STUDIES ON THE FUNCTION OF VITAMIN A IN MUCOPOLYSACCHARIDE BIOSYNTHESIS

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SUMMARY

An *in vitro* system of rat colon segments and of rat colon homogenates was elaborated, which incorporated [^{35}S]sulfate and [^{14}C]glucose into mucopolysaccharide.

Mucopolysaccharide was identified by paper chromatography and paper electrophoresis along with chondroitin sulfate carrier; by co-precipitation and dialysis with chondroitin sulfate; by hydrolysis and identification of labeled sulfate or labeled glucosamine.

The homogenate system required adenosine triphosphate, diphosphopyridine nucleotide, glucose and glutamine. The level of incorporation was lowered to about one-half in colon segments and homogenates from vitamin A-deficient rats. Vitamin A, vitamin A-aldehyde and vitamin A-acid (but no other fat-soluble vitamin tested) restored incorporation to normal. With graded levels of vitamin A added, a maximum of incorporation was reached at 10 μg/6 mg of protein in the homogenates. Using glucosamine or galactosamine instead of glucose and glutamine, or uridine diphosphate acetylglucosamine, uridine diphosphate glucuronic acid, uridine diphosphate glucose and acetylglucosamine, incorporation of labeled sulfate was still dependent on vitamin A. This fact shows that the vitamin may be required either in the polymerisation reaction of the uridine derivatives, or the activation or transfer of sulfate. It was shown that the vitamin A-destroying enzyme lipoxidase can lower or abolish incorporation into mucopolysaccharide.

INTRODUCTION

The problem of the metabolic function of vitamin A, outside of the process of vision' remains as yet unsolved. Observations described in previous papers of this series[1,2] pointed to an involvement of vitamin A in the biosynthesis of the glucocorticoid hormones. An entirely different approach to the problem was prompted by the results obtained by FELL *et al.*[3,4] and by FRAPE *et al.*[5]. The former showed that excess vitamin A, when added to the medium of bone cells grown in tissue culture, inhibited

Abbreviations: MPS, mucopolysaccharides; CS, chondroitin sulfate; TCA, trichloroacetic acid; UDPGA, uridine diphosphate glucuronic acid; UDPAG, uridine diphosphate acetylglucosamine; UDPG, uridine diphosphate glucose; AG, acetylglucosamine; ATP, adenosine triphosphate; DPN, diphosphorpyridine nucleotide.

the synthesis of new cartilage and caused the transfer of sulfate groups bound in chondroitin sulfate to soluble sulfate containing compounds; and that excess vitamin A caused embryonic chick ectoderm to differentiate into mucus-secreting epithelium and inhibited keratinization. FRAPE and his group found that, *in vivo*, vitamin A deficiency caused an increased sulfate uptake into connective tissue mucopolysaccharide, whereas a decrease was obtained when the vitamin was fed. These observations led us to the hypothesis that vitamin A, acting like a hormone, regulates the formation of mucopolysaccharides; excess vitamin A leading to an increase in the mucus type, a deficiency to an increase in the connective-tissue type.

A search was made for a mucus-forming tissue which could serve to test this hypothesis *in vitro*. Rat colon was found to be the most suitable, following the techniques developed by PASTERNAK *et al.*[6] for guinea pig colon. Using this system, it was possible to demonstrate that vitamin A has a direct function in the biosynthesis of colon mucopolysaccharide.

METHODS

Preparation of rats

For the preparation of vitamin A-deficient and pair-fed control rats, the strain, diet and techniques described by WOLF *et al.*[1] were used. The animals were deemed "deficient" at a time after feeding the deficient diet when weight loss had just set in[2].

Incubation of colon segments

After the rats were killed by decapitation, the colons were removed and cleaned of feces with ice-cold 0.9 % saline. They were everted with the aid of a glass rod and cut into segments of about 1 cm long. The segments, weighing about 200 mg per incubation, were incubated with [^{35}S]sulfate (as [^{35}S]sodium sulfate, carrier free, from Abbott Laboratories) and 2 ml of Krebs–Ringer phosphate buffer, pH 7.3, containing glucose, as described by PASTERNAK *et al.*[6], at 37° for 4 h in a Dubnoff shaker in an atmosphere of 95 % oxygen and 5 % carbon dioxide. The [^{35}S]MPS were precipitated from the decanted medium at the end of the incubation by the addition of alcohol-benzene, as described below.

