Vitamin A and Protein Synthesis by Rat Intestinal Mucosa

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SUMMARY

Vitamin A deficiency caused a marked decrease in the number of goblet cells in the small intestine of the rat but no other morphological changes were revealed by electron microscopy. RNA and protein concentrations remained unaffected. Large polyribosomes could be isolated from the mucosa after the common bile duct was ligated. No differences were found in polyribosome stability or the protein-synthesizing activity of free polyribosomes from mucosa of vitamin A-deficient or pair fed normal rats. Rough endoplasmic reticulum, on the other hand, showed a decrease in uptake of a labeled amino acid into protein (to less than one-half of the normal level) under conditions of vitamin A deficiency. The lesion was located in the pH 5 fraction by "crossing-over" experiments, that is, by incubating normal rough endoplasmic reticulum with deficient pH 5 fraction and deficient rough endoplasmic reticulum with normal pH 5 fraction. The mucosal lesion occurs at a very early stage of the deficiency, before the weight plateau stage, however, it was not observable in liver. It is concluded that protein synthesis by membrane-bound but not by free polyribosomes of intestinal mucosa is depressed under conditions of vitamin A deficiency and that the vitamin is therefore involved, directly or indirectly, in protein synthesis at the translational level.

EXPERIMENTAL PROCEDURE

Preparation of Animals—Weanling (50 g) Holtzman strain albino rats were made vitamin A-deficient; pair fed normal control animals received the deficient diet (7) plus 2,000 i.u. of vitamin A acetate per week. The common bile duct was ligated 18 hours prior to killing the animals by decapitation. No food was given during this time.

Preparation of Cell Fractions—Polyribosomes were prepared as reported by Alpers and Isselbacher (8). The small intestine was dissected from the ligament of Treitz to the caecum and immediately rinsed with 100 ml of cold 0.9% NaCl solution con-
taining 1% bentonite. It was then cut open on a glass plate and the mucosa (epithelial cells and lamina propria) was scraped with a microscope slide. The mucosa was homogenized in 6 volumes of ice-cold Medium A of Littlefield and Keller (9) containing 1% bentonite in a Dounce tissue homogenizer with eight strokes of the loose pestle and six strokes of the tight pestle. Intact cells, cell debris, nuclei, and mitochondria were removed by centrifugation at 7,600 × g for 10 min. The supernatant fraction was used for the preparation of the polysomes as described by Wettstein, Stehelin, and Noll (10). The 7,600 × g supernatant fraction was layered on top of a discontinuous sucrose gradient consisting of 3.1 ml of 0.5 M sucrose on top of 2.3 ml of 2.0 M sucrose and centrifuged at 105,000 × g for 3 hours in the R-40 rotor of a Beckman model L ultracentrifuge.

The material at the interface of the 2.0 and 0.5 M sucrose was removed, diluted 1:1 with buffer consisting of Tris (0.05 M), MgCl₂ (5 × 10⁻³ M), KCl (0.025 M, pH 7.6) (Buffer TMK), and recentrifuged at 105,000 × g for 1 hour to pellet the membrane bound polysomes used for RNA and protein estimation. The translucent pellet obtained from the discontinuous gradient was used for studying protein synthesis on free polysomes after thorough resuspension in the standard incubation mixture (see "Analytical Techniques" for composition and concentrations).

Polyribosome patterns were obtained by the method of Britten and Roberts (11) with a linear 15 to 30% sucrose gradient. After centrifugation at 115,000 × g for 75 min in a SW 50 rotor, the gradients were analyzed at 260 nm by forcing them through a continuous flow cell of the Gilford model 2000 spectrophotometer.

Preparation of pH 5 and Microsome Fractions. A portion of the 7,600 × g supernatant fraction (postmitochondrial supernatant fraction) was centrifuged at 105,000 × g for 2 hours to yield a microsomal pellet and a supernatant fraction from which the pH 5 enzymes were precipitated by lowering the pH to 5 with a few drops of 1 N acetic acid and continuous stirring (12). The precipitate was collected by centrifugation at 15,000 × g for 10 min and redissolved in the incubation mixture. The resulting solution was designated as the pH 5 fraction for use in the studies in vitro.

