Mechanism of Action of Vitamin A in Differentiation of Mucus-Secreting Epithelia

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A discussion of the evidence for a mechanism of action of vitamin A at the transcriptional, translational, and posttranslational levels is presented. The conclusion that retinol may be acting at the posttranslational level is based on the following findings. The *in vivo* and *in vitro* biosynthesis of a specific glycopeptide is greatly decreased in vitamin A deficiency. This glycopeptide contains D-fucose, D-galactose, D-glucosamine, D-galactosamine, and D-sialic acid in the molar ratios 1.00:1.73:0.87:1.97:0.49, respectively. By indirect immunofluorescence the glycopeptide was localized in the goblet cell. The number of these cells, as revealed by periodic acid–Schiff staining, was greatly decreased in vitamin A deficiency. When (H^3) retinol and guanosine-diphosphate-mannose (C^{14}) are incubated in the presence of a membrane-rich fraction from rat or hamster liver or intestinal mucosa, a double-labeled mannolipid is isolated. This mannolipid can donate mannose to endogenous acceptors. These findings strongly suggest that retinol may be functioning in the biosynthesis of glycoproteins by carrying monosaccharides.

Though the role of vitamin A in vision is known (Wald, 1968), its mode of action in maintaining mucus cell differentiation is still obscure.

Vitamin A deficiency leads to changes in the differentiative pattern of mucus-secreting tissues. In the small intestinal mucosa of the rat we have found (De Luca *et al.*, 1969) a marked decrease in the number of mucus-secreting goblet cells and simultaneously a decrease in the incorporation of glucosamine-*1*-C^{14} into a specific glycopeptide (De Luca *et al.*, 1970). The intestinal mucosa is unique in that upon vitamin A deficiency no keratinization occurs after cessation of mucus secretion. In the lining of the tracheal and bronchial epithelia (Wong and Buck, 1971), as well as in other tissues (Hayes *et al.*, 1970), foci of keratinization appear in vitamin A deficiency. The biochemical investigation of the keratinized portions is difficult because of the patchy character of the keratinizing processes.

In tackling the problem of the control of cell differentiation, it is of advantage to have biochemical markers for specific cell types, which can be easily assayed for. Since lack of goblet cell formation and synthesis of the fucose–glycopeptide were parallel processes, the fucose–glycopeptide might be a typical constituent of goblet cells and could be used as a marker for mucus cell differentiation.

Another intriguing aspect of the differentiative process in the rapidly turning-over cells of the intestinal mucosa is whether products of differentiation, *i.e.*, glycoprotein in goblet cells, appear *de novo* after cell division or whether the precursor cell contains the same differentiative product, mucus, but perhaps in a precursor form. This precursor could be transformed into the finished product gradually, as the cell acquires its full complement of ultrastructural peculiarities. One is tempted to speculate that this might be the case for goblet cells of the intestine which, as shown by Merzel and Leblond (1969), might arise from oligomucus cells of the crypts of Lieberkühn.

The next and most intriguing question is: at which point does vitamin A act? Does the vitamin have an effect on the precursor cell, and, if so, how does it act molecularly?

Demonstration of the Role of Vitamin A in the Biosynthesis of the Fucose-Containing Glycopeptide. In a study (De Luca *et al.*, 1970) of the incorporation of *1*-C^{14}-glucosamine into glycopeptides, we found that vitamin A deficiency leads to a 50 to 70% decrease in labeling of a specific glycopeptide, fucose–glycopeptide, isolated by DEAE-Sephadex A50 chromatography of the total glycopeptide mixture and subsequent chromatography on Sepharose 4B and 2B of the fraction eluted from DEAE-Sephadex with 0.4 *N* LiCl. The normal incorporation was 16,690 dpm/mg of glycopeptide; the deficient was 5968 dpm/mg of glycopeptide.

Fucose–glycopeptide was shown to have a sedimentation constant of 3.6 in H_2O and to yield only one band in a disc electrophoresis system. Its chemical composition, studied by gas–liquid chromatography, is shown in Table I. Less precise determinations of chemical composition by colorimetric methods have been reported previously (De Luca *et al.*, 1970).

To ascertain that decreased labeling was not due to a higher pool size of glucosamine in vitamin A deficiency, a cell-free synthesis of the fucose–glycopeptide was carried out in the presence of uridine diphosphate-*N*-acetylglycosamine-C^{14}. The findings with the cell-free system agreed very well with the *in vivo* findings, excluding any pool size effect (though one could always imagine pools of intermediates bound to the microsomal membranes, it seems most unlikely that their concentration is so high in deficiency as to simulate a difference in uptake of 50 to 70%).

Moreover, a decrease in the total amount of fucose–glycopeptide is found in severe vitamin A deficiency.

