Vitamin A and the Biosynthesis of Sulphated Mucopolysaccharides

EXPERIMENTS WITH RATS AND CULTURED NEOPLASTIC MAST CELLS

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1. The uptake and incorporation of [^{35}S]sulphate into mucopolysaccharides by colon and duodenum *in vitro* are unaffected by the vitamin A status of the animals.
2. Uptake and incorporation *in vivo* are unaffected at 4 hr. after injection of [^{35}S]sulphate, but at later times are decreased in some tissues of vitamin A-deficient animals.
3. The rate of removal of ^{35}S from blood, its rate of appearance in urine, the plasma concentration of sulphate and the uronic acid content of several tissues are not significantly altered in vitamin A deficiency.
4. These results, and direct measurement of ^{35}S in mucopolysaccharides at various times after injection of [^{35}S]sulphate, suggest that the synthesis of mucopolysaccharides is unaffected but that their turnover is increased in vitamin A deficiency.
5. Neither the growth rate of, nor the incorporation of [^{35}S]sulphate into heparin by, P 815 Y and HC cultured neoplastic mast cells is decreased when the horse serum necessary for growth is treated with ultraviolet light or is replaced by serum from vitamin A-deficient rats.
6. The addition of citral is no more toxic to growth rate or to incorporation of ^{35}S than is the addition of vitamin A itself.
7. It is concluded that neoplastic mast cells in culture do not require vitamin A for growth or for the synthesis of heparin.
8. None of these results is compatible with the view that vitamin A or a derivative is directly involved in the biosynthesis of sulphated mucopolysaccharides.

Wolf & Johnson (1960) suggested that one of the effects of vitamin A deficiency is a decreased synthesis of sulphated mucopolysaccharides. The site of action was said to be the activation of sulphate by ATP sulphurylase (EC 2.7.7.4) (Varadani, Wolf & Johnson, 1960; Subba Rao & Ganguly, 1964; Carroll & Spencer, 1965; Hall & Straatsma, 1966; Sundaresan, 1966), but others (Pasternak, Humphries & Pirie, 1963; Pasternak & Pirie, 1964; Mukherji & Bachhawat, 1967) have been unable to confirm this. Part of the discrepancy may be due to the difficulty of producing deficient and control rats of equal weight, since a symptom of vitamin A deficiency is loss of appetite (Moore, 1957, p. 296). By rearing control rats on retinoic acid, which satisfies most of the animal's requirements for vitamin A (Dowling & Wald, 1960), it is easier to obtain rats of closely corresponding weight and food intake. Using such animals we have been unable to detect any decrease in the synthesis of sulphated mucopolysaccharides and have been encouraged by the report (Levi, Geller, Root & Wolf, 1968) that ATP sulphurylase itself is actually more sensitive to altered protein intake than to vitamin A status.

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Mouse mast-cell tumours contain heparin and continue to synthesize it in cell culture (Green & Day, 1960; Thomas & Pasternak, 1967; Thomas, 1968), remaining biochemically as well as genetically (Green & Day, 1960) differentiated. Such cells seem ideal for studying the relation between vitamin A and the synthesis of sulphated mucopolysaccharides. However, the need of cultured cells for any fat-soluble vitamin has proved difficult to investigate since traces of vitamin are normally present in the serum added to achieve good growth. We have overcome this problem by growing P 815 Y and HC mast cells in horse serum treated with ultraviolet light to destroy vitamin A, and in the serum of rats made deficient in vitamin A. We failed to demonstrate any effect of vitamin A on the growth or metabolism of such cells.

These results have been communicated briefly (Pasternak, 1969).

