Vitamin A in Adrenal Hormone and Mucopolysaccharide Biosynthesis

GEORGE WOLF, D. PHIL.*

WE KNOW OF one function of vitamin A, that in vision, through the researches of Wald and his team. Through their work we know more about what this vitamin does in one particular biochemical reaction sequence than we do about any other fat-soluble vitamin. However, an animal dies of vitamin A deficiency, but not necessarily from blindness. Therefore, vitamin A must have another metabolic function. We began our search for this function in the most generalized way,[1] by comparing the metabolism of labeled acetate in vitamin A deficient and pair-fed normal rats. No differences were discernible in the rate of incorporation of radioactivity into a large variety of metabolic intermediates and products which were investigated. The only product which showed a severe depression in radioactivity incorporation was glycogen. The block caused by the deficiency was localized in the conversion of triose to glucose.[2] Table I shows the progressive loss of the capacity to incorporate radioactivity into glycogen as the deficiency proceeds, and it is to be noted that a severe drop of glyconeogenesis takes place before any other symptoms but weight loss are noticed. It was further shown that this effect was indirect,[2] and due to a lack of adrenal glucocorticoid hormones, as shown in Table II. It can be seen that the lesion is not between glucose and glycogen, but between acetate and glycogen, and that it can be corrected by cortisone injection. Histologically, as had already been indicated by Morton and his co-workers,[3] the adrenocortical lesion was seen to consist of a migration of phospholipid substances from the zona fasciculata to the zona glomerulosa. This change in the cortex begins at a time in the progress of the deficiency before even the effect on glycogen synthesis has started.

We then investigated corticosterone production by quartered adrenal glands *in vitro*. Tetrazolium-blue reducing steroids secreted into the medium were measured. Their value dropped to about one-half when using adrenal glands from animals in the earliest stages of the deficiency, and could be restored to normal by pre-injection of vitamin A as little as six hours prior to killing and excision of the glands.[4]

Next, we used adrenal homogenates incubated with labeled cholesterol and investigated its conversion to various steroids, which were isolated by paper chromatography.[5] Table III shows that a number of steroids on the pathway from cholesterol to corticosterone show lowered incorporation of radioactivity in vitamin A deficiency. If mildly deficient animals were used, however, only the last step

TABLE I

Gross Symptoms and Glycogen Biosynthesis from Acetate in Vitamin A Deficiency

Pair No.	Condition of Vitamin A Deficient Rat	Total Activity of Liver Glycogen (c.p.m.)	
		Normal	Deficient
1	No symptoms	43,500	42,300
2	No symptoms	42,200	37,600
3	Slow growth	11,300	7,060
4	Weight loss (2 days)	12,090	203
5	Severe weight loss	11,480	236
6	Severe xerophthalmia	9,930	0

From the Radiocarbon Laboratory and Division of Animal Nutrition, University of Illinois, Urbana, Illinois.

* Associate Professor of Animal Nutrition.

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TABLE II

Effect of Cortisone Treatment on Glycogen Formation in Livers of Normal and Vitamin A Deficient Rats

Percursor	Cortisone Treatment*	Total Activity of Liver Glycogen (c.p.m.)	
		Normal	Deficient
Acetate-1-C^{14}	−	28,800	2,540
Acetate-1-C^{14}	+	21,100	24,400
Glucose-1-C^{14}	−	121,200	101,200
Glucose-1-C^{14}	+	125,000	120,000

* + = rats were treated with cortisone acetate (5 mg. per day) for four days, then given 300 mg. non-labeled glucose, and thirty minutes later 7.5×10^6 c.p.m. labeled compound intraperitoneally.

