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Effect of Vitamin A Deficiency on Liver Transfer RNA Methylases[1] (38111)

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One of the more widely recognized signs of vitamin A deficiency is the depression of growth. Even though numerous factors can affect growth, sufficient evidence is accumulating to suggest that vitamin A plays a direct role in this process (1). The exact mechanisms by which vitamin A affects growth are not known but recent findings suggest that it may be by regulating or controlling protein synthesis. For instance, DeLuca *et al.* (2) have reported that protein synthesis by the intestinal mucosa is decreased in the deficiency state, while Tryfiates and Krause (3) have demonstrated the opposite effect in liver. Both of these studies have examined protein synthesis in deficient animals at the translational level.

With the discovery of methylated bases as a minor constituent of transfer RNA (tRNA) (4), Borek has suggested that modification of preformed tRNA might serve a regulatory function in protein synthesis (5). Since methylation of tRNA is accomplished by the addition of methyl groups to a preformed tRNA by the methylase enzymes located in the soluble portion of the cell (6), it seems appropriate to use tRNA methylase activity as an indicator of this modification process.

Bradford *et al.* (7) have reported that both the rate and the extent of methylation of tRNA decreased in extracts of vitamin A-deficient bone.

The purpose of this investigation was to determine if there was a change in tRNA methylase activity accompanying the stimulation in liver protein synthesis previously observed in vitamin A deficiency.

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Materials and Methods. Animals. Weanling male albino Wistar rats were made vitamin A-deficient as previously described by Krause *et al.* (8). Rats were judged to be deficient when their weight plateaued and no vitamin A could be detected in either their serum or liver. (This usually required 10–12 wk on the deficient diet.) Control animals were pair-fed and had average serum vitamin A levels of 59 ± 10 μg/100 ml and average liver levels of 462 ± 15 μg/g liver. Control rats had a mean weight of 326 ± 22 g and deficient animals weighed on the average 249 ± 39 g. All animals were fasted 12 hr prior to sacrificing and at approximately the same time of day.

Extraction and determination of vitamin A in serum and liver. Extraction of serum vitamin A was done essentially by the procedures of Dann *et al.* (9) and Kimble (10). The Folch method (11) of lipid extraction was used for retinol extraction from liver. The lipid residues from both serum and liver were dissolved in chloroform and the vitamin A concentration determined by the addition of trifluoroacetic acid (12).

Preparation of crude enzyme extracts. Rats were sacrificed by a blow on the head and then decapitated. Livers were removed and homogenized in 4 vol of cold (0–4°) 0.01 *M* Tris–HCl, pH 7.4, containing 0.01 *M* $MgCl_2$ and 0.005 *M* of mercaptoethanol. The homogenates were centrifuged at 15,000*g* for 10 min and the supernatant fluid was further centrifuged at 105,000*g* for 1 hr. The clear red supernatant liquid was used as the source of the tRNA methylase enzymes. The enzyme protein concentration was determined by the method of Lowry *et al.* (13).

Assay procedures. (a) *tRNA methylase assay*: This procedure was a modification of the

procedures described by Mandel and Borek (14) and Kerr (15, 16). One milliliter of reaction mixture contained 10 m*M* Tris–HCl, pH 8.0, 5 m*M* $MgCl_2$, 5 m*M* mercaptoethanol, 100 μg *E. coli* B tRNA, 59 nCi (sp act 52 mCi/mmole) $^{14}CH_3$-*S*-Adenosylmethionine (SAM), and fixed amounts of enzyme (2.0 mg control or deficient soluble protein). Increasing the amount of $^{14}CH_3$-SAM to 100 nCi resulted in no significant increase in methylation. The concentration of tRNA in the assays measuring total extent of methylation was chosen in order that complete methylation be achieved in the minimum incubation time. Optimal enzyme concentration was that concentration of protein which resulted in the greatest incorporation of $^{14}CH_3$-SAM into tRNA in the assay system used. This mixture was incubated at 37° for 30, 60, 90, and 120 min. At these various intervals, aliquots were withdrawn and placed on Whatman scintillation disks and quickly dried in a stream of hot air. The disks were then immersed in cold 5% trichloroacetic acid (TCA) until all samples were collected, and RNA and protein were precipitated overnight at 4°. All disks were placed between two pieces of filter paper in a Büchner funnel and washed in the following manner: 400 ml 5% TCA, 300 ml ethanol: ether (1:1) and finally with 300 ml ether. Incorporation of radioactivity was measured by placing the washed disks in 10 ml of toluene-base scintillation fluid prepared by dissolving 16 g PPO and 0.4 g POPOP in 4 liters of toluene. To correct for protein methylation, a reaction blank containing 0.1 ml of distilled water was used in place of tRNA for each reaction time, and the counts obtained were subtracted from all tRNA reaction counts. All samples were counted in a Tri-Carb liquid scintillation spectrometer.

