- Chatagnon, C. & Chatagnon, P. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1319.
- Dain, J. A., Weicker, H., Schmidt, G. & Thannhauser, S. J. (1962a). In *Cerebral Sphingolipidoses*, p. 289. Ed. by Aronson, S. M. & Volk, B. W. New York: Academic Press Inc.
- Dain, J. A., Willis, R., Sweet, E. I. & Schmidt, G. (1962b). *Fed. Proc.* **21**, 282.
- Folch, J., Arsove, S. & Meath, J. A. (1951a). *J. biol. Chem.* **191**, 819.
- Folch, J., Ascoli, I., Lees, M., Meath, J. A. & LeBaron, F. N. (1951b). *J. biol. Chem.* **191**, 833.
- Folch, J. & Lees, M. (1959). *Amer. J. Dis. Child.* **97**, 730.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Folch, J., Meath, J. A. & Bogoch, S. (1956). *Fed. Proc.* **15**, 254.
- Jatzkewitz, H. (1961). *Hoppe-Seyl. Z.* **326**, 61.
- Jatzkewitz, H. & Mehl, E. (1960). *Hoppe-Seyl. Z.* **320**, 251.
- Klenk, E. (1942). *Hoppe-Seyl. Z.* **273**, 76.
- Klenk, E. (1951). *Hoppe-Seyl. Z.* **288**, 216.
- Klenk, E. & Gielen, W. (1960). *Hoppe-Seyl. Z.* **319**, 283.
- Klenk, E. & Gielen, W. (1961a). *Hoppe-Seyl. Z.* **323**, 126.
- Klenk, E. & Gielen, W. (1961b). *Hoppe-Seyl. Z.* **326**, 144.
- Klenk, E. & Gielen, W. (1961c). *Hoppe-Seyl. Z.* **326**, 161.
- Kuhn, R. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **1**, 67.
- Kuhn, R. (1959). *Naturwissenschaften*, **46**, 43.
- Kuhn, R., Egge, H., Brossmer, R., Gauhe, A., Klesse, P., Lochinger, W., Röhm, E., Trischman, H. & Tschampel, D. (1960). *Angew. Chem.* **72**, 805.
- Kuhn, R., Weigandt, H. & Egge, H. (1961). *Angew. Chem.* **73**, 580.
- Long, C. & Staples, D. A. (1959). *Biochem. J.* **73**, 385.
- Mangold, H. K. (1961). *J. Amer. Oil Chem. Soc.* **38**, 708.
- Meltzer, H. L. (1958). *J. biol. Chem.* **233**, 327.
- Müldner, H., Wherrett, J. R. & Cumings, J. N. (1962). *J. Neurochem.* **9**, 607.
- Payne, S. N. & Platt, B. S. (1961). *J. Neurochem.* **7**, 5.
- Rosenberg, A. & Chargaff, E. (1958). *J. biol. Chem.* **232**, 1031.
- Rouser, G., Bauman, A. J., Kritchevsky, G., Heller, D. & O'Brien, J. S. (1961). *J. Amer. Oil Chem. Soc.* **38**, 544.
- Stahl, E. (1956). *Pharmazie*, **11**, 633.
- Stahl, E. (1958a). *Chemikerztg.* **82**, 323.
- Stahl, E. (1958b). *Parfüm. u. Kosmet.* **39**, 564.
- Svennerholm, L. (1956a). *Nature, Lond.*, **177**, 524.
- Svennerholm, L. (1956b). *Acta chem. scand.* **10**, 694.
- Svennerholm, L. (1957a). *Biochim. biophys. Acta*, **24**, 604.
- Svennerholm, L. (1957b). *Acta Soc. Med. upsalien.* **62**, 1.
- Svennerholm, L. & Raal, A. (1961). *Biochim. biophys. Acta*, **53**, 422.
- Trams, E. G. & Lauter, C. J. (1962). *Biochim. biophys. Acta*, **60**, 350.
- van Heyningen, W. E. & Miller, P. (1961). *J. gen. Microbiol.* **24**, 107.
- Wagner, H., Hörhammer, L. & Wolfe, P. (1961). *Biochem. Z.* **334**, 175.
- Weicker, H., Dain, J., Schmidt, G. & Thannhauser, S. J. (1960). *Fed. Proc.* **19**, 219.
- Wherrett, J. R. & McIlwain, H. (1962). *Biochem. J.* **84**, 232.