TABLE III

Labeled Steroids Produced by Pig Adrenal Tissue Using Cholesterol-C^{14} as a Precursor*

Steroid	Activity Found in Deficient Tissue (d.p.m.)	Activity Found in Normal Tissue (d.p.m.)
Corticosterone	43,428	88,620
Progesterone	18,312	60,300
DOC	9,908	38,488
17 OH DOC	27,832	. . .†

* Tissue was homogenized and incubated in Sorenson's phosphate buffer, pH 7.2 with the following concentration of additives: sucrose, 0.025 M; KCl, 0.154M; NaCl, 0.154 M; niacinamide, 5 mM; sodium fumarate, 5 mM; $MgSO_4$, 5 mM; glucose, 0.01 M. Cofactors added: ATP, 1 mM; DPN, 0.5 mM; TPN, 0.5 mM. Incubated for two hours under 95 per cent O_2 + 5 per cent CO_2. Values represent an average of three experiments. 1.25 μc. cholesterol-4-C^{14} added. Total volume 3 ml.

† Undetectable.

TABLE V

Corticosterone Produced Under Conditions of Increased TPNH Production*

Addition	Corticosterone Produced in Deficient (d.p.m.)	Corticosterone Produced in Normal (d.p.m.)
Homogenate	2,650	7,548
Homogenate + glucose-6-phosphate	3,104	8,552
Homogenate + glucose-6-phosphate + glucose-6-phosphate dehydrogenase	2,412	9,196

* Conditions of incubation were the same as those given in Table III. Cofactors present at following concentration: ATP, 1 mM; DPN, 0.5 mM; TPN, 0.5 mM; glucose-6-phosphate, 0.5 mM. Three units of glucose-6-phosphate dehydrogenase were added.

in the sequence, the conversion of desoxycorticosterone to corticosterone, is so affected (Table IV). Table V shows that this depression is not due to lack of the essential coenzyme in this reaction, reduced triphosphopyridine nucleotide (TPNH). As is evident from the data in Table VI this lesion can readily be corrected by addition of vitamin A, as well as vitamin A acid, to the homogenate.

To summarize, then, we have traced an abnormal metabolism of acetate in vitamin A deficiency to a participation of the vitamin in the biosynthesis of corticosterone, apparently in the hydroxylation of desoxycorticosterone, and presumably a direct participation, since the vitamin is effective by direct addition to adrenal homogenate *in vitro*.

The outward symptoms of vitamin A de-

TABLE IV

Labeled Steroids Produced when Rat Adrenal Tissue Was Incubated with Cholesterol-C^{14}

Enzyme Source and Addition	Mildly Deficient		Normal	
	Corticosterone (d.p.m.)	Progesterone (d.p.m.)	Corticosterone (d.p.m.)	Progesterone (d.p.m.)
1. Whole homogenate	2,190	. . .	3,660	. . .
1. Mitochondria	. . .	11,538	. . .	9,312
2. Mitochondria	. . .	5,996	. . .	3,160
2. Mitochondria + progesterone	. . .	3,856	. . .	2,094

NOTE: Conditions of incubations as given in Table III.

TABLE VI
Corticosterone Production from Cholesterol-4-C^{14}*

	C^{14} Activity in Corticosterone in Deficient Tissue (d.p.m.)	C^{14} Activity in Corticosterone in Normal Tissue (d.p.m.)
Whole homogenate	9,630	13,706
Whole homogenate + vitamin A	14,998	†
Whole homogenate + vitamin A acid	16,576	†

* Vitamin A added at level of 1.3 μM/incubation. DPN and TPN, 0.5 mM; ATP, 1 mM. 2.2×10^6 d.p.m. cholesterol added to each incubation. Incubated for one hour under 95 per cent O_2, 5 per cent CO_2.
† Not determined.

TABLE VII
Incorporation of $S^{35}O_4^-$ into Mucopolysaccharides by Colon Homogenates

Vitamin A Status	Vitamin A Added (10 μg.)	Mucopolysaccharide (c.p.m.)
−	−	346 (3)
−	+	805 (2)
+	−	936 (4)
−	serum-albumin-ethanol	268 (2)

NOTE: The values are averages of the number of incubations shown in parentheses. The complete system contained 10 μM of ATP, 6 μM of Mg^{++}, 1 μM of glutamine, 3 μM of DPN and 10 μM of glucose: final volume, 1 ml. Each incubation contained 10.6×10^6 c.p.m. of radioactive sulfate and 14 mg. of protein. + = normal rat; − = deficient rat.

