyl and the 7,8-dimethyl tocols. Since these are also the most potent antioxidants among the methyl-substituted tocols, this attribute could perhaps account for their atypical behavior towards ALA dehydratase.

These studies were extended to include a number of synthetic antioxidants (Table VIII). Unlike the tocols, the synthetic antioxidants inhibited only ALA-S both *in vitro* as well as *in vivo*, suggesting a mechanism that is distinctly different from that of vitamin E. On the basis of these observations, it was clear that the mode of action of vitamin E in this system bore no correspondence to that of the synthetic antioxidants.

Other unpublished studies from this laboratory using phenobarbital-2-^{14}C have shown that vitamin E does not significantly interfere with the hepatic uptake of the inducer. From this observation it appears that the action of vitamin E does not involve competition between the vitamin and the inducer for a common carrier.

There is an increasing body of evidence suggesting a role for the fat-soluble vitamins in the regulation of macromolecular synthesis. Our present findings are of conceptual significance, in that certain "trace lipids" have a much greater functional significance in the expression of biological information.

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