Studies on Metabolism of Vitamin A

THE EFFECT OF THE STAGE OF VITAMIN A DEFICIENCY ON SULPHATE ACTIVATION IN RAT LIVER

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1. The synthesis of 'active sulphate' in rat liver was studied at various stages of vitamin A deficiency, with the corresponding pair-fed controls. 2. The activity was significantly decreased even at the onset of the deficiency, and at the acute stage there was further loss. 3. Only at the earlier stages of the deficiency was the addition of retinol, *in vitro*, fully effective in restoring the lost activity; retinoic acid was partially active. No such restoration was possible at the acute deficiency stage.

The possibility of the existence in animals of a relationship between sulphate metabolism and vitamin A has been suggested by various observations made by several workers. Thus, with tissue-culture experiments, Fell, Mellanby & Pelc (1956) demonstrated that the inclusion of excess of vitamin A in the media causes the production of soluble sulphate. In experiments with whole animals, Dziewiatkowski (1954) showed higher concentrations of serum inorganic sulphate in vitamin A-deficient rats, and Balakhovski & Kuznetsova (1958) reported increased urinary excretion of inorganic sulphate.

However, subsequent work at the enzyme level failed to lead to complete agreement on the relationship between vitamin A and sulphate metabolism. Thus Wolf & Varandani (1960) at first showed a decreased uptake of [³⁵S]sulphate by the mucopolysaccharides of the colon homogenates of vitamin A-deficient rats and suggested that this could be due to a lowered synthesis of 'active sulphate' (PAPS†). Later, Varandani, Wolf & Johnson (1960) demonstrated a decrease in the synthesis of PAPS in the colon homogenates of vitamin A-deficient rats that could be restored by the addition of retinol *in vitro*. Sundaresan & Wolf (1963) again claimed that the synthesis of PAPS is depressed in vitamin A-deficient rat tissues, but they failed to restore the lost activity by the addition of retinol or retinoic acid *in vitro*. Instead, they reported that the addition of a metabolite derived from retinoic acid is effective. This was followed by a preliminary announcement by Sundaresan, Elford & Wolf (1964)

claiming the isolation of an enzyme-bound metabolite of vitamin A that is apparently functional in the reactivation of ATP sulphurylase. These observations thus appeared to disagree with the previous findings by Wolf and his co-workers. Simultaneously with those of Sundaresan & Wolf (1963) almost similar claims were made by Rogers, Chang & Johnson (1963) also. In the meantime, Pasternak, Humphries & Pirie (1963) reported that vitamin A deficiency does not affect the synthesis of PAPS in the cornea and colonic mucosa of rats and rabbits. At about the same time we showed that the synthesis of PAPS is depressed in the liver of vitamin A-deficient rats and that the addition of retinol *in vitro* is fully effective in restoring the lost activity, retinoic acid being partially active (Subba Rao & Ganguly, 1964).

Thus, except for Pasternak *et al.* (1963), there has been general agreement that PAPS synthesis is decreased in vitamin A deficiency and the point of disagreement has been on the ability of retinol to restore the lost activity. We have therefore reinvestigated this question and show below, in confirmation of our previous results, that the synthesis of PAPS is decreased in the liver of vitamin A-deficient rats and that it can be reactivated by the addition of vitamin A derivatives *in vitro*; it is also shown below that such reactivation is possible only at the milder stages of the deficiency.

MATERIALS AND METHODS

The sources of the various materials used, the preparation of the particle-free rat-liver supernatant that was used as the source of the enzymes synthesizing PAPS, and the methods of isolation and assay of PAPS were as described by Subba Rao & Ganguly (1964).

The vitamin A-deficient rats were obtained as described

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† Abbreviation: PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963). The period of the deficiency was arbitrarily divided into three stages as follows: (i) The weight-plateau stage, which signified the period when the rats maintained a constant weight for 3–4 days. (ii) The stage of mild deficiency, where the animals started losing weight. It immediately followed the weight-plateau stage and lasted for 4–6 days, during which time the animals lost 4–8 g. (iii) The stage of severe deficiency, which followed the mildly deficient stage; during this period the rats rapidly lost weight and were highly infected. At all the three stages of the deficiency the enzymic activities of the deficient rats were compared with those of corresponding pair-fed controls.

We have found that pure retinol is unstable both in the crystalline state and in solution, even in the presence of antioxidants. Therefore, for the additions *in vitro*, instead of using synthetic retinol as supplied by the manufacturers we always freshly prepared it enzymically from synthetic retinyl acetate. A sample of retinyl acetate was purified by chromatography on an alumina column by the method of Thompson, Ganguly & Kon (1949) and then hydrolysed by the procedure of Murthy, Mahadevan, Seshadri Sastry & Ganguly (1961), with acetone-dried preparations of rat pancreas as the source of vitamin A ester hydrolase. The alcohol formed was separated from the ester by column chromatography, after which its spectrum, measured in a

Beckman model DU spectrophotometer, was always that characteristic of retinol.

