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VITAMIN A AND MUCOPOLYSACCHARIDE BIOSYNTHESIS BY CELL-FREE PARTICLE SUSPENSIONS*

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SUMMARY

In continuation of previous work from this laboratory, a system was developed consisting of particles from colon homogenates sedimenting at 20000 × *g* (after removal of whole cells and debris at 1000 × *g*), which could effect net synthesis of mucopolysaccharide-bound hexosamines from glucose 6-phosphate, glutamine, ATP, UTP, DPN and Mg^{2+}. Whereas the whole homogenate had an absolute requirement for, at least, ATP, UTP and DPN, the particles required at least glucose 6-*P* and Mg^{2+}. The average of mucopolysaccharide-bound hexosamines formed was 2.8 μmoles/100 mg protein for whole homogenate, 7.3 μmoles for the 20000 × *g* particle suspension, and 0.6 μmole for the cell-sap and microsome fraction on incubation for 3 h at 37°. Histological examination showed the particles to be cell-free, bacteriological tests (after incubations with chloromycetin, which gave normal mucopolysaccharide synthesis) revealed no bacterial contamination. The formation of mucopolysaccharide-bound hexosamines showed an approximately linear relationship with the amount of protein in the particles incubated. Mucopolysaccharide synthesis was lower with UDP-glucuronic acid and UDP-acetylglucosamine as substrates in place of glucose 6-*P*. The level of mucopolysaccharide-bound hexosamines formed by whole homogenates of vitamin A-deficient rats was reduced to 0.27 μmole/100 mg of protein, by vitamin A-deficient particles to less than 0.1 μmole. Complete restoration of the latter to the normal level of mucopolysaccharide synthesis with added vitamin A was not possible, but could be so restored by adding a metabolite derived from vitamin A acid.

INTRODUCTION

Previous work from this laboratory has demonstrated the effect of vitamin A on the biosynthesis of mucopolysaccharides in rat colon[1-4]. In order to make an approach towards the identification of the reaction step or steps in which the vitamin partici-

Abbreviation: MPS, mucopolysaccharide.

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pates, a technique was developed for the biosynthesis of MPS by colon homogenates, measured by a net increase in MPS-bound hexosamines[4]. It was shown that in vitamin A deficiency, this synthesis was severely depressed, and could be partially restored specifically by the addition of small amounts of vitamin A.

In the present study, a fractionation of colon homogenate was carried out in order to obtain that cell-fraction in which MPS is synthesized. In view of previous results from our laboratory[2], and the fact that several of the known enzymes of MPS synthesis occur in the cell-sap, it was surprising to us to find that MPS synthesis took place in cell particles similar to (or identical with) mitochondria.

EXPERIMENTAL

Materials, methods and animals used were exactly identical with those described previously[4], with the following exceptions. About 2 g of cleaned rat colon was homogenized in buffer, as described[4]. Whole cells were removed from the homogenate by centrifugation at 1000 × *g* for 10 min, and the supernatant suspension was incubated for 3 h at 37° in air as described[4]. At the end of the incubation period, the reaction was stopped by heating to 100° for 3 min. Dialysis and hydrolysis followed[4], and the assay of the resulting hexosamines was done as described previously[4].

For experiments in which particle suspensions were used, the supernatant obtained from the centrifugation of the homogenate at 1000 × *g* was re-centrifuged at 20000 × *g* for 20 min. The resulting pellet was incubated after re-suspension in the same volume of buffer solution. The supernatant of the centrifugation at 20000 × *g* was used as the "microsome and cell-sap" fraction.

A microscopic examination of the particle suspension after staining with methylene blue revealed no whole cells. Many whole cells could be seen in the pellet from the 1000 × *g* centrifugation. The particle suspension, after incubation with chloromycetin (7.5 μg/ml) showed no bacterial growth upon incubation on nutrient agar plates for 48 h.

