Ineffectiveness of Factor 3-Active Selenium Compounds in Resorption-Gestation Bioassay for Vit. E. (23846)

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Factor 3, described in 1951 as a new dietary agent(1), has recently been obtained in purified form.* It has been characterized as an organic substance containing selenium as an integral constituent of the molecule(2). The factor is very different from tocopherol: it is of low molecular weight, strictly water soluble, and stable against oxidation and acid hydrolysis(3). It was extremely effective in prevention of some fatal deficiencies, most of which have hitherto been attributed to lack of Vit. E. Evidently, these various diseases develop only if lack of both of these two essential factors coincides.† Thus far, it has been demonstrated that Factor 3 prevents liver necrosis in the rat; multiple necrotic degeneration (heart, liver, muscle, and kidney necrosis) in the mouse(4); exudative diathesis in the chick(5) and in the turkey(6); as well as dietary liver necrosis in the pig(7). Necrotic liver degeneration in the rat has been used as an assay system during the investigation of the factor. In preventing this fatal disease, 0.7 μg of selenium in form of α-Factor 3‡ per 100 g of diet afford 50% protection (8). Approximately 1,000 times as much Vit. E gives the same effect. It seemed important to investigate the influence of Factor 3 on fetal resorption in the pregnant rat since this classical symptom of Vit. E deficiency is the basis of the official bioassay procedure for determining Vit. E activity. The establishment of the International Standard and the International Unit of Vit. E is based upon it (9), as is the National Formulary X factor of 1.36 for relative potency of natural *vs.* synthetic α-tocopherols(10). Using the Mason-Harris modification(11) of Evans original resorption-gestation bioassay for Vit. E(12), and selenocystine and selenite as Factor 3-active supplements(8), we find that Factor 3-active compounds do not affect the Vit. E assay even when supplied in large excess.

Materials and methods. Two Factor 3-active compounds (selenocystine HCl and Na_2SeO_3) were bioassayed at levels supplying a total of 1, 10, or 100 μg of selenium (Table I). An additional level of 1 mg Se as H_2SeO_3 was also tested. The test substances were administered orally in aqueous solution by stomach tube, as well as intraperitoneally in doses of 0.2 ml on each of 5 consecutive days of pregnancy. The reference standard *d*-α-tocopheryl acetate, diluted in olive oil, was fed orally from a calibrated dropper in total doses of 0.2, 0.4, and 0.8 mg on the 4th through 8th days of pregnancy. Assay animals were female rats raised from weaning on diet low in Vit. E(11,13). When they attained a weight of 150 g they were mated with normal male rats, allocated into comparable groups, supplemented with either test or standard substances, and killed on the 20th day for examination of uterine contents. Only animals which had at least 4 implantation sites were used. Those which had at least 1 live fetus *in utero* were considered positive responses. Those with fetuses all dead or resorbed were negative responses. The proportion of positive to total responses in each group represents litter efficiency. The dosage which induces a 50% litter efficiency is known as the median fertility dose (MFD). The Factor 3 activity of selenocystine HCl and sodium selenite was determined, using the

* Schwarz, K., *et al.*, to be published. Since various compounds of selenium show greatly different biological activity, the term "Factor 3" is used to designate the biologically active, selenium-containing component, or components, present in nutrients and other materials of natural origin.

† The protective effect of L-cystine is caused by trace contamination with Factor 3-active selenium. (Schwarz, K., to be published).

‡ In acid hydrolysates of kidney powder, at least 2 chemically different but related components with Factor 3 activity have been detected. These have been designated α- and β-Factor 3. (Schwarz, K., Mason, L. H., and Foltz, C. M., to be published).

TABLE I. Vitamin E Bioassay of Factor 3-Active Selenium Compounds (8-10 Rats/Group).

Supplement	Mode of admin.	Total dose per animal	Amt of selenium per dose	Factor 3 (Fischer u)* per dose	Litter efficiency (%)	MFD (mg)
d-α-tocopheryl acetate	Oral	.2 mg			7	.39
		.4			50	
		.8			94	
D,L-selenocystine hydrochloride	Oral & I.P.	2.6 μg	1 μg	6.9	0	
		26	10	69	0	
		260	100	690	0	
Sodium selenite (Na_2SeO_3)	*Idem*	2.2	1	7.3	0	
		22	10	73	0	
		218	100	730	0	
Selenious acid (H_2SeO_3)	"	2.18 mg	1 mg	(5000)†	0	

* 1 Fischer unit is amount required daily/animal by Fischer strain of rats to achieve 50% protection against liver necrosis. For details concerning these tests see reference 8.