Incubation of colon homogenates

All steps were carried out at 4°. The rats were killed, the colons were removed and cleaned as described, and homogenized with 3.5 volumes of calcium and magnesium-free Krebs–Ringer phosphate buffer (0.01 *M*, pH 7.3), containing nicotinamide (0.3 *M*), for 2 min in a motor-driven, loose-fitting glass Potter-Elvehjem homogenizer. Debris and nuclei were removed by centrifugation at 600 × *g* for 15 min. The resulting supernatant suspension was used for incubation, by mixing with the co-factors and [^{35}S]sulfate or [^{14}C]glucose, described in the Tables to a final volume of 1 ml. The incubation was carried out at 37° for 2.5 h in an atmosphere of oxygen–carbon dioxide (95:5) in a Dubnoff shaker. The [^{35}S]MPS were isolated by the double precipitation method described below.

Isolation of mucopolysaccharides

The MPS formed during the incubations were isolated by two methods:

Single precipitation: After incubation, 9 volumes of alcohol–benzene (1:1, v/v)[7] were added to the incubation mixture, which was then left overnight at 4°, when a precipitate appeared. The mixture was centrifuged and the supernatant solution discarded. The precipitate was washed 3 times with the alcohol–benzene mixture and dissolved in 0.5 ml distilled water. To this was added 0.5 ml of an 8 % aqueous solution of TCA. The precipitated proteins were removed by centrifugation and the MPS in the supernatant solution was purified by descending paper chromatography. An aliquot of 0.2 ml of the solution was applied as a band to the baseline of a sheet of Whatman No. 1 filterpaper and developed in 1 *M* ammonium acetate–ethanol (2:5, v/v)[8]. MPS remained at the origin, whereas free sulfate moved with an R_F of 0.23. Similarly, during electrophoresis in barbital buffer at pH 8.8, MPS remained at the origin; in citrate buffer (0.05 *M*, pH 5.6), MPS moved 7 cm in 7 h (300 V).

Double precipitation: After precipitation and deproteinization as described above, the MPS were again precipitated from the remaining solution by addition of 9 volumes of the alcohol–benzene mixture, and left overnight at 4°. The precipitate was washed three times with alcohol–benzene and paper chromatographed as described.

Identification of mucopolysaccharides

The MPS were identified by paper chromatography and paper electrophoresis, as described above along with known samples of chondroitin sulfate, and agreement of the radioactive spots with the color spots given with the toluidine blue spray[9], was obtained.

Further, a sample of [^{14}C]MPS was hydrolyzed as described for CS by KORN[10] and the hexosamines isolated on a Dowex-50 column, along with carrier glucosamine, by the method of BOAS[11]. On paper chromatography, the spot of radioactive glucosamine coincided with the ninhydrin-color given by the carrier glucosamine.

A sample of the radioactive glucosamine was transformed into the osazone[12], along with carrier glucosamine, which was then recrystallized to constant specific activity.

A sample of [^{14}C]MPS (1.002 μC) isolated as described, was precipitated by cetylpyridinium bromide together with carrier CS (50 mg) (obtained from Nutritional Biochemical Corp.), as described by KORN[10], and the precipitate dialyzed, whereupon the radioactivity was retained in the non-dialyzable cetylpyridinium bromide complex of CS.

A further sample of [^{35}S]MPS, isolated as described, was hydrolyzed with 4 *N* hydrochloric acid at 100° for 2 h[8]. The hydrolysed solution was evaporated to dryness *in vacuo* over solid KOH and paper chromatographed in the ammonium acetate–ethanol system described above. All the radioactivity traveled with free sulfate (R_F 0.23) (R_F of methionine, 0.70; of cystine, 0.36)[8].