TABLE VIII
Effect of Vitamin A on the Incorporation of $S^{35}O_4^-$ into Mucopolysaccharides by Colon Homogenates Containing UDP-Derivatives

Vitamin A Status	Addition of Vitamin A (10 μg.)	Mucopolysaccharides (c.p.m.)
+	−	832 (2)
−	−	270
−	+	681

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 1 μM of UDPGA, 1 μM of UDPG, 1 μM of glutamine, 8 μM of AG (all obtained from Sigma Chemical Corporation), 1 μM of ATP and 10 μM of Mg^{++}. Each incubation contained 8.5×10^6 c.p.m. of activity and 14 mg. of protein. + = normal rat; − = deficient rat.

TABLE IX
Incorporation of S^{35}-Sulfate Into Mucopolysaccharide by Subcellular Fractions and pH 5 Enzymes of Pig Colon Mucosa

Subcellular Fraction	Mucopolysaccharide (c.p.m./mg. protein)
Supernatant free from mitochondria and nuclei	1,293 (4)
Supernatant free from mitochondria, nuclei and microsomes	1,521 (4)
Supernatant free from mitochondria, nuclei, microsomes and pH 5 enzymes	326 (1)
Microsomes	516 (4)
pH 5 Enzymes	1,728 (4)
pH 5 Enzymes and microsomes	1,770 (3)

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 10 μM ATP, 6 μM $MgCl_2$, 3 μM DPN, 1 μM glutamine and 10 μM glucose. The radioactivity added per incubation, 9×10^6 c.p.m. made to final volume of 1 ml. with phosphate buffer, pH 7.4.

ficiency show no similarity to those found in cases of adrenal insufficiency, and, indeed, the only detectable consequence of the glucocorticoid hormone lesion is the decreased capacity for glycogen synthesis. Therefore, the adrenal cortex disturbance must be only one of a number of divergent symptoms of vitamin A deficiency, the most apparent of which is the deterioration of mucous membranes. We were led, therefore, to an investigation of the influence of vitamin A on the metabolism of mucopolysaccharides, which are the characteristic constituents of mucus. Already in 1953,[6,7] H. B. Fell had shown that the profound effect of vitamin A on another type of mucopolysaccharide, that of bone and cartilage, is direct and not mediated through a hormone. We found the most promising tissue for *in vitro* synthesis of MPS (mucopolysaccharide) to be the mucosa of colon. Investigating the uptake of labeled sulfate into MPS, we observed,[8] both in colon segments and homogenates, a depression of MPS synthesis in vitamin A deficiency, restorable specifically by vitamin A added to the medium (Table VII). Figure 1 shows the pathway of MPS synthesis, as now accepted. By using, as substrates in MPS synthesis, intermediates closer to the

TABLE X
Effect of Preincubation with Lipoxidase on the Incorporation of $S^{35}O_4^-$ Into Mucopolysaccharide Synthesized by pH 5 Enzymes

Lipoxidase (0.3 mg./ml.)	Added Vitamin A (10 μg.)	Mucopolysaccharide (c.p.m./mg. protein)
–	–	2,706 (4)
0.1	–	1,213 (4)
0.1	+	2,188 (4)

NOTE: The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 10 μM of glucose, 1 μM of glutamine, 3 μM of DPN and $S^{35}O_4^-$, 12 × 10^6 c.p.m., made up to 1 ml. with phosphate buffer, pH 6.8.