METHODS

Diet. Rice starch (British Drug Houses Ltd., Poole, Dorset) (400 g.), 200 g. of sucrose, 200 g. of casein [Fisons Pharmaceuticals Ltd., Loughborough, Leicestershire; low vitamin content, washed twice with ethanol-ether (3:1, v/v) and once with ether, and dried at room temperature], 50 g. of salt mixture (containing 104 g. of NaCl, 166 g. of MgSO_4 ,

204g. of Na_2HPO_4 , 272g. of KH_2PO_4 , 324g. of CaHPO_4 , 70g. of ferric citrate pentahydrate, 780g. of calcium lactate pentahydrate, 2g. of KI, 2g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 5g. of MnCl_2 , 150ml. of arachis oil (British Drug Houses Ltd.), water-soluble vitamins (400mg. of choline chloride, 100mg. of *meso*-inositol, 50mg. of *p*-aminobenzoic acid, 48mg. of calcium pantothenate, 20mg. of nicotinamide, 8mg. of riboflavin, 8mg. of pyridoxine chloride, 4mg. of thiamine and 0.088mg. of biotin), fat-soluble vitamins [40mg. of *DL*- α -tocopherol, 0.3mg. of calciferol and 1.0mg. of retinoic acid (Eastman-Kodak Co., Rochester, N.Y., U.S.A.) where indicated, in glycerol tributyrate] and water (300–400ml.) were thoroughly mixed to a paste and stored at 3° until required.

Rats. Male Wistar rats (approx. 3 weeks old) were transferred at weaning to the above diet containing retinoic acid. After 2–3 months the weight of the rats, which appeared perfectly healthy, began to reach a plateau (200–300g.). Retinoic acid was then withheld from half the rats (deficient group). Within a week these began to lose appetite and their weight dropped. Xerophthalmia and associated eye infections set in and some rats developed diarrhoea. By 3 weeks several had died. During this period the food intake of the other half of the rats (control group) was restricted by pair-feeding to that of the deficient group. Extra retinoic acid (50 μg . in 0.4ml. of glycerol tributyrate/rat/day) was given as the food intake dropped below 5g./day. The control rats lost weight but remained otherwise healthy. At about 3 weeks, when the animals were used, deficient and control rats were of equivalent weight.

Uptake of [^{35}S]sulphate in vitro. Groups of rats were stunned and killed by decapitation. The intestine was removed, rinsed with ice-cold 0.9% NaCl and placed on ice. Duplicate contiguous segments of colon and duodenum, each 4cm. long, were everted with a plastic cannula and ligated distal to the cannula. The segments were half-filled with warm Krebs improved Ringer III buffer (Dawson & Elliott, 1959), which was introduced through the plastic cannula with a syringe. The segments were ligated at the proximal end, removed from the cannula and placed in a conical flask at 37° containing 9ml. of warm Krebs–Ringer buffer which was gassed with $\text{O}_2 + \text{CO}_2$ (95:5). After they had been shaken for 5 min., 10 μC of $\text{Na}_2^{35}\text{SO}_4$ in 1ml. of Krebs–Ringer buffer was added, and shaking was continued for a further 30 min. The everted sacs were removed, cut open, rinsed in ice-cold Krebs–Ringer buffer, blotted and dried at 110° to constant weight (6–8 hr.). Total and incorporated ^{35}S were measured by the rivanol method described below.

Uptake of [^{35}S]sulphate in vivo. Rats were lightly anaesthetized with ether and injected intraperitoneally with $\text{Na}_2^{35}\text{SO}_4$ (1mc/ml.; 2–10 μC /g. body wt.). After 5 hr. they were injected with unlabelled Na_2SO_4 (0.5M; 1ml./200g. body wt.). Rats were killed as above at various times after injection. Blood was collected in small beakers previously rinsed with heparin solution and samples (0.01 and 0.02ml.) were plated for radioactivity assay. Tissues were removed, rinsed in tap water, separated from adhering fat, blotted and either heated at 110°, as above, followed by rivanol fractionation (see below), or weighed and homogenized (Silverson homogenizer) in 5ml. of cold 5% (w/v) trichloroacetic acid and treated as described below.