Preparation of the Rough Endoplasmic Reticulum Fraction—The method of Moulé, Rouiller, and Chauveau (13) was used as described for rat liver. The mucosal scrapings were homogenized in 6 volumes of 0.88 M sucrose in Buffer TMK containing 1% bentonite. The homogenate was centrifuged at 20,000 × g for 20 min to remove cells, nuclei, and mitochondria, and the resulting supernatant was centrifuged for 1 hour at 82,000 × g to yield a pellet of rough endoplasmic reticulum and a supernatant fraction. This last named fraction was used to prepare the pH 5 fraction either by immediate precipitation at pH 5 or upon removal of free polysomes and smooth endoplasmic reticulum by dilution with Buffer TMK to 0.44 M sucrose, centrifugation at 105,000 × g for 2 hours, and subsequent precipitation at pH 5.

Analytical Techniques—Ribonuclease activity was examined as described by Usunomiya and Roth (14). RNA content was determined by the method of Schneider (15) and protein by the method of Lowry et al. (16). Amino acid incorporation into protein, both on free and membrane-bound polyribosomes, was studied with the system of Maxwell (17). The standard incubation mixture contained, in a volume of 0.5 ml: 0.5 mg of ribosomal or rough endoplasmic reticulum protein, 1 mg of pH 5 fraction protein, 5 μmoles of P-enolpyruvate, 126 μmoles of succrose, 1 μmole of ATP, 0.1 μmole of GTP, 20 μg of crystalline pyruvate kinase, 25 μmoles of Tris, 5 μmoles of MgCl₂, 30 μmoles of KCl, 1 μmole of mercaptoethanol, and 0.05 μmole of each of the 19 L-amino acids (minus leucine, cysteine) and uniformly labeled L-leucine-¹⁴C (0.3 μC; specific activity, 250 mC per mmole). When rough endoplasmic reticulum preparations were used for the amino acid incorporating study, 0.5 μmole of UDP-N-acetylglucosamine was added. Reaction mixtures were incubated at 37° for 45 min. Proteins were precipitated with an equal volume of 10% trichloroacetic acid and the aminoacyl-tRNA was solubilized at 90° for 15 min. The precipitated protein was collected on filter discs (0.45 μm pore size) and dried. The discs were then suspended in toluene 2,5-diphenyloxazole (PPO) (0.3%) scintillation medium and counted in a Nuclear-Chicago liquid scintillation counter. When samples with more than 0.15 mg of protein were counted, NCS was used as a dissolving medium and Bray’s (or Bruno’s) solution was used for counting.

Materials: ATP, GTP, phosphoenolpyruvate, and phosphoenolpyruvate kinase were purchased from Sigma. Uniformly labeled L-leucine-¹⁴C was obtained from New England Nuclear. Bentonite (U. S. P.) was purchased from Fisher. Rats were obtained from Holtzman. The vitamin A-deficient diet was obtained from General Biochemicals, and prepared according to the method of Wolf, Lane, and Johnson (7). All deficient animals were used 2 days after the end of the weight plateau stage, unless indicated otherwise.

RESULTS

Fig. 1, A and B, shows the sharp decrease, caused by the vitamin A deficiency, in the number of goblet cells in the crypts of the intestinal mucosal epithelium at the level of the common bile duct. The figure shows a standardized area, with crypt areas approximately equal in control and experimental animals. No difference in height of epithelium was found in a series of random measurements. A count of goblet cells per crypt in 100 crypts was made for a normal (17.3 ± 1.3) and a deficient (11.0 ± 1.9) animal (difference statistically significant at the level P < 0.01).

It should be noted that in all experiments reported in this work the normal control rats were pair fed to the vitamin A-deficient rats such that food intake of the two groups was at all times the same. The histological appearance suggests that the tissue is quite unaffected by the vitamin deficiency, except in the severe reduction in the number of mucus-secreting cells. This conclusion was confirmed by electron microscopy of the tissue. This showed no morphological changes in vitamin A-deficient cells. Subcellular structures were unchanged. The amount of rough endoplasmic reticulum was about the same in normal (Fig. 2A) and deficient (Fig. 2B) mucosal cells. The deficient goblet cells, although fewer in number than normal goblet cells (Fig. 3A), were as richly endowed with rough endoplasmic reticulum (Fig. 3B).

Analysis of the RNA and protein concentrations in the cell fractions involved in protein and glycoprotein synthesis again showed no differences in mucosal tissue from normal and deficient animals (Table I).

1 The authors are grateful to Dr. P. M. Newbome, Department of Nutrition and Food Science, Massachusetts Institute of Technology, for preparing the histological sections shown and for performing the counts of goblet cells.