final product, such as galactosamine, glucosamine 6-phosphate, uridine diphosphoglucuronic acid, and uridine diphosphoacetylglucosamine, we could still obtain the effect of vitamin A, as is illustrated, for instance, in Table VIII. It was obvious, therefore, that the vitamin was not involved in the biosynthesis of any of these intermediates. We were able to trace the capacity for MPS synthesis to an enzyme fraction obtainable from pig colon mucosa,[9] where it resided in the protein fraction of the supernatant solution after high speed centrifugation, precipitable at pH 5.2 (Table IX). This enzyme fraction is again susceptible to vitamin A deficiency, as well as to lipoxidase treatment (Table X). Lipoxidase is known to destroy vitamin A, and it lowers MPS synthesis in the pig colon mucosal enzyme fraction. The activity can again be restored by added vitamin A.

Up to this point we have been investigating only the incorporation of infinitesimally small amounts of labeled substrates (sulfate or glucose) into MPS. It was necessary to determine whether net synthesis also was affected by vitamin A. We were able to show that colons of vitamin A deficient rats contained 40 per cent less total hexosamines than those of normal rats and that this was due to a deficiency of galactosamine rather than of glucosamine.[10] We next developed a cell-free mucosal homogenate system[11] which, with glucose 6-phosphate, uridine and adenosine triphosphates and diphosphopyridine nucleotide, gave net synthesis of total and of mucopolysaccharide-bound hexosamines. Net synthesis was about 1 μM MPS-bound hexosamine per 100 mg. protein, lowered to about 0.3 μM in deficiency and restorable with added vitamin A (but no other fat soluble vitamin).

The minimum effective amount was determined to be 1.25 × 10^{-2} μM vitamin A per incubation.

As mentioned above, by the use of substrates in MPS synthesis which were closer to the final product, an analysis could be made of the particular reaction which would be susceptible to vitamin A deficiency. None was found, until incubations were attempted with S^{35}-labeled phosphoadenosine phosphosulfate (PAPS, "active sulfate") instead of with sulfate (see Figure 1). It could be shown[12] that, whereas vitamin A deficiency severely lowered sulfate incorporation into MPS from free sulfate, no such depression was obtained with the use of PAPS. As shown in Table XI, vitamin A deficiency causes lowered PAPS synthesis, a reaction

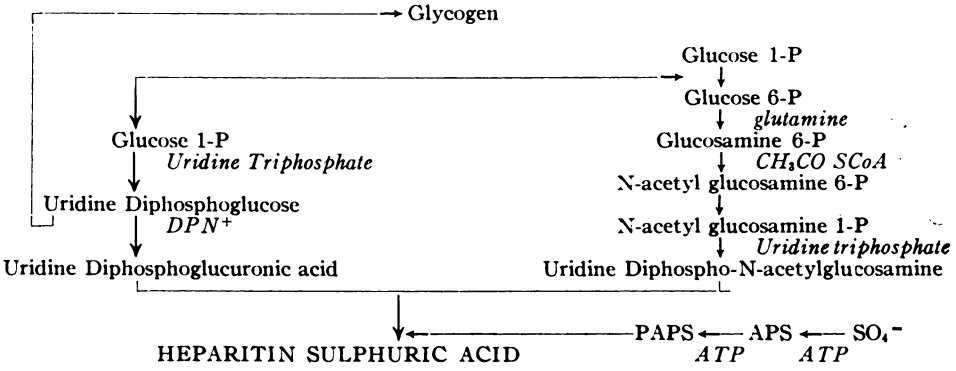

FIG. 1. Possible pathway of mucopolysaccharide biosynthesis (P, phosphate; CH_3CO-SCoA, acetyl coenzyme A; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate APS, 5′-adenosinephosphosulfate).