Vitamin A solution

Two mg of crystalline vitamin A were dissolved in 0.3 ml of absolute alcohol, followed by grinding of the solution with 1.7 ml of 0.2 % crystalline bovine plasma albumin in 0.1 *M* phosphate buffer, pH 7.4, in a glass homogenizer for approx. 2 min. The suspension was then centrifuged for 5 min at 500 × *g*. The supernatant solution was used after spectrophotometric assay of its vitamin A content. Vitamin A aldehyde and acid were dissolved similarly.

Protein determination

The protein content of the homogenate was determined by turbidity measurement. Suitable aliquots of homogenate were diluted to 3 ml with distilled water and mixed. To each tube, 3 ml 10% TCA were added, mixed and read after 10 min at 420 mμ in a Coleman colorimeter. A standard curve was made with crystalline bovine plasma albumin.

Estimation of hexosamine

The MPS formed, isolated by paper chromatography, was eluted from the paper with water. The eluate was evaporated to dryness in a stream of nitrogen on the steambath. To the dry residues, 1 ml of 3 *N* HCl was added and the MPS was hydrolyzed by refluxing for 4 h. Hexosamine was determined by the ELSON AND MORGAN procedure as modified by NEUHAUS AND LETZRING[13].

Measurement of radioactivity

A radioactivity scanning apparatus ("Actigraph", Nuclear Chicago Corporation) was used to determine ^{35}S and ^{14}C-activity on paperstrips. The paper chromatograms were passed beneath the counting tube of the scanner and peaks corresponding to radioactive spots were obtained on the chart of an automatic recorder. The paper under the peak corresponding to MPS was cut out and weighed. The weight of the paper was proportional to, and converted into, counts/min with the help of a calibration curve prepared by chromatographing and counting known quantities of [^{35}S]sulfate.

RESULTS

Effect of vitamin A-deficiency on the incorporation of [^{35}S]-sulfate into MPS

By rat colon segments: It was necessary to determine, whether there are differences between the synthesis of MPS in vitamin A deficient compared to normal rats. For this purpose, the colon segments from deficient and normal rats were prepared and incubated with [^{35}S]sulfate in Krebs–Ringer phosphate buffer, pH 7.4, as described. The MPS synthesized and secreted into the medium were isolated by the single precipitation method, purified by paper chromatography and counted by the scanning apparatus, as also previously described. The results are presented in Table I.

The incorporation of [^{35}S]sulfate into MPS by deficient colon segments was found to be about one-half compared to that of normal colons. When a suspension con-

TABLE I

INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON SEGMENTS

The values are averages of the number of incubations shown in parentheses. Activity added, $19 \cdot 10^6$ counts/min; incubation period 4 h; —, deficient rat; +, normal.

Vitamin A status	*Mucopolysaccharide counts/min*
+	4247 (3)
—	1629 (3)

TABLE II

EFFECT OF ADDED VITAMIN A ON INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON SEGMENTS

The values are averages of the number of incubations shown in parentheses. Incubation period 3.5 h, activity added $19 \cdot 10^6$ counts/min; +, normal rat; —, deficient rat.

Vitamin A status	*Vitamin A added*	*Mucopolysaccharide counts/min*
—	—	1279 (4)
—	10 μg (alcohol)	3515 (4)
—	10 μg (aldehyde)	3001 (4)
+	—	2685 (4)

TABLE III

INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Each incubation contained $13.5 \cdot 10^6$ counts/min of activity and 16 mg of protein.

ATP 10 μmoles	*Mg^{++} 6 μmoles*	*Glutamine 1 μmole*	*DPN 3 μmoles*	*Glucose 10 μmoles*	*Mucopolysaccharide counts/min*
+	+	+	+	+	1,307
+	+	+	+	—	268
+	+	+	—	—	0
+	+	—	—	—	0
+	+	+	—	+	536
+	+	—	+	+	0
—	—	+	+	+	0
—	+	+	+	+	0

taining 10 μg of vitamin A was added to the incubation medium of the deficient colons, the incorporation was raised to the level of the normal (Table II). Both vitamin A and vitamin-A-aldehyde (retinene) were effective.