To obtain the active metabolite of vitamin A acid, five vitamin A-deficient rats were each injected with 6.1 mg [^{14}C]vitamin A acid (2.27 μC) (obtained through courtesy of Professor O. WISS, of Hoffmann-La Roche Co.) intraperitoneally in 0.5 ml of saline. The rats were killed after 24 h, the intestines removed, cleaned with cold 0.9% saline solution and ground up with anhydrous sodium sulfate. The mixture was then extracted 5 times with small portions of petroleum ether (Skelly F). The combined extracts were extracted twice with 5 ml 6% sodium bicarbonate solution. The aqueous extract was then carefully acidified with 2 N HCl, and extracted 5 times with the same volume of ether. The ether solution was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The residue (from all five rats) (0.0044 μC) showed an ultraviolet absorption spectrum with inflections at 310–315 and 335 mμ. It had been shown previously by esterification of the acids from the intestine with diazomethane and fractionation of the esters on a column of de-activated alumina, that vitamin A acid is absent from this material. It was dissolved in dilute sodium bicarbonate solution, the pH adjusted to 7.2, and aliquots were added to the particle suspension as shown in Table IX.

RESULTS AND DISCUSSION

In order to carry out a cell-fractionation of rat colon with respect to MPS-synthesizing capacity, the system previously developed[4] was used, with removal, first, of the nuclei, whole cells and debris. Table I shows that under these circumstances MPS-synthesis, as measured by net increase in MPS-bound hexosamines, continues to function. In fact, if calculated per 100 mg of protein, it increased from about 1.0 to about 2.5 μmoles of MPS-bound hexosamines formed. Some of the requirements of this

TABLE I

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON HOMOGENATES: REQUIREMENTS

Rat-colon homogenates were prepared and incubated with co-factors (total volume, 2 ml; protein per incubation as shown) for 3 h at 37° in air as described in the text. Zero-time samples were heated to boiling and dialyzed, etc. without incubation. Co-factors added to each sample: glucose 6-*P*, 20 μmoles; L-glutamine, 30 μmoles; ATP, 2 μmoles; UTP, 4 μmoles; DPN, 1 μmole; Na_2SO_4, 1 μmole; $MgCl_2$, 10 μmoles. Each numbered experiment refers to a colon from a separate animal. Data within each numbered experiment refer to replicate determinations of zero-time values or values after incubation, each made on separate aliquots of the same homogenate. CS signifies "complete system". Values in table refer to total mucopolysaccharide-bound hexosamines per sample. In Expts. 1a and 2a, increases in hexosamines upon incubation statistically significant, $p < 0.01$.

Expt.	*Additions*	*Protein (mg)*	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
							(μg)	*(μmoles)*	
1a	CS	11.0	284.0		305.6				
				273.5	353.6	329.2	55.7	0.312	2.84
			262.4		305.6				
2a	CS	9.4	257.6		300.0				
			252.0	253.9	304.8	289.8	35.9	0.201	2.14
			252.0		300.0				
					252.8				
2b	−UTP				241.6	—	0	0	0
	−DPN				257.6	—	3.7	0.027	0.29
	−ATP				252.8	—	0.8	0.005	0.05
	−Glutamine				300.0	—	46.1	0.257	2.77

TABLE II

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON HOMOGENATES FROM VITAMIN A-DEFICIENT RATS

Methods used, co-factors added and data expressed identical with those shown in Table I. Values in table refer to total mucopolysaccharide-bound hexosamines per sample.

Protein (mg)	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamine formed*		*Hexosamines formed per 100 g protein (μmoles)*
					(μg)	*(μmoles)*	
8.5	72.6		88.6				
	111.0	95.7	102.0	99.6	3.9	0.022	0.27
	103.2		100.2				
			109.2				
			98.4				

TABLE III

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES

Methods used, co-factors added and data expressed identical with those in Table I, except that a particle suspension (20000 × *g*) instead of whole homogenate was incubated. Values in table refer to total mucopolysaccharide-bound hexosamines per sample.

Protein (mg)	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
					(μg)	*(μmoles)*	
3.4	90.8	87.4	151.2	148.0	60.6	0.34	10.0
	76.8		106.8				
	78.8		153.6				
	103.2		185.6				
			160.8				
			132.0				

TABLE IV

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON MICROSOMES AND CELL-SAP

Methods used, co-factors added and data expressed identical with those shown in Table I, except that the microsome and cell-sap fraction was used. Values in table refer to total mucopolysaccharide-bound hexosamines per sample.