Extraction of tissues. (a) Rivanol method (Whitehouse & Boström, 1961). Dried tissues (10–40mg.) were digested in

0.1M-sodium acetate buffer, pH 5.6 (1–4ml.), containing papain (2mg./ml.; previously activated by heating it in 5mM-cysteine for 45 min. at 60°), cysteine (5mM) and EDTA (5mM), at 60° for 16 hr. After boiling and centrifuging, the residue was redigested for a further 16 hr. Alternatively, dried tissues (10–40mg.) were digested in 0.1M-sodium phosphate buffer, pH 7.5 (1–4ml.), containing Nagarse (Teikoku Chemical Industry Co. Ltd., Osaka, Japan; 0.1mg./ml.), benzylpenicillin (Glaxo Laboratories Ltd., Greenford, Middx.; 200i.u./ml.) and streptomycin sulphate (Glaxo Laboratories Ltd.; 50 μg /ml.) at 36° for 24 hr. Samples of the supernatant obtained after boiling and centrifuging were plated for radioactivity assay for total ^{35}S . The percentage of total ^{35}S that was in [^{35}S]mucopolysaccharide was found to be very reproducible for samples of any tissue at a given time (see also Tables 1, 2 and 3), and this allows one to equate total ^{35}S with ^{35}S actually taken up by the tissue. The pH of the supernatants was adjusted to 1.5 with 1N-HCl, they were then centrifuged, and an excess of 3% (w/v) rivanol (6,9-diamino-2-ethoxyacridine; Bayer Products Ltd., Surbiton-on-Thames, Surrey) was added to the supernatant. Precipitated sulphated mucopolysaccharides were removed by centrifugation after being kept at 3° overnight, and samples of the supernatant were plated for radioactive assay for soluble ^{35}S . No further precipitate was obtained on addition of rivanol. The difference between total ^{35}S and soluble ^{35}S was taken as a measure of [^{35}S]mucopolysaccharide. This was confirmed by dissolving the precipitate in 2M-ammonium formate and passing it down Sephadex G-50. All the radioactivity was eluted as a single peak immediately after the void volume. By passing the soluble ^{35}S fraction down Sephadex G-25, it was shown to contain a mixture of inorganic [^{35}S]sulphate and some higher-molecular-weight substances containing ^{35}S .

(b) Trichloroacetic acid method. The trichloroacetic acid extract (total ^{35}S) was centrifuged, and the precipitate was extracted with ether and dissolved in 5ml. of 0.5N-NaOH by shaking at 37° overnight. Samples were removed for radioactive assay for [^{35}S]mucopolysaccharide.

Both methods gave essentially similar results. In some experiments animals were placed in metabolism cages and the urine was collected over 24 hr. Total ^{35}S was measured and inorganic [^{35}S]sulphate precipitated with BaCl_2 (Folin, 1905). The residual radioactivity was taken as a measure of ester [^{35}S]sulphate.

Cell culture. P815Y and HC (a line rich in heparin-containing granules, kindly given by Dr G. A. Fischer) cells were grown as described by Wheldrake & Pasternak (1968). Incorporated ^{35}S (sulphate) or ^{14}C (glucosamine) was measured after samples had been filtered through Oxoid membranes (0.45 μ pore size) and washed with medium containing an excess of unlabelled substrate. Retinal (Distillation Products Industries, Rochester, N.Y., U.S.A.), retinol acetate (Roche Products Ltd., London, W. 1), citral (British Drug Houses Ltd.), geraniol and citronellal (Fluka A.-G., Buchs, Switzerland), citronellol (British Drug Houses Ltd.), dihydrocitronellal (kindly given by Boake, Roberts and Co. Ltd., London, E. 15), β -ionone (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.) and crotonaldehyde (British Drug Houses Ltd.) were dissolved in redistilled ethanol (which did not affect cell growth at final concentrations below 0.2%).

Treatment with ultraviolet light. Samples (20ml. in sterile

Petri dishes placed on top of crushed ice) of horse serum were irradiated for 8 hr. at a distance of 6 in. from an ultraviolet lamp housed in a sterile chamber. The vitamin A content at the end of irradiation was difficult to detect since over 50% had been destroyed.

Determination of vitamin A. Dr A. T. Diplock kindly carried out the determination of vitamin A by the method of Moore (1957, p. 586).

