between groups were rather small, the effect of both pantothenic acid and vitamin E in liver confirmed previous findings with these two substances. Although one or two anomalies can be observed, it seems the two vitamins work in opposite senses, pantothenic acid depressing and vitamin E elevating the concentrations of ubiquinone and ubichromenol. The effects in brain, in general, supported these conclusions. Gray, Chisholm & Lee Peng (1960) have recently shown that synthesis of coenzyme A by rat liver is increased by dietary supplementation with tocopherol. In both the present experiments involving single pantothenic acid deficiency (see results for groups G and O) tocopherol concentrations in the liver were increased to some extent, probably significantly. If, as Gray *et al.* suppose, tocopherol is an intermediate in coenzyme A biosynthesis, then pantothenic acid deficiency may lead to a certain sparing action on liver vitamin E. This would be in accordance with the observed increases in liver ubiquinone.

SUMMARY

1. Groups of male and female rats were maintained on thiamine-deficient and riboflavin-deficient diets for 38 days, after which they were killed

and their hearts, livers and spleens were analysed for ubiquinone, ubichromenol and α -tocopherol. The concentrations were compared with those found in tissues from control animals, supplemented with the required vitamin. Although certain changes occurred in heart and liver, there appear to be secondary manifestations of severe weight changes during deficiency. Administration of thiamine and riboflavin did not appear to influence ubiquinone or ubichromenol concentrations directly.

2. In pantothenic acid deficiency, groups of rats showed elevated ubiquinone concentrations in liver, but not in heart. A secondary effect, through vitamin E, may be involved.

We wish to thank Mrs W. S. Miller for technical assistance.

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Studies on Vitamin E

8. VITAMIN E, UBIQUINONE AND UBICHROMENOL IN THE RABBIT

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It has been shown by Edwin, Diplock, Bunyan & Green (1961) that vitamin E has a marked effect on the concentrations of ubiquinone and ubichromenol in the tissues of the rat and it was suggested that a biosynthetic relationship existed between the three substances. The rat is an unusual mammal with respect to ubiquinone in that most of the quinone of its tissues consists of ubiquinone 45 (Rüegg *et al.* 1959), whereas the ubiquinone in other mammals so far studied has been found to be ubiquinone 50 (Lester & Crane, 1959). It was therefore of some interest to study the effect of vitamin E in another species, and the rabbit was used as an experimental animal. Edwin *et al.* (1961) drew attention to the significant response of the uterus in the rat to tocopherol administration,

compared with that of skeletal muscle, and showed that, in young females, a rapid increase in the tocopherol content of the uterus after dosage resulted in a marked rise in the ubiquinone concentration. It was suggested that these findings might be related to the sensitivity of the female rat to reproductive failure in vitamin E deficiency, and, as a corollary, it was speculated whether the specific symptom of vitamin E deficiency in other species could be correlated with local deficiencies of tocopherol in the tissues. From this point of view the rabbit provides a useful contrast to the rat, for in this species, gestation-resorption does not occur, but the chief deficiency syndrome is a marked muscular dystrophy, which is fatal if untreated. The purpose of this study therefore was to investigate the

distribution of vitamin E in the rabbit, the response of some of the more important tissues to administration of tocopherol and the relationship, if any, between vitamin E and ubiquinone.

EXPERIMENTAL

Animals. Sixteen mature cross-bred female rabbits were purchased from a commercial supplier.

Stock diet. This had the following composition (%): soya-bean meal, 18; fine linseed, 4; grass meal, 42; wheat bran, 14; barley meal, 13; salt, 5; oats, 4.

Vitamin E-deficient diet. This had the following composition: casein (Genatosan Ltd., 'low-vitamin content'), 15%; sucrose, 36.3%; maize starch, 36%; lard, 3%; cod-liver oil, 6%; McCollum's salt mixture, 3%. The following were added to each kg. of diet: choline tartrate, 1 g.; inositol, 1 g.; nicotinic acid, 200 mg.; pyridoxine, 5 mg.; thiamine, 5 mg.; riboflavin, 5 mg.; calcium pantothenate, 10 mg.; folic acid, 5 mg.; menaphthone, 250 μ g.; vitamin B₁₂, 70 μ g.

Experimental plan. The rabbits were divided into four equal groups. Group A was fed with the stock diet and group B with the deficient diet. Group C was given the deficient diet and in addition received oral doses of α -tocopheryl acetate equivalent to 2 mg./kg. body wt./day for 49 days, and after that until the end of the experiment 4 mg./kg./day. The animals in group D were given the deficient diet and were each given two oral doses of 50 mg. of α -tocopheryl acetate 48 and 24 hr. before they were killed. The experiment lasted 15 weeks. The rabbits in groups B, C and D were examined continuously for signs of muscular dystrophy and their urine was examined weekly for signs of creatinuria. One rabbit in group B displayed the symptoms of dystrophy and creatinuria before the end of the experiment. It was killed, dissected at once and the organs were stored at -20° until the end of the experiment. Two rabbits in group D also showed signs of dystrophy before the end of the experiment, and they were given their two doses of vitamin E and then killed; their organs were stored at -20° until the end of the experiment. After 15 weeks, all the remaining rabbits were killed by cervical dislocation and the required tissues taken. Those from each group were pooled and analysed as described previously by Edwin *et al.* (1961).