By rat colon homogenates: The conditions under which the incorporation of [^{35}S]-sulfate into MPS by rat-colon homogenates would take place, were first determined. The rat-colon homogenates, prepared as described, were mixed with the co-factors, as indicated in Table III, and incubated. The MPS were isolated by the double precipitation method, purified by paper chromatography and counted in the scanning apparatus as described. The incorporation was highest when the medium contained ATP, DPN, glucose and glutamine. When ATP or glutamine were omitted, no incorporation could be obtained, and on deletion of glucose or DPN, the incorporation was lowered. In all the experiments subsequently described, all the above co-factors were added to the incubation medium, and this will be referred to as "complete system".

Identification of MPS

Hexosamine contents, determined as described, of MPS before and after incubation were 3.8 μg and 17.4 μg per incubation, respectively, indicating net MPS-synthesis by rat-colon homogenates. Incubating [^{14}C]glucose, instead of [^{35}S]sulfate,

[^{14}C]mucopolysaccharide was isolated, presumably by formation of labeled hexosamine and glucuronic acid moieties of MPS.

As shown in Table IV, after hydrolysis of the [^{14}C]MPS obtained, the hexosamines, isolated by means of carrier glucosamine, retained constant radioactivity through recrystallizations of the osazone, as well as on paper chromatography. For further identification of the MPS, the toluidine spray of HAMERMAN[9] was used on the paper chromatograms and after paper electrophoresis, of the isolated MPS. A pink spot against a blue background, coinciding with the radioactive spot and with a CS spot, proved the presence of labeled MPS.

When [^{14}C]MPS, isolated in the usual manner, was mixed with carrier CS and precipitated with cetylpyridinium bromide, the complex thus formed retained radioactivity which, moreover, was not dialysable.

A sample of [^{35}S]MPS, when hydrolyzed under conditions leading to hydrolysis of the sulfate group only[8], yielded free radioactive sulfate. The same level of radioactivity incorporation was obtained whether incubation of the colon homogenate was carried out in presence or absence of chloromycetin.

Homogenates were then prepared from vitamin A-deficient and normal rat colons and incubated with the complete system. The results are summarized in Table V. The [^{35}S]sulfate incorporated into MPS by homogenates from deficient colons was once again about one-half that of normal colons. The addition of vitamin A-suspension to the deficient homogenates restored incorporation to normal. The suspending medium alone (serum albumin-ethanol) had no effect (Table V).

Specificity of vitamin A

To determine the specificity of vitamin A in reversing the decrease in the synthesis of MPS due to vitamin A-deficiency, suspensions of vitamins D, E and K_1 were added to the complete system of vitamin A-deficient colon homogenates. No fat-soluble vitamin except vitamin A could completely reverse the decrease in the synthesis of MPS. Vitamin E reversed partially. If vitamins E and A were added together, the increase in MPS synthesis went beyond that obtained with vitamin E alone. The results are shown in Table VI. Recently DOWLING AND WALD[14] have demonstrated that rats on a diet containing vitamin A-acid instead of vitamin A grew normally, although they developed night blindness. In our system also, vitamin A-acid, when added to the incubation medium of deficient homogenates, restored to normal the decrease in incorporation of [^{35}S]sulfate into MPS (Table VI).

Effect of addition of graded levels of vitamin A

It was of some interest to find out the effect of the addition of graded levels of vitamin A on restoring the decrease in the incorporation of [^{35}S]sulfate into MPS due to vitamin A-deficiency. The results in Table VII show that the maximum incorporation could be obtained when the amount of vitamin A added was 10–15 μg/6 mg of protein.