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The Activation of Sulphate by Extracts of Cornea and Colonic Mucosa from Normal and Vitamin A-Deficient Animals

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(Received 12 September 1962)

Mammalian cornea can incorporate [^{35}S]sulphate into mucopolysaccharide *in vivo* (Odeblad & Boström, 1952; Dohlman, 1957) and *in vitro* (Aurell, Dohlman & Rodén, 1956; Wortman & Strominger, 1957; Wortman, 1960, 1961), and therefore presumably contains the enzymes adenosine triphosphate-sulphate adenylyltransferase (EC 2.7.7.4) and adenosine triphosphate-adenylylsulphate 3'-phosphotransferase (EC 2.7.1.25), which catalyse the activation of inorganic sulphate by ATP (Robbins & Lipmann, 1956) to form adenosine 3'-phosphate 5'-sulphatophosphate ('active sulphate'). We have now demonstrated the activation of sulphate in extracts of calf, rabbit, guinea-pig and rat corneal epithelium and stroma.

An early sign of vitamin A deficiency is keratinization of the corneal epithelium followed by liquefaction of the corneal stroma; when Varandani, Wolf & Johnson (1960) reported that the activation of sulphate by colonic mucosa was decreased in rats made deficient in vitamin A and that activity could be restored by addition of vitamin A *in vitro*, we examined corneal extracts for a similar effect. We have been unable to detect any significant decrease, as a result of vitamin A-deficiency, in the activation of sulphate by extracts of rat cornea, rat colonic mucosa, rabbit corneal epithelium or rabbit colonic mucosa. The activation of sulphate by rabbit corneal stroma was possibly decreased, but could not be restored by the addition of vitamin A *in vitro*.

MATERIALS AND METHODS

Calf. The eyes were obtained from the slaughterhouse within 1–2 hr. of death.

Guinea pig. The animals were normal laboratory stock killed by the intraperitoneal injection of Nembutal.

Rat. Weanling albino Wistar rats were given a diet containing: starch, 63%; casein (fat- and vitamin-free), 21%; salt mixture (British Drug Houses Ltd.), 5.5%; hardened vegetable oil, 10.5%. To 1 kg. of diet 250 g. of dried yeast was added to supply vitamins other than vitamin A. The experimental procedure was the same as that of Varandani *et al.* (1960). All rats were maintained on the diet for approx. 4 weeks, at which time corneal changes were beginning. Half of the rats were then given a single dose of 6500 i.u. of vitamin A per rat and all were killed 3 days later. At this time the liver vitamin A of the deficient rats was too low to estimate, whereas that of the control rats was 530–660 i.u./g. The corneas from three or more rats were combined.

Rabbit. Litters of Dutch rabbits were started on a diet of crushed oats plus bran (6%), CaCO_3 (1.5%) and water *ad libitum* (Mann, Pirie, Tansley & Wood, 1946) at 3–5 weeks of age with the doe. After weaning at 6 weeks the litter was divided into two groups, one of which continued on the deficient diet while the other was given this diet plus 1000 i.u. of vitamin A in Haliverol by mouth twice weekly. All rabbits received 1 drop of wheat-germ oil by mouth twice weekly to supply vitamin E. Those on the deficient diet failed to grow well and showed lesions of the corneal epithelium and almost complete loss of vitamin A from the liver in 3–6 months.

The eye lesions in both rats and rabbits were assessed by slit-lamp examination and weights were checked weekly.

The rabbits were killed when keratinization of the central cornea was easily visible to the naked eye, but there was some variation between them. At this time vitamin A content of liver had fallen to between 2 and 10 i.u./g. in comparison with 100–150 i.u./g. in the control litter mates.

Liver vitamin A was estimated by the method of Moore (1957).

Preparation of tissue extracts. The eyes were removed and in most cases the corneal epithelium was scraped off with a sharp scalpel. Pieces of conjunctiva, the corneal stroma and the retina were then cut from the eye and suspended in ice-cold 0.20 M-sucrose–0.01 M-cysteine–0.01 M-EDTA buffer brought to pH 7.5 with NaOH. The buffer:tissue ratio used was about 5:1 (v/w) for stroma and about 40:1 (v/w) for epithelium. Pieces from two or more eyes were pooled in every case. Mucosal scrapings (Kent & Pasternak, 1958) of rat and rabbit colon previously washed free of faecal matter were suspended in 4–5 vol. of the same buffer. All tissues were disrupted in a Potter–Elvehjem-type glass or stainless-steel homogenizer, centrifuged at 105 000g for 1 hr. in a Spinco model L refrigerated centrifuge and the supernatant fractions used.