Demonstration of the Presence of Fucose–Glycopeptide in the Goblet Cell of the Rat Small Intestinal Mucosa by Immunofluorescence. An antibody to fucose–glycopeptide was prepared in chickens and the indirect immunofluorescent method of Coons and Kaplan (1950) was followed to localize fucose–glycopeptide. Parallel staining of the same section with PAS (periodic acid–Schiff reagent) was conducted for goblet cells. The goblet cells stained with PAS and they alone fluoresced (De Luca *et al.*, 1971). Deep in the crypts of Lieberkühn, where oligomucus cells are found, there was a faint fluorescence, indicating the presence of fucose–glycopeptide in trace amounts or a very similar crossreacting molecule. The mucus-secreting cells of rat conjuctiva, a tissue very responsive to vitamin A status, also showed the fluorescence indicative of fucose–glycopeptide; hamster intestine also contained fluorescence localized in the goblet cells. Other mucus-secreting tissues are now in the process of being tested. If fucose–glyco-

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peptide is a constituent of all mucus-secreting epithelia, it could be used as a marker for mucus cell differentiation.

The steps by which vitamin A controls the synthesis of fucose–glycopeptide are unknown.

One could hypothesize that the vitamin acts on the DNA of the precursor cell and somehow evokes the correct messengers for differentiation of goblet cells and synthesis of fucose–glycopeptide. These messengers should be relatively short-lived since every cell is replaced every 18 hr in the gut of the rat.

Another possibility is that there is a pool of preexisting stem cells which become differentiated goblet cells only if certain aminoacyl–tRNA's become available.

A body of evidence is accumulating to indicate that this mechanism of control of cell differentiation is operating in several tissues of different organisms (Strehler *et al.*, 1967; Ilan *et al.*, 1970). Finally vitamin A may be acting on cell differentiation by controlling the synthesis of glycoproteins at a posttranslational stage.

We will next present evidence for each one of these hypotheses both from our own work and from the work of others.

Evidence for a Mode of Action of Vitamin A at the Transcriptional Level. Several groups of investigators have shown that RNA metabolism is altered in vitamin A deficiency and that the administration of vitamin A to deficient animals increases the incorporation of radioactive precursors into RNA.

Johnson *et al.* (1969) have reported that rat intestinal mucosa and liver RNA labeling is increased after vitamin A injection *in vivo;* an increase in RNA labeling is observed 5 min after the administration of the vitamin. The effect was most marked on nuclear RNA.

Analogous results were obtained by Zile and De Luca (1970) in the intestinal mucosa of the rat. Unfortunately, in both cases no measurements of the sizes of the endogenous pools of precursors were attempted.

We have found in our laboratory (Kleinman *et al.*, 1971) that vitamin A deficiency leads to a decreased incorporation of both uridine-H^3 and orotic acid-5-C^{14} into RNA. Pool sizes of precursors were measured and found to be consistently smaller in vitamin A deficiency. All classes of RNA examined were found to be affected by vitamin A deficiency to the same extent.

The morphological events occurring in vitamin A deficiency *in vivo* and those observed in culture of chick ectodermal explants in the presence of 10 μg/ml of culture medium (Fell, 1957) indicate that vitamin A gives the signal for the production of mucus and its deficiency evokes the appearance of keratinizing cells.

This has been confirmed at the electron microscope level in the parotid duct (Hayes *et al.*, 1970) and in the trachea (Wong and Buck, 1971). However, though one sees bundles of keratin in vitamin A deficiency, no one has been able to show that the morphological changes are linked to or concomitant with the appearance of a new mRNA and a new protein. Moreover, one cannot exclude the possibility that changes in the morphology of tissues and appearance of bundles of keratin may be due to posttranscriptional events.

Evidence for the Mode of Action of Vitamin A at the Translational Level. Some work has been done in our laboratory on the action of vitamin A at the translational level. De Luca *et al.* (1969) prepared rough endoplasmic reticulum and pH 5 enzyme from normal and vitamin A-deficient rat intestinal mucosa after ligation of the common bile duct to remove pancreatic hydrolases. A 50 to 70% decrease in the rate of uptake of leucine-C^{14} into proteins was found in vitamin A deficiency. Interestingly, this decrease was found to be caused by a lower aminoacyl–tRNA charging activity in the vitamin A deficient preparation (Kleinman *et al.*, 1971). The possibility of specific isoaccepting species of tRNA being preferentially affected by vitamin A deficiency was overruled at least for leucine and threonine. This was achieved by preparing leu-tRNA, labeled with C^{14}-leucine for the normal and H^3-leucine for the deficient, mixing the two preparations, and chromatographing the extracted aminoacyl–tRNA's on Freon columns. It was important to determine next whether the lower charging was due to lower enzyme activities or different availability of tRNA in vitamin A deficiency. We prepared normal and vitamin A-deficient tRNA and, by adding either one to the deficient pH 5 enzyme fraction, we were able to restore the rate of incorporation of C^{14}-leucine into leu-tRNA to normal levels.