Rat serum. Groups of rats were reared on Oxoid pasteurized breeding diet (normal) or the defined diet described above with or without retinoic acid (control and deficient). Rats were killed when they had reached a growth plateau; blood was collected and allowed to clot. It was centrifuged and the clear supernatant was sterilized by filtration through Ford grade SB Sterimats.

Other methods. Radioactive solutions (0.01–0.1 ml.) were either evaporated on aluminium planchets and assayed with a low-background automatic sample-changing counter (Isotope Developments Ltd., Beenham, Berks.; efficiency, 5%; negligible self-absorption) or were added to 7 ml. of a solution containing either 8 g. of 2-(4-*tert.*-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole [Ciba (A.R.L.) Ltd., Duxford, Cambs.] and 80 g. of naphthalene in 1 l. of dioxan or 10 g. of 2,5-diphenyloxazole and 0.5 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene [both from Nuclear Enterprises (G.B.) Ltd., Edinburgh] in 1 l. of toluene-methylcellosolve

(1:1, v/v), and assayed in a Beckman liquid-scintillation spectrometer (efficiency, 87%). Radioactive filters were assayed with an end-window probe fitted to a Panax D 657 scaler (efficiency, 1.3%). Uronic acid (Dische, 1947) and plasma sulphate (Dodgson & Spencer, 1953) were measured by standard techniques.

RESULTS AND DISCUSSION

Experiments with rats. Table 1 shows that the uptake of [^{35}S]sulphate and its incorporation into mucopolysaccharides by everted sacs of colon and duodenum are not decreased in vitamin A deficiency. Incorporation by several tissues (colon, ileum, lung and kidney) *in vivo* was (despite large S.E.M. values) less in deficient animals when measured 24 hr. after injection of the label (Table 2), but at 4 hr. (Table 3) this was not so; in fact, incorporation by colon was actually greater, as found *in vitro* (Table 1). This suggests that increased turnover, as opposed to decreased synthesis, may be a symptom of deficiency. The results of Table 4 confirm this view, though the differences in turnover rate were not large. Indeed, the closer the correspondence

Table 1. *Uptake and incorporation of [^{35}S]sulphate by rat tissues in vitro*

Duplicate everted intestinal sacs were incubated for 30 min. and analysed by the rivanol technique, as described in the Methods section. Values are means $\pm$ S.E.M. from ten separate experiments.

	[^{35}S]Mucopolysaccharides			
	Control		Vitamin A-deficient	
	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)
Colon	45 $\pm$ 14	35 $\pm$ 10	64 $\pm$ 21	36 $\pm$ 7
Duodenum	74 $\pm$ 36	35 $\pm$ 7	90 $\pm$ 26	35 $\pm$ 5

Table 2. *Uptake and incorporation of [^{35}S]sulphate in vivo by the tissues of rats killed 24 hr. after injection of the label*

Groups of rats (numbers in parentheses) received $10\mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4/\text{g. wt.}$ 24 hr. before being killed. Tissues were analysed by the rivanol technique as described in the Methods section. Values are means $\pm$ S.E.M.

	[^{35}S]Mucopolysaccharides			
	Control		Vitamin A-deficient	
	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)
Colon	3380 $\pm$ 380 (7)	73 $\pm$ 5	1610 $\pm$ 190 (7)	71 $\pm$ 6
Ileum	2160 $\pm$ 1430 (7)	62 $\pm$ 14	1050 $\pm$ 520 (8)	54 $\pm$ 16
Duodenum	1330 $\pm$ 990 (5)	57 $\pm$ 10	1430 $\pm$ 880 (6)	58 $\pm$ 11
Liver	350 $\pm$ 120 (5)	44 $\pm$ 11	230 $\pm$ 130 (5)	54 $\pm$ 13
Lung	430 $\pm$ 320 (6)	36 $\pm$ 8	220 $\pm$ 160 (7)	33 $\pm$ 14
Xiphisternal cartilage	470 $\pm$ 100 (4)	71 $\pm$ 7	470 $\pm$ 170 (5)	73 $\pm$ 8
Kidney	590 $\pm$ 190 (4)	46 $\pm$ 9	350 $\pm$ 140 (5)	49 $\pm$ 11