Assay of adenosine 3'-phosphate 5'-sulphatophosphate formation. The method and assay mixture of Pasternak (1962) was used. The amount of sulphatophosphate synthesized was calculated on the assumption that all ^{35}S -labelled material formed during the incubation was adenosine 3'-phosphate 5'- ^{35}S -sulphatophosphate. Crystalline vitamin A alcohol (Roche Products Ltd., 15 Manchester Square, London, W. 1, or Nutritional Biochemicals Corp.,

Cleveland, Ohio, U.S.A.) was added as a solution in propylene glycol (1 mg./ml.) or a suspension [1 mg./ml. of aq. 2% serum albumin-ethanol (19:10, by vol.)]. Radioactivity and protein were measured as described by Pasternak (1962). The identity of the ^{35}S -sulphatophosphate synthesized by rabbit corneal epithelium was confirmed by co-chromatography on Dowex 1 (formate form; 200–400 mesh; X4) with an authentic non-radioactive sample, prepared enzymically with an extract of *Escherichia coli* ATCC 9723 (Pasternak, 1962), with the ammonium formate elution system described by Pasternak (1960, 1962).

RESULTS AND DISCUSSION

A compound having the electrophoretic mobility of adenosine 3'-phosphate 5'- ^{35}S -sulphatophosphate was synthesized from ^{35}S -sulphate and ATP by all the corneal extracts examined (Table 1). With rabbit corneal epithelium the compound was identified as adenosine 3'-phosphate 5'- ^{35}S -sulphatophosphate by subjecting the products of a reaction mixture containing 45 μmoles of ATP, 56 μmoles of magnesium chloride, 4.5 μmoles (150 μC) of potassium ^{35}S -sulphate and 2.1 ml. of supernatant enzyme to ion-exchange chromatography in the presence of 1 μmole of added carrier adenosine 3'-phosphate 5'-sulphatophosphate. The carrier sulphatophosphate appeared in the same effluent fractions (tubes 73–77) as the radioactive sulphatophosphate (6.3 $\mu\text{m-moles}$) synthesized during the incubation. The only other radioactive peak was unchanged ^{35}S -sulphate (tubes 54–59). Wortman (1961) has shown that epithelial, stromal and endothelial extracts of ox and rabbit cornea are able to synthesize a ^{35}S -containing compound having the electrophoretic mobility of chondroitin sulphate from ATP and ^{35}S -sulphate. Since this compound lost approximately 50% of its ^{35}S content when treated with 0.1 N-hydrochloric acid

Table 1. *Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by corneal extracts*

The extracts were prepared and assayed as described in the text. The values of enzymic activity are given as means $\pm$ s.e. where applicable, the numbers of experiments performed being given in parentheses.

Extract	Enzymic activity ($\mu\text{m-moles}$ of sulphatophosphate synthesized/mg. of protein/45 min.)
Calf corneal epithelium	1.00 (2)
Calf corneal stroma	0.35 (2)
Rabbit corneal epithelium	0.65 $\pm$ 0.08 (12)
Rabbit corneal stroma	0.57 $\pm$ 0.12 (12)
Guinea-pig corneal epithelium	0.30 (1)
Guinea-pig corneal stroma	0.28 (1)
Rat corneal epithelium	0.30 (1)
Rat corneal stroma	0.44 (1)

Table 2. *Synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by extracts from normal and vitamin A-deficient animals*

The animals for each experiment were litter mates, half of which were made deficient in vitamin A as described in the text. The values of enzymic activity are given as means $\pm$ S.E. where applicable, the number of experiments performed being given in parentheses.