TABLE XI
Effect of Vitamin A Deficiency on $PAPS^{35}$ Synthesis

Vitamin A Status of Rats	Addition	$PAPS^{35}$ (c.p.m./mg. protein)	
		Experiment 1	Experiment 2
Adequate	...	19,200	59,000
Deficient	...	8,440	28,700
Deficient	Vitamin A, 20 μg. in propylene glycol, 5 μl.	24,300	54,100
Deficient	Propylene glycol, 5 μl.	...	31,400

Activity added: Experiment 1, 8.4×10^5 c.p.m.; Experiment 2, 16.8×10^5 c.p.m.

which is an essential step in the formation of sulfated MPS. The lesion was again restorable to normal with added vitamin A. It seems, therefore, that the capacity of vitamin A to affect MPS synthesis lies in its function in the activation reactions of sulfate. Preliminary evidence would implicate the first step; i.e., the reaction of adenosine triphosphate with sulfate to give adenosine phosphosulfate, as being vitamin A dependent. Isolation of the enzyme involved, and a search for vitamin A or a derivative thereof in the enzyme, is now under way.

It is possible, albeit somewhat speculative, to generalize from these observations. As Fell has shown, excess vitamin A causes a dissolution of the MPS part of cartilage matrix, presumably by a breakage of the MPS-protein bound in cartilage. On the other hand, vitamin A stimulates the formation of MPS of mucus. We are led to the hypothesis that the activation of sulfate is the rate limiting step in MPS synthesis. Sufficient vitamin A leads to a maximum rate of MPS synthesis. Large amounts of MPS, in some unexplained way, cause dissolution of the MPS-protein bound in cartilage and the increased formation of MPS bound in mucus. The concentration of MPS itself may be the regulatory factor determining whether it is to be bound in cartilage or in mucus. Future research will show whether or not these hypotheses are borne out in fact.

SUMMARY

Vitamin A deficiency was found to cause an abnormal metabolism of acetate, and reduced glyconeogenesis. This was traced to a malfunction of the adrenal cortex and, ultimately, to an interruption of adrenal glucocorticoid hormone (corticosterone) biosynthesis in the vitamin A deficient rat. In adrenal cortex homogenates from vitamin A deficient animals, the reduced corticosteroid biosynthesis could be restored to normal by adding vitamin A or vitamin A acid *in vitro*. The influence of the vitamin on one of the enzymes of corticosteroid biosynthesis seems, therefore, to be a direct one.

Another lesion caused by vitamin A deficiency, the degeneration of mucous membranes, was found to be due to a requirement for vitamin A in mucopolysaccharide biosynthesis. This was shown by lowered incorporation of labeled sulfate or glucose into mucopolysaccharide in rat colon homogenates, restorable specifically by added vitamin A. Similarly, net synthesis of mucopolysaccharides in rat colon homogenates, as measured by an increase in mucopolysaccharide-bound hexosamines, is partially dependent on vitamin A. This effect was traced to an enzyme fraction and finally to a single reaction in the biosynthesis of mucopolysaccharide, the activation of sulfate to form adenosine phosphosulfate.

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DISCUSSION

Dr. John. A. Lucy (*Madison, Wisconsin*): Dr. Wolf has demonstrated that the *in vitro* incorporation of (^{35}S) sulphate into mucopolysaccharide by segments and homogenates of colon from vitamin A deficient rats is reduced as compared with the incorporation observed in control experiments with tissue from normal animals. Furthermore, the point of action of the vitamin appears to be at the stage of polymerisation of uridine diphosphate derivatives or in the transfer of sulphate to the polymer. In an investigation on the effect of excess vitamin A on sulphate incorporation in a different tissue, embryonic cartilage, Fell, Mellanby and Pelc found that limb-bone rudiments of the chick cultured *in vitro* in the presence of vitamin A first ceased to take up labeled sulphate and then lost that already incorporated. The loss of (^{35}S) sulphate from the matrix was accompanied by the disappearance of metachromasia. Dr. Honor B. Fell, John T. Dingle and I have been studying the mode of action of this effect of excess vitamin A on cartilage and have obtained evidence that loss of metachromasia results from an effect on the protein of the tissue rather than on the metabolism of the polysaccharide.