2 The authors are grateful to Dr. G. Millonig, Department of Biology, Massachusetts Institute of Technology, for preparation of the electron micrographs.
In order to make an analysis of the elements of the protein-synthesizing mechanism of mucosal cells, we began by sucrose gradient analysis of the polyribosomes. The method included bentonite addition to inhibit destruction of the aggregates by ribonuclease. Surprisingly, the preparation from vitamin A-deficient mucosa showed a pattern resembling that obtainable from liver (Fig. 4d), with heavy polyribosomes (10 ribosomes or more) predominating, whereas normal mucosa yielded predominantly small aggregates of two to four ribosomes (Fig. 4a). The recent work of Alpers and Isselbacher (8) suggested that ligation of the common bile duct would result in preservation of polyribosomes with larger numbers of ribosomes. When ligation was performed 18 hours prior to the killing of the animals, identical polyribosome patterns from deficient and normal rat mucosa (Fig. 4, c and d) were obtained. Both consisted mainly of heavy aggregates of 10 ribosomes or more. From this point

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### Fig. 1
Section of duodenum of normal, pair fed control (A), and vitamin A-deficient (B) rats showing crypts of Lieberkühn, stained with hematoxylin and eosin. Unstained areas are goblet cells. CL, crypt of Lieberkühn; gc, goblet cell. A, X 1400. B, X 1470.

### Fig. 2
Electron micrographs of normal (A) and vitamin A-deficient (B) rat intestine. The tissue was fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.5) and 5% sucrose overnight, rinsed in 5% sucrose with 1% osmium tetroxide and 0.5% NaCl in 0.1 M sodium phosphate buffer (pH 7.5) for 14 hours, and rinsed again in 0.5% NaCl. The standard dehydration and Epon embedding technique was used (18). Sectioning was done on a Porter-Blum MT-2 ultramicrotome with a diamond knife and staining by the method of Millonig (19). Tissue preparations were examined in the Hitachi HU-11A electron microscope. mw, microvilli; cm, cell membrane; m, mitochondria; rer, rough endoplasmic reticulum. A, X 21,000. B, X 13,000.
on, all animals used in this work had their common bile duct ligated 18 hours before use.

The protein-synthesizing activity of the free polyribosomes was then tested. The requirements for protein synthesis were found to be the same as those described by Alpers and Isselbacher (8). However, it was necessary to determine the optimum amount of pH 5 fraction needed per incubation for incorporation of labeled amino acids into protein, since this amount might differ for polyribosomes from deficient and normal tissues. Fig. 5 shows a titration of polyribosomes against pH 5 fraction. No difference was found, at least for the optimum value, although for higher amounts of pH 5 fractions the activity of the normal polyribosomes declined while that of the deficient appeared to plateau.

The kinetics of incorporation of a labeled amino acid into protein by polyribosomes is shown in Fig. 6: a slight decrease (about 12%) in deficient polyribosomes was found. Upon repeating this experiment several times, however, this decrease often disappeared, and in no case was it found to be greater than that shown in Fig. 6. We concluded that the rate of protein synthesis on polyribosomes is not affected by vitamin A deficiency.

We next attempted to determine whether protein synthesis on microsomes was affected by vitamin A deficiency. Microsomes were prepared as described (17). A decrease in activity was occasionally observed with microsomes from deficient animals, but the results were variable; frequently, no such difference was found. With the technique for microsome preparation used it was obviously not possible to obtain precise and reproducible control of the proportions of free and membrane-bound polyribosomes constituting “microsomes.” As shown above, protein synthesis on free polyribosomes is not affected by the vitamin A status and an uncontrolled increase in the amount of free polyribosomes in the mixture obscures the effect seen with membrane-bound polyribosomes.

The use of microsomes, prepared as described, was therefore deemed unsatisfactory. In consequence, the same experiments were performed with “clean” rough endoplasmic reticulum, prepared according to the method of Moule et al. (13). The cofactor requirements were the same as for free polyribosomes. We found a partial requirement for UDP-N-acetylglucosamine in experiments with microsomes, but none for rough endoplasmic reticulum. Nonetheless, the compound was added to all incubations with rough endoplasmic reticulum because occasionally an improved level of incorporation of amino acid into protein could be observed in the presence of UDP-N-acetylglucosamine both for deficient and normal systems.