Experiments with hexosamines

The foregoing experiments have established that, under the conditions studied, the *in vitro* synthesis of MPS by rat colon is enhanced by vitamin A. The synthesis of MPS is complex and not all the steps are as yet known. The next object, therefore,

TABLE IV

INCORPORATION OF [^{14}C]GLUCOSE INTO HEXOSAMINES OF MPS SYNTHESIZED BY COLON HOMOGENATES

The complete system consisted of 10 μmoles ATP, 6 μmoles of $MgCl_2$, 1 μmole glutamine, 3 μmoles of DPN, 20 μmoles of Na_2SO_4 and 1,000,000 counts/min of [^{14}C]glucose

	Counts/min
Total [^{14}C]MPS isolated	15,200
Total [^{14}C]hexosamine obtained therefrom	6,232
Specific activity of hexosamine	*Counts/min/mmole*
After addition of carrier (54 μmoles)	5,000
After 2nd crystallization of osazone	6,800
After 3rd crystallization of osazone	6,750

TABLE V

INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

The values are averages of the number of incubations shown in parentheses. The complete system contained 10 μmoles of ATP, 6 μmoles of Mg^{++}, 1 μmole of glutamine, 3 μmoles of DPN and 10 μmoles of glucose — final volume 1 ml. Each incubation contained $10.6 \cdot 10^6$ counts/min of radioactive sulfate and 14 mg of protein. +, normal rat; —, deficient rat.

Vitamin A status	*Vitamin A added 10 μg*	*Mucopolysaccharide counts/min*
—	—	346 (3)
—	+	805 (2)
+	—	936 (4)
—	Serum-albumin-ethanol	268 (2)

TABLE VI

INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES: SPECIFICITY OF VITAMIN A

The values are averages of the number of incubations, shown in parentheses. The complete system was that shown in Table V. Each incubation contained $9.78 \cdot 10^6$ counts/min of activity and 14 mg of protein.

Status of animal	*Addition (10 μg)*	*Mucopolysaccharide counts/min*
Normal	—	896 (2)
Deficient	—	483 (2)
Deficient	Vitamin A alcohol	841 (2)
Deficient	Vitamin A acid	805 (1)
Deficient	Vitamin D	232 (1)
Deficient	Vitamin E	608 (2)
Deficient	Vitamin E + vitamin A	752 (2)
Deficient	Vitamin K_1	435 (2)

TABLE VII

EFFECT OF ADDITION OF GRADED LEVELS OF VITAMIN A ON THE INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES

The values are averages of the number of incubations, shown in the parentheses. The complete system of Table V was used. Each incubation contained $10.6 \cdot 10^6$ counts/min of activity and 16 mg of protein.

Vitamin A status	*Vitamin A added* μg	*Mucopolysaccharide counts/min*
Deficient	—	265 (2)
Deficient	5	409 (2)
Deficient	10	574 (2)
Deficient	15	584 (2)
Deficient	20	505 (2)
Deficient	30	571 (2)
Normal	—	562 (2)

TABLE VIII

EFFECT OF ADDITION OF HEXOSAMINES ON THE INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

The values are averages of the number of incubations, shown in parentheses. The complete system was that shown in Table V. In Expts. 2 and 3 glucosamine and galactosamine were substituted for glucose and glutamine. Each incubation contained $9.7 \cdot 10^6$ counts/min and 14 mg of protein.

Vitamin A status	*Addition* 11 *µmoles*	*Mucopolysaccharide counts/min*
Deficient	—	250 (2)
Deficient	Glucosamine	215 (1)
Deficient	Galactosamine	233 (2)
Normal	—	562 (2)

was to localize the function of vitamin A in a specific step in the synthesis of MPS.

It was found that the vitamin is not involved in the formation of hexosamine, because glucosamine or galactosamine, when substituted for glucose and glutamine in the incubation medium of deficient homogenates, could not restore the decrease in incorporation (Table VIII).

Experiments with uridine diphosphate derivatives

Many workers have presented evidence that the UDP-derivatives are intermediates in the synthesis of MPS[15,16]. It was decided, therefore, to find out whether vitamin A is required in a step before or after the involvement of UDP-derivatives.

At first, a system, requiring UDP-derivatives and capable of incorporating [^{35}S]-sulfate into MPS by rat-colon homogenates had to be developed. Table IX shows that the maximum incorporation of [^{35}S]sulfate in MPS occurs, when the incubation medium contained UDPAG, UDPGA, UDPG, AG, glutamine and ATP. This system shows an absolute requirement for glutamine, UDPAG and UDPGA, and partial requirement for UDPG and AG.