Expt.	*Protein (mg)*	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
						(μg)	*(μmoles)*	
1	3.6	42.4	39.6	44.8	43.6	4.0	0.022	0.61
		34.8		40.4				
		34.0		48.8				
		47.2						
2	6.8	49.6	49.7	58.0	58.1	8.4	0.047	0.69
		43.2		56.8				
		49.6		52.8				
		54.4		62.0				
		52.8*		61.2				

* Incubated without co-factors.

TABLE V

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES IN PRESENCE OF CHLOROMYCETIN

Methods used, co-factors added and data expressed as shown in Table IV. Chloromycetin (15 μg) was added to each incubation. Values in table refer to total mucopolysaccharide-bound hexosamines per sample. At the end of the incubation, agar plates were inoculated with incubation mixtures. After 24 h insignificant bacterial growth (150 cells/ml). After 48 h 134 cells/ml were present.

Protein (mg)	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
					(μg)	*(μmoles)*	
2.1	183.2	166.1	209.2	224.0	57.9	0.323	15.4
	153.2						
	162.4		240.4				

system, for UTP, DPN and ATP, but not for glutamine, which is presumably already present, are also shown in Table I. As illustrated in Table II, the system is vitamin A-dependent, in that the value of hexosamines formed by colon homogenates from deficient rats dropped practically to zero.

As previously[4], the high zero-time value for MPS-bound hexosamines, due to the high MPS content of colon, was a cause for concern. In order to eliminate the uncertainty due to this circumstance, at least three and usually a larger number of determinations were carried out on separate aliquots of the same homogenate or particle suspension, before (zero-time value) and after incubation. Statistical tests showed that the increases in MPS-bound hexosamines were highly significant.

Cell fractionation revealed that, contrary to expectation, the MPS-synthesizing activity of the preparation resided in the fraction containing particles sedimenting at 20000 × *g*. (Table III) rather than in the microsome and cell-sap fraction (Table IV). That this result was not due to contamination of the particle suspension by whole cells was demonstrated by the absence of whole cells from the preparation after staining, under conditions when whole cells were easily seen in the fraction sedimenting at 1000 × *g*. Bacterial contamination was excluded by carrying out the incubation of the particles in presence of chloromycetin (Table V), when a similar level of MPS synthesis was achieved, but no bacterial growth was detectable after plating out the suspension on nutrient agar.

TABLE VI

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES: REQUIREMENTS

Methods used, co-factors added and data expressed as shown in Table III. Values in table refer to total mucopolysaccharide-bound hexosamines per sample. CS signifies "complete system"

Expt.	*Additions*	*Protein (mg)*	*Hexosamines zero-time (μg)*	*Averag (μg)*	*Hexosamines after incubation (μg)*	*Averag (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
							(μg)	*(μmoles)*	
1	CS	2.3	56.4 66.4 48.0	56.9	80.0 90.8 72.0 86.8	82.4	25.5	0.142	6.2
	$-MgCl_2$				62.4 51.2	56.9	0	0	0
2	CS	1.6	24.0 28.8 28.0	26.2	43.2 48.4	45.8	19.6	0.109	6.8
	$-MgCl_2$				32.0 32.0	32.0	5.8	0.032	2.0
3	CS	3.3	112.0 117.6 92.4	107.2	135.6 135.6 132.0	133.8	26.6	0.149	4.5
	—G-6-*P*				102.0 107.6 128.0	112.2	5.0	0.028	0.8

A study of the requirements of the particle system (Table VI) showed that at least Mg^{2+} and glucose 6-phosphate are needed. Fig. 1 illustrates the approximately linear relationship between protein incubated and MPS synthesized by the particle suspension. When comparing the activity of whole homogenate (Table I) and that of the particle suspension (Tables III, V and VI), it can be seen that, on a basis of the amount of protein incubated, a 2- to 4-fold purification of the system has been obtained.