The optimum amount of pH 5 fraction remained to be determined (Fig. 7). The difference in protein-synthesizing capacity between deficient and normal rough endoplasmic reticulum was marked. The results of crossing-over experiments are shown in Fig. 8 for a normal and a deficient animal at the stage of deficiency 3 days beyond the weight plateau stage (severe deficiency). Similar results were found for animals at a stage of deficiency before they had begun to lose weight (2 days at the weight plateau stage) (Fig. 9). In all cases, the lesion produced by the vitamin deficiency was located in the pH 5 fraction and not in the rough endoplasmic reticulum. Finally, the beginning of a difference between deficient and normal animals was detected before the weight plateau stage had been reached, in other words, before any overt symptoms of vitamin A deficiency had appeared (Fig. 10). This was done by making the usual group of 10 rats vitamin A-deficient from weaning and killing those to be used for the experiment while they were still gaining in weight, at a time when the first two animals of the group had reached the plateau
RNA and protein content and ratios in various cell fractions from vitamin A-deficient and normal rat intestinal mucosa

The cell fractions were prepared as described under "Experimental Procedure." RNA and protein estimation on the pellets was done after suspension in Buffer TMK.

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Normal control</th>
<th>Vitamin A-deficient</th>
<th>RNA</th>
<th>Protein</th>
<th>RNA/protein</th>
<th>RNA</th>
<th>Protein</th>
<th>RNA/protein</th>
<th>% total</th>
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<tr>
<td>Whole homogenate</td>
<td>3.50</td>
<td>0.054</td>
<td>2.39</td>
<td>0.04</td>
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<tr>
<td>1000 × g supernatant</td>
<td>1.12</td>
<td>0.050</td>
<td>1.18</td>
<td>0.042</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000 × g pellet</td>
<td>2.30</td>
<td>0.048</td>
<td>1.27</td>
<td>0.057</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free polysomes</td>
<td>0.81</td>
<td>0.048</td>
<td>0.53</td>
<td>0.051</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interface (RER + SER)</td>
<td>0.34</td>
<td>0.124</td>
<td>0.213</td>
<td>0.167</td>
<td>0.197</td>
<td></td>
<td>0.157</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

* Per cent of 6000 × g supernatant fraction.

Vitamin A-deficient

**TABLE I**

![Fig. 4](image) Fig. 4. Polysome profiles from normal (a) and vitamin A-deficient (b) rat intestinal mucosa. Postmitochondrial supernatant fraction was prepared without bentonite and layered on a 5-ml linear sucrose gradient as described under "Experimental Procedure." Polysome profiles from normal (c) and vitamin A-deficient (d) intestinal mucosa were prepared 18 hours after ligation of the common bile duct, with 1% bentonite in the rinse-NaCl solution and homogenizing mediums.

![Fig. 5](image) Fig. 5. Uptake of uniformly labeled L-leucine-14C into protein with a changing ratio of ribosomal to pH 5 fraction protein. The polysome and pH 5 fractions were prepared as described under "Experimental Procedure," with their ratio varying as noted in the graph. Incubation and precipitation of aliquots were carried out as described in the text. Both vitamin A normal and deficient polysomes were incubated with normal and deficient pH 5 fractions, respectively.

It had previously been observed that 2 to 3 days after the first animal in a group reaches the plateau stage of deficiency, all others follow. One can therefore safely assume that those animals whose protein-synthesizing capability of intestinal
Fig. 6. Kinetics of uniformly labeled L-leucine-14C uptake into protein by polysomes. Membrane-free polysomes and pH 5 fractions were prepared as described under "Experimental Procedure" and incubated with leucine-14C. Incubation and precipitation of aliquots were as described under "Experimental Procedure."

Fig. 7. Rough endoplasmic reticulum (RER), prepared according to Moulé et al. (13), and pH 5 fractions (prepared as described under "Experimental Procedure") were incubated with varying protein ratios to determine the optimum ratio of rough endoplasmic reticulum to pH 5 fraction protein.

Rough endoplasmic reticulum is illustrated in Fig. 10 were at a stage of vitamin A deficiency 2 to 3 days before the plateau stage.

A control experiment was necessary to show that the observed

Fig. 8. Crossing-over experiment with rough endoplasmic reticulum (RER) prepared according to Moulé et al. (13). ND, normal rough endoplasmic reticulum, deficient pH 5 fraction; NN, normal rough endoplasmic reticulum, normal pH 5 fraction; DN, deficient rough endoplasmic reticulum, normal pH 5 fraction; DD, deficient rough endoplasmic reticulum, deficient pH 5 fraction.