The system, containing UDP-derivatives, when incubated with deficient colon

TABLE IX

INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES CONTAINING UDP-DERIVATIVES

Each incubation contained $8.5 \cdot 10^6$ counts/min and 14 mg protein. The complete system was that described in Table V, with the UDP-derivatives substituted for glucose.

UDPGA 1 μmole	*UDPAG* 1 μmole	*UDPG* 1 μmole	*Glutamine* 1 μmole	*AG* 8 μmoles	*ATP* 1 μmole	*Mg* 10 μmoles	*Mucopolysaccharides counts/min*
+	+	+	+	+	+	+	895
+	+	+	+	+	+	+	770
—	+	+	+	+	+	+	0
+	—	+	+	+	+	+	0
+	+	—	+	+	+	+	286
+	+	+	—	+	+	+	0
+	+	+	+	—	+	+	376

TABLE X

EFFECT OF VITAMIN A ON THE INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES CONTAINING UDP-DERIVATIVES

The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 1 μmole of UDPGA, 1 μmole of UDPAG, 1 μmole of UDPG, 1 μmole of glutamine, 8 μmoles of AG, (all obtained from Sigma Chemical Corporation) 1 μmole of ATP and 10 μmoles of Mg^{++}. Each incubation contained $8.5 \cdot 10^6$ counts/min of activity and 14 mg of protein; + normal rat; —, deficient rat.

Vitamin A status	*Addition of vitamin A 10 μg*	*Mucopolysaccharides counts/min*
+	—	832 (2)
—	—	270
—	+	681

TABLE XI

EFFECT OF LIPOXIDASE ON $^{35}SO_4^{=}$ INCORPORATION INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Complete system was that shown in Table V. Each incubation contained $10.6 \cdot 10^6$ counts/min of activity and 14 mg of protein. Crystalline soybean lipoxidase was obtained from Nutritional Biochemical Corporation.

Preincubation for 1 h with lipoxidase (3 mg/ml)	*Addition of vitamin A 10 μg*	*Mucopolysaccharide counts/min*
0.01 ml	—	305
0.01 ml	+	538
0.04 ml	—	268
0.04 ml	+	0
0.07 ml	—	0
0.07 ml	+	0
0.10 ml	—	0
0.10 ml	+	0

homogenates, showed once again the lower incorporation of [^{35}S]sulfate into MPS (Table X). This decrease in the incorporation could be restored by the addition of vitamin A. It can, therefore, be inferred that vitamin A functions at some step beyond the synthesis of the UDP-derivatives.

Preincubation with lipoxidase

In an attempt to achieve independence from the use of vitamin A-deficient animals, and to find a means of destroying vitamin A *in vitro*, the colon homogenates were pre-incubated for 1 h with lipoxidase, an enzyme known to destroy vitamin A. This pre-incubation, as shown in Table XI, lowered or abolished the incorporation of [^{35}S]sulfate into MPS. However, addition of vitamin A restored incorporation only at low concentrations of lipoxidase, presumably because the added vitamin was destroyed by the excess lipoxidase present in the incubation medium.

DISCUSSION

Vitamin A-deficiency has for long been known to result in abnormal epithelial cells, leading to keratinization. Epithelia of mucus secreting cells, as for instance the salivary gland duct cells, are the first to be affected. Upon administration of vitamin A, these cells return to normal, both in morphology and function. In the work presently described, it could be shown that vitamin A is required for the synthesis of MPS of the type secreted by colon. It represents the first instance, apart from the reactions of the visual cycle, in which added vitamin A functions in a biochemical reaction sequence in a cell-free, *in vitro* system.