Thus, it is clear that no qualitative changes occurred due to vitamin A deficiency in the isoaccepting species of tRNA for leucine, but that the availability of the tRNA for charging was reduced in vitamin A deficiency. The question of an action of vitamin A at the translational level therefore becomes identical with that of the synthesis of RNA.

Evidence for a Function of Vitamin A at the Posttranslational Level. We have shown that the synthesis of a specific glycopeptide, fucose–glycopeptide, is reduced in vitamin A deficiency. This glycopeptide was found to be located in the goblet cells of the rat and hamster small intestine by immunofluorescence (De Luca *et al.*, 1971). Corneal epithelium and tracheal epithelium also seem to contain the same or a cross-reacting glycopeptide. Thus we have found a biochemical lesion, which seems to be common to different species and different organs within the same species, caused by vitamin A deficiency. Moreover, oral administration of vitamin A restores the biosynthesis of fucose–glycopeptide and the number of goblet cells to normal levels within 18 hr (De Luca *et al.*, 1970).

Our interest lies now in discovering how the vitamin controls the synthesis of the fucose–glycopeptide in epithelial tissues and whether the fucose–glycopeptide is merely a product of differentiation or its trigger.

Experiments conducted in our laboratory have shown that the biosynthesis of a lipid containing mannose made from GDP–mannose-C^{14} by a cell-free system consisting of a membrane-rich fraction is greatly reduced in vitamin A deficiency (De Luca *et al.*, 1970). By adding retinol *in vitro* we could restore the biosynthesis of this mannolipid to normal levels, while the normal level of incorporation of C^{14}-mannose from GDP–mannose-C^{14} was identical in the presence or absence of retinol. In addition, when the same membrane fraction was incubated in the presence of H^3-retinol and GDP–mannose-C^{14}, a double-labeled mannolipid was obtained by DEAE–cellulose and silicic acid chromatography (De Luca *et al.*, 1971). The products of the hydrolysis of this compound are being identified. Preliminary experiments in our laboratory have shown that the mannolipid can function as a sugar donor for

Table I. Chemical Composition of Fucose–Glycopeptide

	Percent	Molar ratio
Fucose	11.8	1.00
Glucosamine	22.5	1.73
Galactosamine	11.1	0.87
Galactose	25.5	1.97
N-Acetyl–neuraminic acid	10.9	0.49

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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LITERATURE CITED

Coons, A. H., Kaplan, M. H., *J. Expt. Med.* **91,** 1 (1950).
Dankert, M., Wright, A., Kelley, W. S., Robbins, P. W., *Arch. Biochem. Biophys.* **116,** 425 (1966).
De Luca, L., Little, E. P., Wolf, G., *J. Biol. Chem.* **244,** 701 (1969).
De Luca, L., Rosso, G., Wolf, G., *Biochem. Biophys. Res. Commun.*, **41,** 615 (1970).
De Luca, L., Rosso, G., Wolf, G., *Fed. Proc.* **30,** 1134 (1971a).
De Luca, L., Schumacher, M., Nelson, D. P., *J. Biol. Chem.* **246,** 5762 (1971b).
De Luca, L., Schumacher, M., Wolf, G., *J. Biol. Chem.* **245,** 4551 (1970).
Fell, H. B., *Proc. Roy. Soc. (London) Ser. B* **146,** 926 (1957).
Foster, D. W., Ginsburg, V., *Biochem. Biophys. Acta* **54,** 376 (1961).
Hayes, K. C., McCombs, H. L., Faherty, T. P., *Lab. Invest.* **22,** 81 (1970).
Ilan, J., Ilan, J., Patel, N., *J. Biol. Chem.* **245,** 1275 (1970).
Johnson, B. C., Kennedy, M., Chiba, N., *Amer. J. Clin. Nutr.* **22,** 1048 (1969).
Kleinman, H. K., De Luca, L., Wolf, G., *Arch. Biochem. Biophys.* **145,** 332 (1971).
Merzel, J., Leblond, C. P., *Amer. J. Anat.* **124,** 281 (1969).
Strehler, B. L., Hendley, D. D., Hirsch, C. P., *Proc. Nat. Acad. Sci. U.S.* **57,** 1751 (1967).
Wald, G., *Nature (London)* **219,** 800 (1968).
Wolf, G., De Luca, L., The Fat Soluble Vitamins, DeLuca, H. F., Suttie, J. W., The University of Wisconsin Press, Madison, Milwaukee, and London, 1970, p 257.
Wong, Y. C., Buck, R. C., *Lab. Invest.* **24,** 55 (1971).
Zile, M., DeLuca, H. F., *Arch. Biochem. Biophys.* **140,** 210 (1970).

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Vitamin E. Regulation of the Biosynthesis of Porphyrins and Heme

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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

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