Table 3. Uptake and incorporation of [^{35}S]sulphate in vivo by the tissues of rats killed 4 hr. after the injection of label

Groups of rats (numbers in parentheses) received $2\mu\text{C}$ of $\text{Na}_2^{35}\text{SO}_4/\text{g. wt.}$ 4 hr. before being killed. Tissues were analysed by the rivanol technique as described in the Methods section. Values are means $\pm$ s.e.m.

	[^{35}S]Mucopolysaccharides			
	Control		Vitamin A-deficient	
	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)	(counts/min./mg. dry wt.)	(% of total ^{35}S in tissue)
Colon	850 ± 220 (5)	48 ± 5	1400 ± 410 (4)	53 ± 4
Duodenum	630 ± 350 (5)	24 ± 3	660 ± 310 (4)	27 ± 5
Lung	130 ± 50 (5)	17 ± 3	140 ± 70 (4)	12 ± 7
Kidney	200 ± 50 (5)	18 ± 2	190 ± 60 (4)	20 ± 5

Table 4. Turnover of intestinal [^{35}S]mucopolysaccharides

Rats (two to five per group) were killed 4, 24, 48, 72 and 98 hr. after intraperitoneal injection of [^{35}S]sulphate and the incorporated radioactivity was measured by the trichloroacetic acid technique as described in the Methods section. The results were plotted on semi-log paper, and half-lives ($t_{\frac{1}{2}}$) were read off. Values of $t_{\frac{1}{2}}$ are means $\pm$ s.e.m. of three separate series of experiments.

	$t_{\frac{1}{2}}$ (hr.)	
	Control	Vitamin A-deficient
Colon	29 ± 0.3	25 ± 0.7
Ileum	24 ± 0.9	22 ± 1.2
Duodenum	23 ± 0.5	21 ± 1.2

in weight and food intake between control and deficient rats, the less the difference appears to be. This agrees with the observation (Levi *et al.* 1968) that protein consumption affects the synthesis of ATP sulphurylase more than does the vitamin A status of rats.

The fact that the proportion of the total label within the tissue that is incorporated into mucopolysaccharide is the same in deficient and control animals (Tables 1, 2 and 3) raises the possibility that the lowered incorporation of label (Table 2) is due to an increase in the concentration of endogenous sulphate, the actual rate of synthesis being unaltered. Though increased plasma sulphate concentrations in vitamin A deficiency were reported by Dziewiatkowski (1954), we could detect no significant difference between control ($109 \pm 22\mu\text{g.}$ of sulphate/ml.; mean $\pm$ s.e.m. of ten rats) and deficient ($115 \pm 19\mu\text{g.}$ of sulphate/ml.; mean $\pm$ s.e.m. of ten rats) animals. Nor was the total uronic acid content of ileum, duodenum or kidney different. The rate of removal of ^{35}S from blood

was also the same (initial $t_{\frac{1}{2}}$ approx. 6 hr., followed by a lower rate, $t_{\frac{1}{2}}$ approx. 35 hr.), as was the rate of appearance of ^{35}S in urine, 8% of which was present as ester sulphate in control and deficient rats; this is in contrast with earlier reports (Balakhovskij & Kuznetsova, 1958; Subba Rao, Seshadri Sastry & Ganguly, 1963) of altered urinary excretion of ^{35}S in vitamin A deficiency. Histological examination of intestinal tissues and kidney, as well as radioautography of mesenteric mast cells, revealed no significant differences in the mucopolysaccharide material of control and deficient animals. These results are consistent with the hypothesis that it is the turnover and not the content of mucopolysaccharide that is affected in vitamin A deficiency. The reports of Mukherji & Bachhawat (1965) and of Perumal, Ananthasamy, Lakshmanan, Jungalwala & Rama Rao (1966) of marked loss of [^{35}S]sulphate incorporation by various tissues of deficient rats at 24 and 48 hr. respectively, accompanying smaller variations in mucopolysaccharide content, may be interpreted in the same light. Increased turnover may also account for reports such as that of Frape, Allen, Speer, Hays & Carton (1959) of greater [^{35}S]sulphate incorporation by deficient animals at relatively early times after injection of label.