The assay used for MPS synthesis in the present work was the uptake of labeled sulfate from the medium into the final product of a many-step synthesis. Hence, the requirement of vitamin A for any particular step can only be derived by inference. For a more direct analysis, a dissection of the reaction sequence into constituent enzymic steps, and the influence of the vitamin thereon, is under way in this laboratory and will be the subject of a subsequent publication. In the meantime, it is possible to infer, albeit tentatively, from the present results, that the vitamin is not required for the following known steps: in the conversion of glucose to hexosamines; in the acetylation of hexosamines; in the conversion of hexosamines and glucose into their UDP derivatives; in the oxidation of UDPG to UDPGA. These conclusions could be reached because, starting with any of the above intermediates in place of glucose and glutamine, sulfate incorporation into MPS was still dependent on vitamin A. By a process of elimination, then, one could conclude that vitamin A functions either in the polymerisation of the UDP derivatives, or the activation or transfer of sulfate to the polymer. Confirmation of either of these alternatives must await the isolation of the separate reaction steps involved.

The very low level of incorporation (between 0.0022 and 0.025 %) of the added labeled sulfate needs some comment, since it might call into question the identity of the MPS produced. The following considerations support the contention that labeled MPS was synthesized: (a) The isolation procedure is designed specifically for MPS; (b) coincidence of a specific color reaction and radioactivity on paper after paper chromatography and paper electrophoresis in two solvent systems, and coincidence

with the spot of carrier CS; (c) coincidence of ^{35}S and ^{14}C-radioactivity on paper chromatography and paper electrophoresis, after incubation with [^{35}S]sulfate and [^{14}C]glucose, respectively; (d) upon hydrolysis of the [^{14}C]MPS and isolation of the hexosamines with carrier glucosamine on a Dowex-50 column, the glucosamine carried radioactivity upon paper chromatography, as well as after conversion to, and recrystallization of, the osazone derivative; (e) the [^{14}C]MPS is precipitable, with carrier CS, by the specific MPS precipitation reagent, cetylpyridinium bromide. The precipitated complex retains radioactivity upon dialysis; (f) the [^{35}S]MPS can by hydrolyzed under conditions known to hydrolyze the sulfate group of MPS, producing free radioactive sulfate, identified by paper chromatography. Any sulfur-containing amino acids would have traveled at greatly different rates.

To exclude the possibility that the incorporation of labeled sulfate or glucose might be caused by bacterial infection of the homogenates, incubations were carried out in presence and absence of chloromycetin. No difference in incorporation was detectable.

The great differences in per cent incorporation of the added labeled sulfate from experiment to experiment requires some comment. The level of incorporation of labeled sulfate into colon MPS depends on the nutritional state of the animal. Although care was taken to use rats only at the point in the progress of their deficiency at which the weight-curve began to level off, the actual weights and nutritional states differed in each experiment. The animals for each separate experiment, on the other hand, were litter mates, and the deficient and normal were pair-fed, to ensure equality in weight, the only difference being due to the deficiency. Hence, each experiment is self-consistent, with its own normal controls, with which the results obtained from its own deficient animals should be compared.

That vitamin A is required in MPS synthesis could not be claimed unless evidence were obtained that the vitamin is present in colon mucosa. In fact, the unsaponifiable fraction of pig colon mucosa gave a positive Carr-Price reaction, indicating 3.7 μg of vitamin A/100 g of mucosa.

In view of the recent results of DOWLING AND WALD[14], it is of interest to note that vitamin A-acid as well as vitamin A and vitamin A-aldehyde are active in the present system, whereas other fat-soluble compounds are inactive. Using graded levels of vitamin A, a maximum response was obtained. Moreover, the vitamin A-destroying enzyme lipoxidase lowered or abolished MPS synthesis.

In order to achieve independence of the use of vitamin A-deficient animals, attempts were made to lower mucosal activity by isoctane extraction, by irradiation at 326 mμ, and by oxidation. In all three methods, sulfate incorporation by mucosal homogenate was lowered and could be raised to normal after addition of vitamin A. However, this was found to be non-specific, since vitamins E and K_1, and cholesterol achieved the same result.

In a different series of experiments carried out in this laboratory[17], it was found that corticosterone production by rat adrenal cortex *in vitro* was lowered in vitamin A-deficiency, and restored to normal by addition of TPNH (or TPNH generators such as glucose-6-phosphate or glycogen). In order to obtain a link between these, so far disconnected, functions of vitamin A, the above co-factors were added to deficient colon homogenate incubations, but failed to restore lowered MPS synthesis to normal.

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