Extract	Enzymic activity (μ m-moles of sulphatophosphate synthesized/mg. of protein/45 min.)	
	Normal animals	Vitamin A-deficient animals
Rabbit corneal epithelium	0.67 $\pm$ 0.13 (8)	0.76 $\pm$ 0.10 (8)
Rabbit corneal stroma	0.62 $\pm$ 0.16 (8)	0.39 $\pm$ 0.10 (8)
Rabbit retina	0.75 (1)	0.67 (1)
Rabbit conjunctiva	0.59 (1)	0.42 (1)
Rabbit colonic mucosa	3.82 (3)	4.52 (3)
Rat cornea (whole)	0.28 (4)	0.50 (4)
Rat retina	0.44 (1)	0.47 (1)
Rat colonic mucosa	1.32 (4)	1.37 (4)

at 38° for 10 min., it was probably largely adenosine 3'-phosphate 5' [35 S]-sulphatophosphate, which has the same mobility as chondroitin sulphate. Both sets of results indicate that corneal stroma, as well as epithelium, contains the sulphate-activating enzymes. The partial failure of rat corneas deprived of their epithelium to incorporate [35 S]sulphate into mucopolysaccharides *in vivo* (Smelser, 1960) cannot therefore be due to lack of these enzymes.

The effect of vitamin A deficiency on the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate by rat and rabbit tissues is shown in Table 2. Only with rabbit corneal stroma was there possibly a decrease. The addition of vitamin A to the incubation medium (final concn. 63 μ g./ml.) did not increase the synthesis of sulphatophosphate. The results are thus at variance with those of Varandani *et al.* (1960), who found that the activation of sulphate was decreased in the colons of vitamin A-deficient rats and that activity could be restored by the addition of vitamin A *in vitro*. Wolf & Moretti (1961), using extracts of rat colonic mucosa, have reported that it is the synthesis of mucopolysaccharide rather than that of sulphatophosphate which is controlled by vitamin A *in vivo* and *in vitro*. It is possible that the 35 S-labelled material synthesized by stromal extracts in the present experiments contains 35 S-sulphated mucopolysaccharide in addition to [35 S]sulphatophosphate,

and that the lowered incorporation of 35 S by stromal extracts of vitamin A-deficient rabbits reflects a decreased conversion of adenosine 3'-phosphate 5'-sulphatophosphate into sulphated mucopolysaccharides.

SUMMARY

1. The enzymes (adenosine triphosphate-sulphate adenyllyltransferase, EC 2.7.7.4, and adenosine triphosphate-adenyllylsulphate 3'-phosphotransferase, EC 2.7.1.25) which catalyse the activation of sulphate have been shown to be present in extracts of several parts of the eye from calf, rabbit, guinea pig and rat, and of colonic mucosa from rabbit and rat.

2. Vitamin A deficiency does not lead to a decrease in the activation of sulphate by extracts of rabbit colonic mucosa, rabbit corneal epithelium, rat colonic mucosa or rat cornea.

3. The activation of sulphate by extracts of rabbit corneal stroma is possibly decreased by vitamin A deficiency but could not be restored by the addition of vitamin A *in vitro*.

We are indebted to Mr H. C. Robinson for help with certain experiments. This work was supported by grants from the Rockefeller Foundation and the National Institutes of Health, Education and Welfare, United States Public Health Service.

REFERENCES

- Aurell, G., Dohlman, C.-H. & Rodén, L. (1956). *Acta ophthalm., Kbh.*, **34**, 281.
- Dohlman, C.-H. (1957). *Acta ophthalm., Kbh.*, **35**, 115.
- Kent, P. W. & Pasternak, C. A. (1958). *Biochem. J.* **69**, 453.
- Mann, I., Pirie, A., Tansley, K. & Wood, C. (1946). *Amer. J. Ophthalm.* **28**, 801.
- Moore, T. (1957). *Vitamin A*, p. 582. Amsterdam: Elsevier Press.
- Odeblad, E. & Boström, H. (1952). *Acta path. microbiol. scand.* **31**, 339.
- Pasternak, C. A. (1960). *J. biol. Chem.* **235**, 438.
- Pasternak, C. A. (1962). *Biochem. J.* **85**, 44.
- Robbins, P. W. & Lipmann, F. (1956). *J. Amer. chem. Soc.* **78**, 2652.
- Smelser, G. K. (1960). In *The Transparency of the Cornea*, p. 125. Ed. by Duke-Elder, S. & Perkins, E. S. Oxford: Blackwell Scientific Publications.
- Varandani, P. T., Wolf, G. & Johnson, B. C. (1960). *Biochem. biophys. Res. Commun.* **3**, 97.
- Wolf, G. & Moretti, A. (1961). *Fed. Proc.* **20**, 162.
- Wortman, B. (1960). *Amer. J. Physiol.* **198**, 779.
- Wortman, B. (1961). *J. biol. Chem.* **236**, 974.
- Wortman, B. & Strominger, J. L. (1957). *Amer. J. Ophthalm.* **44**, 291.