Fig. 9. Crossing-over experiment as in Fig. 8, with animals 2 days at the weight plateau stage of deficiency. Designations are the same as in Fig. 8. RER, rough endoplasmic reticulum.
FIG. 10. Experiment as in Fig. 9, with animals before the onset of the plateau stage of deficiency, as described under "Results." Designations are the same as in Fig. 8. A similar experiment at an even earlier stage of deficiency gave 1100 cpm/0.1 mg of protein for the normal preparation and 900 cpm/0.1 mg of protein for the deficient preparation after 30 min of incubation. RER, rough endoplasmic reticulum.

FIG. 11. Uniformly labeled L-leucine-14C incorporation into proteins with a system identical with that described under "Experimental Procedure" for intestinal cell fractions, except with liver rough endoplasmic reticulum and pH 5 fractions.

As has already been observed by Wolbach and Howe (20), the parenchymal cells of the liver are not affected by vitamin A deficiency. In fact, as shown in Fig. 11, the difference in protein synthesis observed with liver rough endoplasmic reticulum and pH 5 fraction was slight. Actually, the protein-synthesizing system from liver of deficient rats showed higher incorporating activity than that from normal rats.

**DISCUSSION**

The results show that protein synthesis by membrane-bound but not by free polyribosomes of intestinal mucosa is depressed under conditions of vitamin A deficiency. Therefore, vitamin A is involved, directly or indirectly, in the translation process, at least in intestinal mucosa. The lesion caused by the deficiency is located in the pH 5 fraction, and could be present in the tRNA, the amino acid-activating enzymes, or other enzymes involved in protein synthesis. tRNA and amino acid-activating enzymes have both been suggested as being implicated in the control of differentiation at the translational level (21, 22).

It is important to note that, apart from the effect on the mucous-secreting cells, the deficient mucosa cells are histologically and biochemically like normal cells. The same amount of rough endoplasmic reticulum is seen by electron microscopy, and even the goblet cells, although much fewer in number, are in appearance identical with normal goblet cells. Protein and RNA concentrations are not affected by vitamin A deficiency, and free polyribosomes show no differences with respect to intactness of pattern and function.

The technique of ligation of the common bile duct to obtain large aggregates of ribosomes similar to those found in liver preparations was introduced by Alpers and Isselbacher (8). Unlike these workers, we could not use bentonite in place of ligation to obtain improved polyribosome patterns. While they state that "very large polyribosomes (i.e. greater than 10) were not observed," we could obtain very large polyribosomes (Fig. 4c) provided that ligation was done 18 hours prior to the killing of the animals.

The stability of the large polyribosomes isolated from intestinal mucosa of vitamin A-deficient rats (Fig. 4b) deserves some comment. It is most probably a result of the atrophy of pancreatic glands or keratinization of pancreatic ducts, or both, which occur in vitamin A deficiency, as described by Wolbach and Howe (20). In consequence, there is less secretion of pancreatic ribonuclease into the intestine. Alpers and Isselbacher (8) showed that polyribosome instability in normal intestinal mucosa was due to pancreatic ribonuclease. On the other hand, although we assayed ribonuclease of intestinal mucosal homogenates and confirmed the high value found by Alpers and Isselbacher (10), we found no difference between vitamin A-deficient and normal animals.

As pointed out above, the control animals in all experiments reported were pair fed. As discussed in detail in an earlier report (23), pair feeding of a normal (20%) protein diet to normal and vitamin A-deficient rats results in depression of protein intake in the pair fed normal control animal, since in severe deficiency its food intake is depressed, being limited to that of the vitamin-deficient rat. The comparison, therefore, between a pair fed normal control and a vitamin A-deficient rat is somewhat obscured by the superimposition of the effect of depressed food levels, and especially of depressed protein intake. It was therefore gratifying to find the lesion due to vitamin A deficiency detectable at the plateau stage of deficiency (Fig. 9), that is, before food intake had declined severely, and even more so to find it detectable at the preplateau stage (Fig. 10), when food intake was still normal.

This study also shows a specificity of the action of the vitamin...
on protein synthesis with respect to tissue through the fact that protein synthesis on liver rough endoplasmic reticulum with pH 5 fraction showed no decrease in deficiency.

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