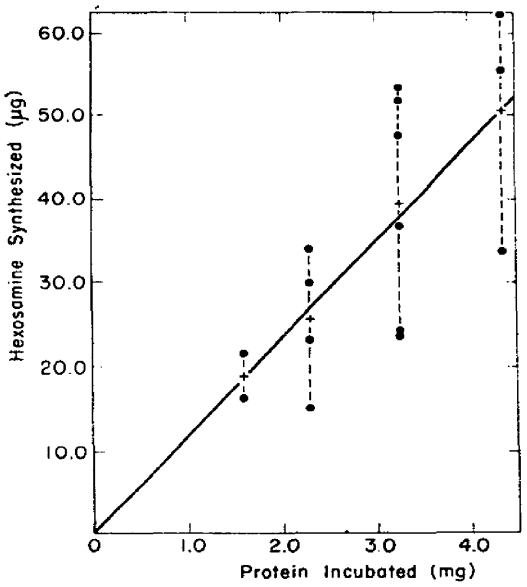

Fig. 1. Experiment showing the variation of MPS-bound hexosamines formed with protein incubated in the particle suspension under conditions described in Table III. ●, the hexosamines synthesized for each level of protein incubated; +, the averages of each incubation.

In previous work from this laboratory, it had been found that upon fractionation of pig-colon mucosa, an enzyme fraction could be obtained in the supernatant solution after centrifugation at 105000 × *g*, which catalyzed the incorporation of labeled sulfate and glucose into MPS, and was influenced by vitamin A (ref. 2). Our present results differ from this work, in that MPS synthesizing activity, measured by net increase of MPS-bound hexosamines, is found in the particle suspension sedimenting at 20000 × *g*. No explanation has been found for this difference beyond the fact that in the previous work the particle suspension was not tested by itself, and may have shown higher activity than the supernatant solution. There is, of course, the possibility of a species difference, since only the rat was used in the experiments presently reported whereas pig colon was used before. It is noteworthy that SCHILLER *et al.*[5] found a particle suspension sedimenting at 20000 × *g* from rat fetus skin to effect hyaluronic acid synthesis.

A further difficulty is due to the fact that most of the enzymes which synthesize the precursors of MPS are known to be located in the cell-sap, *e.g.*, the UDP-glucuronic acid forming enzyme[6] and the sulfate activating enzyme[7] (sulfurylase). It is possible that these enzymes also exist in the MPS-synthesizing particles of colon,

TABLE VII

MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES WITH URIDINE NUCLEOTIDES

Methods used and data expressed as in Table IV. Co-factors added to each incubation: UDP-acetylglucosamine, 1 μmole; UDP-glucuronic acid, 1 μmole; acetylglucosamine, 1 μmole; ATP, 2.5 μmoles; DPN, 2.5 μmoles; UTP, 1 μmole; $MgCl_2$, 10 μmoles; Na_2SO_4, 1 μmole. Values in table refer to total mucopolysaccharide-bound hexosamines per sample. CS signifies "complete system".

Expt.	Additions	Protein (mg)	Hexosamines zero-time (μg)	Average (μg)	Hexosamines after incubation (μg)	Average (μg)	Average hexosamines formed (μg)	Average hexosamines formed (μmoles)	Hexosamines formed per 100 mg protein (μmoles)
1	CS	2.0	65.6 68.4 69.2	67.6	78.4 72.0 76.8 82.0	77.5	9.9	0.055	2.5
2	CS	1.0	26.6 26.0 22.8	24.4	32.0 30.0 30.0 33.2	31.7	7.3	0.041	4.1
	$-Na_2SO_4$				31.2 31.2 27.6	29.4	5.0	0.028	2.8

TABLE VIII

THE INFLUENCE OF VITAMIN A ON MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES

Methods used, co-factors added and data expressed as shown in Table III. Colons were from vitamin A-deficient rats. Vitamin A added ($2.5 \cdot 10^{-3}$ μmoles) in suspension in bovine serum albumin. Values in table refer to total mucopolysaccharide-bound hexosamines per sample. CS signifies "complete system".