We have observed that after six days' cultivation in medium containing added vitamin A(10 i.u./ml.) the hexosamine content of the limb-bone rudiments is reduced to half that of the controls grown on normal medium. It was also found that degradation of the protein of the culture medium (plasma: embryo extract clot) was greater in the vitamin A treated cultures than in the control cultures. Experiments with normal embryonic cartilage demonstrated that treatment of the rudiments with distilled water at 4°c. for one hour followed by incubation in buffer for two hours at 37°c. at pH 3-5 results in a loss of metachromatic staining properties and liberation of polymeric hexosamine-containing components into the buffer. During the water treatment, a proteolytic enzyme that had an acid pH optimum was released from cytoplasmic particles of the cartilage tissue. In experiments with rat liver lysosomes that were suspended in 0.25 M. sucrose, it has been demonstrated that a proteolytic enzyme is released on the addition of vitamin A; the pH optimum of the enzyme liberated was the same as that of the enzyme obtained by hypotonic treatment. These observations indicate that a possible mechanism for the action of excess vitamin A on cartilage may involve alteration of the permeability of lysosomes and other intracellular particles. Proteolytic enzymes may be released in this way which then degrade cartilage matrix with a resultant loss of polysaccharide.

While Dr. Wolf has observed that addition of vitamin A stimulates the incorporation of sulphate into mucopolysaccharide by rat colon tissue, we have found that excess vitamin A appears to act on cartilage by stimulating proteolytic degradation of the protein-polysaccharide complex of the matrix. The difference in response of these two tissues to the vitamin in our respective experiments seems to be of a qualitative rather than quantitative nature and this difference may reflect two distinctive patterns of enzyme distribution in these tissues. The response of a particular tissue that is susceptible to vitamin A may depend upon the relative

Table I

Reproduction in the Vitamin A Deficient Rabbit

Weeks on Diet	Treatment	No. Mated	Average Young		Sites	Vitamin A	
			Living	Dead		Liver (γ/gm.)	Plasma (γ/100 ml.)
12	None	14	0.9	0.6	1.5	1.1	4.4
12	Progesterone	14	4.8	2.9	0.1	1.1	5.7

TABLE II

Reproduction in Rats Fed Only Sucrose and Distilled Water during Gestation

Diet	Treatment	No.	Average Young	
			Living	Dead or Sites
Control	None	108	10.3	0.4
Sucrose	None	35	1.2	7.7
Sucrose	Progesterone	33	6.2	2.9
Sucrose	Prolactin	5	7.8	2.2

quantities of certain enzymes in the tissue which are stimulated by the vitamin either directly, or indirectly by a mechanism such as that proposed for the action of excess vitamin A on cartilage.

DR. RAY L. HAYS: In the broader aspects of the effect of vitamin A or the lack of it on the well being of the animal, it was interesting to find exogenous progesterone an aid in alleviating some of the effects of a vitamin A deficiency on reproduction in the rabbit. Table I shows the results of our experiments.[1] It can be noted that while the liver and plasma vitamin A values are extremely low, reproduction was greatly improved by the daily injection of 8 mg. of progesterone. In the progesterone treated animals there were more living fetuses and even those which died had lived longer than those of the untreated females.

Table II shows the result of further work we have done in this area. When rats were placed on a diet of sucrose and distilled water for the duration of pregnancy, the average number of living young was reduced to 1.2.[2,3] This detrimental effect of the severely restricted diet was mostly alleviated by daily injections of 5 mg. of progesterone or 4-6 I.U. of prolactin.[4]

It would seem that the feeding of only sucrose caused a deficiency in the secretion of prolactin by the anterior pituitary, which caused a decrease in the progesterone secretion and the resultant loss of fetuses. It is possible that a similar mechanism is involved in vitamin A deficiency. On the other hand, the level of vitamin A in the blood may have been too low to permit normal essential physiologic function and that progesterone in some way compensated for it.

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