(b) *Methylation rate assay.* In order to insure an excess quantity of substrate over enzyme, 500 μg of *E. coli* B tRNA was used along with varying amounts of SAM, e.g., 1×10^{-6}–1×10^{-5} *M* as suggested by Moore and Smith (17). In the present assay system, 500 μg of *E. coli* B tRNA was also saturating since higher tRNA concentrations did not result in a higher rate of methylation. The stock concentration of SAM used was 1.12×10^{-4} *M* and additions varied from 10 λ to 100 λ.

All rate assays were performed at optimal enzyme concentration, and a reaction blank was carried out for each determination.

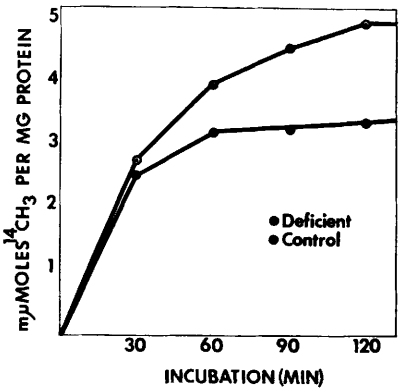

FIG. 1. Comparison of liver tRNA Methylase activity in control and vitamin A-deficient rats. Assays were performed as described in Materials and Methods. Each point represents the mean of duplicate determinations on four animals.

Preliminary experiments indicated that the incorporation of labeled methyl groups was linear for 30 min; thus, a 20-min reaction time was selected for all rate studies.

Results and Discussion. In order to compare control and vitamin A-deficient tRNA methylase activity it was necessary that methylation be complete in each methylase assay experiment. In Fig. 1 it may be seen that methylation was essentially complete after 1 hr of incubation for the control enzymes while 2 hr were required for the deficient enzymes. A comparison of the final extent of methyl incorporation by the enzymes from control and deficient liver on the basis of nmoles $^{14}CH_3$ incorporated into tRNA/mg protein/2 hr incubation reveals a 1.5-fold increase in the methylation of tRNA by deficient liver. These results may indicate that tRNA methylases from vitamin A-deficient livers are capable of methylating novel sites in heterologous tRNA which are not accessible to the control liver enzymes.

Since control animals were pair-fed, it can be assumed that the observed increase in methylation is not the result of decreased food consumption by the deficient animals. We have observed that under the experimental conditions of producing the deficient rat, the food consumption never decreases over 20% of that for the control rat.

A decrease in methyl incorporation into

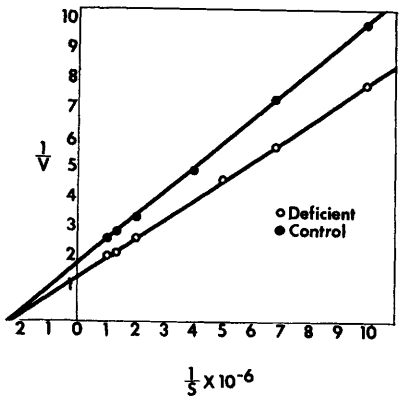

FIG. 2. Lineweaver–Burk plot of kinetic data for control and vitamin A-deficient liver tRNA methylase. The modified assay for rate determinations is described in Materials and Methods. Each point is the mean of duplicate determinations on three animals. The substrate varied is $^{14}CH_3$-SAM.

tRNA beyond optimal protein concentrations was observed for both methylases from control and deficient livers. A similar finding has been reported by Kerr (16) for normal rabbit livers. This has been interpreted as indicating the presence of an inhibitor in the crude extracts. The significance of this explanation is being investigated in our laboratory.

In order to simplify the bisubstrate kinetics encountered with this class of enzymes, the assay system was saturated with *E. coli* B tRNA so that only the effect of SAM concentration on enzyme rate was studied. Figure 2 is a Lineweaver–Burk plot of the experimental rate data. Michaelis Menten constants ($K_{M\text{-SAM}}$) proved to be 4.2×10^{-4} *M* and 4.0×10^{-4} *M* for control and deficient enzymes, respectively. These values are comparable to those reported by Moore and Smith (17) for *E. coli* B tRNA methylases.

A 30% increase in maximum velocity (V_{max}) was observed with deficient liver enzymes. Since $K_{M\text{-SAM}}$ values did not differ significantly for the control and deficient enzymes, the increased rate may well not be related to the binding of SAM to the enzymes. The increased rate could be related to an increase in the number of sites available to vitamin A-deficient tRNA methylases as evidenced by the increase extent of tRNA methylation by deficient enzymes.

The mechanism by which tRNA methylation is stimulated by a lack of vitamin A is unknown, as is the functional result of methylated bases in tRNA. It has been suggested (18) that more highly methylated species of tRNA are less specific with regard to their ability to be aminoacylated. This might account for the previously reported increased activity of the pH 5 fraction of liver in cell free protein synthesis (3).

The opposite effect of vitamin A deficiency on bone methylases (7) may well illustrate that the deficient state does not exert the same effect on all target organs.

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