† Calculated from values obtained with SeO_2(8).

prophylactic rat assay against necrotic liver degeneration and the inbred Fischer 344 strain of rats. Values reported for Factor 3 were the result of series of assays at various dose levels(8). The pre-depletion method was used. Results are expressed in daily units for the Fischer animal, a unit constituting the amount required per day to afford 50% protection against liver necrosis.§

Results. In the resorption-gestation test, the calculated MFD for the Vit. E standard was 0.39 mg *d*-α-tocopheryl acetate (Table I). The results for Factor 3 preparations at all levels of supplementation were negative. Although the i.p.-administered Factor 3 preparations were completely absorbed, they exerted no tocopherol-like activity. The results show clearly that Factor 3-active selenium compounds do not influence the standard assay for Vit. E. However, these results do not imply that gestation is normal in the absence of Factor 3. Under conditions of the experiment, an ample amount of this agent is supplied by "vitamin-free" casein in basal diet, since this material is a good source of Factor 3(3).

The finding clearly supports the concept that Factor 3 and Vit. E do not replace each other but are independent, essential dietary agents. A possible explanation of the phenomenon that simultaneous lack of both agents is the cause of certain fatal deficiency diseases has been proposed. It is based on the assumption that Vit. E and Factor 3 participate independently in *alternate* pathways of oxidation-reduction, *i.e.*, electron transfer, in intermediary metabolism(14).

Summary. Factor 3-active selenium compounds, (selenocystine, sodium selenite and selenious acid) were bioassayed for Vit. E activity using standard rat resorption-gestation technic. These substances, administered in excess either orally or intraperitoneally, do not affect the bioassay of Vit. E even though they are highly effective against a variety of other deficiencies preventable by tocopherol, such as dietary necrotic liver degeneration and exudative diathesis.

§Factor 3 requirement of Fischer strain is 2.1 times as much as that of previously used Sprague-Dawley strain of rats, *i.e.*, 1 Fischer unit is equivalent to 2.1 Sprague-Dawley units.

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On the Mechanisms of Calcification.* (23847)

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Studies of the mechanism of calcification have been dominated by a single approach, that formulated by Robison(1): calcification involves some booster mechanism by which the product of the concentrations of calcium and phosphate is elevated locally so as to induce a precipitation of bone salts from saturated or slightly undersaturated tissue fluids. An important weakness in this approach is the necessary assumption that the tissue fluids are not already supersaturated with respect to bone salt. Actually, the older literature(2,3, 4) and recent results(5,6) have demonstrated rather conclusively that the serum and, most probably, the extracellular fluids are *supersaturated* with respect to the mineral phase of bone. More recently, in recognition of this supersaturated state of body fluids, attention has turned to the possibility that the mechanism of calcification may involve the crystal seeding or a surface-catalyzed induction of crystal formation. Indeed, quite some time ago(7), it was clearly shown that bone mineral would itself induce crystal formation from normal serum ultrafiltrates. These findings were extended and the ability to induce the formation of apatite crystals was shown also to reside in the collagen molecule, although specificity of collagen for such crystal induction was only crudely demonstrated(5, 8). More recently, however it has been claimed, on evidence from electronmicroscopy and X-ray diffraction technics, that only *one* crystallographic form of the collagen molecule is capable of inducing apatite crystal formation from supersaturated solutions of calcium and phosphate(9). Collectively, these studies make the *principle* of catalytic crystal seeding an attractive hypothesis at present. However, several questions arise: Do all collagens catalyze equally well? All connective tissues contain collagen, why don't all connective tissues calcify? Is the same mechanism, crystal seeding, operative in both osteoid *and* cartilage? As stressed recently(10), there are differences between calcification in osteoid and in the provisional zone of calcification in cartilage.

To obtain partial answers to some of these questions, an attempt was made to prepare purified, reconstituted(11) collagen fibers from a number of different tissues. It was hoped that these various collagen preparations could be compared quantitatively for their ability to induce crystal formation from supersaturated solutions of calcium and phosphate. Good yields of relatively pure, reconstituted fibers were obtained from skin and tendon. However, the usual reagents(11) employed in dissolving collagen from other tissues failed to dissolve collagen from bone sections previously demineralized with ethylene diamine tetra acetic acid (EDTA) at pH 7.4. Whether the EDTA-treated bone had been altered by the demineralizing procedure

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