Expt.	Additions	Protein (mg)	Hexosamines zero-time (μg)	Average (μg)	Hexosamines after incubation (μg)	Average (μg)	Average hexosamines formed (μg)	Average hexosamines formed (μmoles)	Hexosamines formed per 100 mg protein (μmoles)
1	CS	1.0	70.8 65.6	68.3	61.6 69.6	65.6	0	0	0
	+Vitamin A				75.6 75.6 75.6 70.8	74.3	6.0	0.033	3.3
2	CS	1.6	61.2		62.8 59.2 73.2	65.1	3.9	0.022	1.4
	+Vitamin A				62.0 68.0 57.6 61.2	62.3	1.1	0.006	0.4

a possibility currently being investigated in this laboratory. Preliminary data indicate that particles sedimenting at 20000 × *g* as well as the supernatant solution contain sulfurylase activity (unpublished observations by the authors). It is of interest to note that the particle suspension does not yield a greater amount of MPS in presence

TABLE IX

THE INFLUENCE OF A VITAMIN A ACID DERIVATIVE ON MUCOPOLYSACCHARIDE SYNTHESIS BY COLON PARTICLES

Methods used, co-factors added and data expressed as shown in Table III. Colons were from vitamin A-deficient rats. "Cpd. III" represents the metabolite of vitamin A acid referred to in the text. CS signifies "complete system". Values in table refer to total mucopolysaccharide-bound hexosamines per sample.

Expt.	*Additions*	*Protein (mg)*	*Hexosamines zero-time (μg)*	*Average (μg)*	*Hexosamines after incubation (μg)*	*Average (μg)*	*Average hexosamines formed*		*Hexosamines formed per 100 mg protein (μmoles)*
							(μg)	*(μmoles)*	
1	CS	1.4	49.6 62.0 54.0	55.1	62.8 54.0	58.3	3.2	0.018	1.3
	+Cpd. III (8 μg)				80.0 68 4 64.0 67.2	69.4	14.3	0.079	5.7
2	CS	0.94	70.4 80 4	75.4	72.8 81.2 76.0	76.6	1.2	0.007	0.7
	+Cpd. III (8 μg)				100.0 90.4	95.2	19.8	0.109	11.6
	+Cpd. III (16 μg)				95.6 80.4 83.2 84.4	85.9	10.5	0.058	6.2

of UDP-actylglucosamine and UDP-glucuronic acid than merely glucose 6-phosphate, as shown in Table VII. It is likely that colon MPS contains moieties other than those directly derived from UDP-glucuronic acid and UDP-acetylglucosamine, such as sialic acid and fucose. Therefore, the system would be expected to be less efficient in incorporating the sialic acid and fucose moieties of MPS when supplied with UDP-glucuronic acid and acetylglucosamine, than with glucose 6-phosphate.

Particles from vitamin A-deficient rats showed much reduced MPS synthesis. With whole homogenate[4], it has been possible to restore this activity to normal, or even above, by adding vitamin A. With the particle suspensions presently used, restoration of activity was low or absent (Table VIII). This suggests that the vitamin itself acts not as such but is first changed into an "active form", a change effected by another cell-fraction than the particles. That this is actually the case is demonstrated in Table IX. The active metabolite was obtained by injecting vitamin A-deficient rats with labeled vitamin A acid, and extracting intestinal lipids with ether. Labeled acidic material was re-extracted from the ether solution with bicarbonate as described. Upon incubation, this acidic lipid material free from vitamin A acid but derived from it, completely reactivated deficient particle suspensions to normal (Table IX). Further work regarding the nature of this active metabolite is in progress.

In conclusion, as can be seen from the summary Table X, it has been found that the MPS-synthesizing ability of colon homogenates resides in a particle preparation sedimenting at 20000 × *g*, and that this preparation is strongly dependent on the vitamin A status of the animal, though not on vitamin A itself, but probably a metabolite of it.

TABLE X

MUCOPOLYSACCHARIDE SYNTHESIS BY CELL FRACTIONS FROM RAT COLON

Cell fraction	*Mucopolysaccharide-bound hexosamines formed per 100 mg protein incubated for 3 h at 37° (μmoles)*
Whole homogenate (normal)	2.84; 2.14; 2.77
Whole homogenate (deficient)	0.27
Microsomes + cell-sap (normal)	0.61; 0.60
Particles (normal)	10.0; 6.2; 6.8; 4.5
Particles (deficient)	1.4; 1.3; 0.7; 0
Particles (deficient + vitamin A)	3.3; 0.4
Particles (deficient + vitamin A acid metabolite)	5.7; 11.6; 6.2

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