For establishing the identity of the PAPS synthesized, the criteria, e.g. its total hydrolysis by 0.1 N-HCl at 37° for 30 min. and its ability to donate about 80% of the sulphate group to *p*-nitrophenol enzymically, were employed, as described by Subba Rao & Ganguly (1964). In addition to these, the compound formed had an absorption maximum at 260 m μ , and when subjected to electrophoresis it moved 20 cm., whereas inorganic sulphate moved 28 cm., which is in agreement with Varandani *et al.* (1960).

RESULTS AND DISCUSSION

The results of the experiments where the synthesis of PAPS was followed at the three stages of the deficiency (Table 1) show that in rat liver it is affected by vitamin A deficiency. However, the loss of the activity, and the ability of added retinol or retinoic acid to restore it, seem to depend on the stage of the deficiency. Thus, though there was some loss at the first two stages (weight-plateau and mildly deficient), which could fully be restored by retinol and partially by the acid, further loss took

Table 1. *Effect of the stage of vitamin A deficiency on the synthesis of [³⁵S]PAPS*

The reaction mixtures and the assay of enzymic activities were as described by Subba Rao & Ganguly (1964). The given amounts of retinol or retinoic acid were added to the reaction mixture. All values are averages from three rats; the ranges are given in parentheses.

Status of the rats	Addition		[³⁵ S]PAPS formed (counts/min./mg. of protein/hr.)
Weight-plateau stage	Pair-fed	None	37720 (36320–39600)
	Deficient	None	27600 (26900–28430)
	Deficient	5 μ g. of retinol in 0.01 ml. of 1% ethanol	36830 (35810–38600)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	32670 (31260–34150)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	32670 (31260–34150)
Mildly deficient	Pair-fed	None	33270 (32100–34650)
	Deficient	None	20870 (19000–23600)
	Deficient	5 μ g. of retinol in 0.01 ml. of 1% ethanol	32200 (31000–34440)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	23930 (22600–25000)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	23930 (22600–25000)
Severely deficient	Pair-fed	None	31330 (30700–32050)
	Deficient	None	16530 (14830–18000)
	Deficient	5 μ g. of retinol in 0.01 ml. of 1% ethanol	17060 (14800–20080)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	16910 (14600–20000)
	Deficient	5 μ g. of retinoic acid in 0.01 ml. of phosphate buffer, pH 10.0 (0.2 M)	16910 (14600–20000)

place at the severely deficient stage, which could not be reactivated to any extent by the vitamin A derivatives.

There could be several sources of error leading to the conflicting claims, e.g. the identification of the product. That the compound isolated in the present work is PAPS was established by several criteria (cf. the Materials and Methods section). Moreover, since retinol is rather unstable, we have taken extreme care to make sure that the compound used for the additions *in vitro* is pure retinol, and for this purpose we always prepared it freshly from purified retinyl acetate. We have even avoided saponification with ethanolic potassium hydroxide, because peroxides might cause some destruction or structural modification of retinol, and have instead used a much milder method, namely enzymic hydrolysis.

As indicated in the introduction, though there has been general agreement that ATP-sulphurylase activity is decreased during vitamin A deficiency, the point of disagreement was on the ability of retinol to restore *in vitro* the lost activity. However, much of this information (Sundaresan & Wolf, 1963; Sundaresan *et al.* 1964; Rogers *et al.* 1963) has appeared in the form of abstracts, and therefore, in the absence of experimental details, it is difficult for us objectively to discuss these apparently conflicting reports. Nevertheless, in spite of these limitations our present results can offer some explanation. Thus if in their earlier work these workers had used rats that were mildly deficient, whereas in their later experiments their animals were severely deficient, their failure to reactivate with the added retinol in the later trials would agree with our present results, and therefore the claims would cease to be contradictory. Further, if any metabolite of retinol were to participate in this reaction, at the earlier stages of the deficiency the enzyme system responsible for the formation of such a metabolite could have been still active, whereas at the acute

stage it might have been totally lost, leading to no formation of the hypothetical metabolite. Similarly, the reported failure to obtain any reactivation by adding retinoic acid or retinol to the purified ATP sulphurylase (Sundaresan & Wolf, 1963) can also be explained on the basis that the enzyme(s) that converts these compounds into the metabolite was probably removed during the process of purification. The present data as well as the present interpretations have been discussed by Subba Rao (1964).

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