Our results suggest that, whatever alterations in sulphate metabolism may occur, they are slight and probably of secondary importance. The increased turnover of colonic mucopolysaccharide (Table 4), for example, may itself be a consequence of stimulated lysosomal activity (Dingle, Sharman & Moore, 1966) and may reflect a general alteration in the behaviour of epithelial cells.

Experiments with cultured mast-cell tumours. Although the vitamin A content of the horse serum added to the growth medium was found to be remarkably low (approx. $18\mu\text{g.}$ of retinol/100 ml. of serum), it was thought desirable to decrease it even further by ultraviolet-light treatment. This had

Table 5. *Effect of serum variations on growth and [³⁵S]sulphate incorporation by cultured neoplastic mast cells*

P815Y cells were grown for three generations in the presence of Na₂³⁵SO₄ (20 µC/ml.) and serum as indicated. HC cells were grown for four to five generations in the presence of serum as indicated and Na₂³⁵SO₄ (2 µC/ml.) was then added for 3 hr. Incorporated ³⁵S was measured by filtration as described in the Methods section. All values are means of two or more experiments.

Cell line	Serum	Division time (hr.)	Incorporation of ³⁵ S (counts/min./10 ³ cells)
P815Y	Horse	11	3.4
P815Y	Horse, u.v.-light-treated	12	2.9
HC	Horse	17	1.2
HC	Horse, u.v.-light-treated	15	1.2
P815Y	Rat, normal	20	3.2
P815Y	Rat, control (retinoic acid)	16	4.1
P815Y	Rat, vitamin A-deficient	17	3.8

Table 6. *Toxicity of vitamin A (retinol) and its derivatives to P815Y cells*

Cells were grown for three to four generations in the presence of the compounds listed at various concentrations, and the '50% toxicity' was determined graphically.

	Concn. required to inhibit cell growth by 50% (µM)
Retinal	8
Retinol	63
Citral	21
Geraniol	89
Citronellal	15
Citronellol	98
Dihydrocitronellal	13
β-Ionone	14
Crotonaldehyde	4

no effect on growth rate or incorporation of ³⁵S by P815Y or HC cells (Table 5). Replacement of horse serum by rat serum resulted in a somewhat lower growth rate, but there was no specific effect of vitamin A status (Table 5). Essentially similar results were obtained with HC cells. In no case is it likely that growth or incorporation of ³⁵S is due to traces of intact horse serum present in the inoculum, since cells were passaged for three generations or more through the appropriate serum, and omission of serum prevented growth or incorporation of [³⁵S]sulphate.

Citral has been implicated as a specific antagonist of vitamin A in cultured chick epithelial cells (Ayedelotte, 1963a,b), and accordingly its action on P815Y mast-cell tumours was studied. It proved to be equally toxic to growth and to [³⁵S]sulphate or [¹⁴C]glucosamine (Thomas, 1967) incorporation. The inhibition could not be prevented by simultaneous or prior addition of vitamin A or its aldehyde. Indeed retinal was more toxic than citral itself (Table 6). Results with other derivatives showed that toxicity was not specific to vitamin A, but was attributable chiefly to the presence of an aldehyde group attached to an unsaturated carbon chain, crotonaldehyde being the most toxic compound tested (Table 6).

One may conclude that vitamin A is required neither for the growth of mammalian mast-cell tumours in culture nor for their synthesis of heparin. This substantiates an earlier view (Paul, 1960) that fat-soluble vitamins are not required for vertebrate cell growth *in vitro*. Concurrently with this work J. T. Dingle (personal communication) has found that [¹⁴C]glucosamine incorporation by cultured fibroblasts is similarly insensitive to lack of vitamin A.

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