RESULTS AND DISCUSSION

The 15-week experimental period is considerably longer than that normally used to produce muscular dystrophy in the rabbit. This was necessary since we particularly wanted to examine the uterus and mature females had to be used. The starting weights of our animals therefore were higher than usual, and not all the deficient animals showed clinical signs of dystrophy or creatinuria even after 15 weeks. The analyses from seven tissues are recorded in Table 1.

The pattern of vitamin E distribution in the rabbits on the stock diet showed certain differences from that observed previously in the rat. Nerve,

heart and adipose tissue contained as much tocopherol as the corresponding tissues in the rat, but uterus and skeletal (hind-leg) muscle contained much less. It is clear that the concentrations of tocopherol in the tissues of the rabbit are influenced by the character of the diet (as they are in the rat). Thus the concentration in heart was much lower and in liver much higher in group C animals than in group A animals.

Two significant observations emerge from the analyses of tissues from group B animals. First, although these animals were given the deficient diet for a long time (one was killed *in extremis*) their body fat apparently still contained large amounts of tocopherol. Secondly, skeletal muscle from this group contained one of the lowest concentrations of tocopherol so far observed by us in any animal tissue (0.9 μ g./g.). The administration of 100 mg. of α -tocopheryl acetate to deficient animals (group D) seemed to produce a rather smaller effect in terms of increased tissue concentrations than a dose of 10 mg. did in rats (the dosage being approximately equivalent on a body-weight basis), although, in both uterus and skeletal muscle, tocopherol concentrations approximating to those in group A animals were reached. The remarkable concentrations of tocopherol in nerve, and the avidity with which this tissue retains tocopherol during dietary depletion, resembles closely the behaviour of nerve tissue in the rat, discussed by Edwin *et al.* (1961).

Ubiquinone and ubichromenol concentrations in rabbit tissues also showed differences from those in the rat. The concentration of ubiquinone in heart was considerably higher than in any other tissue and was of the same order as that found in rat heart. The ubiquinone concentrations in most of the other rabbit tissues, though, particularly in liver, were generally considerably lower than in the rat. The adipose tissue in the rabbit, however, contained more ubiquinone than was observed in the rat. Ubichromenol was found in every tissue except fat and skeletal muscle from animals in groups A, B and D.

The influence of vitamin E on concentrations of ubiquinone and ubichromenol in the rabbit paralleled that already observed in the rat, but the changes took place at a lower concentration. Comparing tissues from groups B and C, for example, with the exception of brain, in which there was little change, supplementation with tocopherol increased the concentration of ubiquinone and usually increased that of ubichromenol. If the previous estimates of variance made by Edwin *et al.* (1961) in their studies on the rat can be taken as a guide, it would seem that the changes in heart, liver and muscle are significant. Similarly, comparing group B with group A, ubiquinone

concentrations are considerably higher in the heart, liver, uterus and muscle of the group A animals, there being little difference in nerve, brain or fat. Administration of tocopherol to group D animals 48 hr. before they were killed restored the ubiquinone to normal and usually lowered the ubichromenol concentrations in the tissues examined. Again, from our previous estimates, it seems that the differences in heart, liver, uterus and muscle are significant. These observations support the idea that there is a biosynthetic relationship between the three substances in the rabbit as well as in the rat.

A study of the data suggests that the rabbit appears to have more difficulty in the mobilization of tocopherol than does the rat. For example, the heavy concentration of tocopherol in the fat of animals in the deficiency state is striking, and supports the suggestion of Edwin *et al.* (1961) that the clinical nature of the vitamin E-deficiency syndrome, which varies from species to species, might be related to local deficiencies of the vitamin.

In confirmation of this, the concentration of α -tocopherol in rabbit muscle is only about one-third of that in rat muscle, and, on depletion, exceptionally low concentrations of tocopherol are produced in the former tissue. Also, the rabbit uterus, unlike the rat uterus, does not contain high concentrations of tocopherol. This again reflects the remarkable differences in the nature of the deficiency syndrome in the two species.

A general conclusion from a study of both species can now be made. It appears that local tissue deficiencies of vitamin E may occur in animals (even to the point of causing death), although no absolute state of deficiency exists in the organism as a whole. It follows therefore that the analyses of tissues that may be quite unrelated to the clinical picture of deficiency (such as, for example, the commonly used serum or liver analyses) are only of limited importance in contributing towards an understanding of the role of vitamin E. It is already fairly clear that neither the total amount of tocopherol in the animal organism nor a calculation

Table 1. *Distribution of vitamin E, vitamin A, ubiquinone and ubichromenol in the female rabbit*

Four groups of four rabbits were used. Group A was on the stock diet, group B on the vitamin E-deficient diet and group C on the supplemented diet. Group D was given the deficient diet and each animal in this group received 100 mg. of α -tocopheryl acetate 48 hr. before death. Average rabbit weights in each group at death were: group A, 2.565 kg.; group B, 2.565 kg.; group C, 2.518 kg.; group D, 2.585 kg. n.d., Not detected.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μ g./g.)	Ubichromenol (μ g./g.)	α -Tocopherol* (μ g./g.)	Vitamin A (i.u./g.)
Heart	A	5.83	123	22.6	35.2	2.5
	B	6.34	59	13.5	6.6	1.5
	C	7.39	71	15.3	10.5	1.8
	D	5.82	93	10.0	9.5	2.5
Liver	A	67.2	27.7	8.9	12.1	180
	B	84.7	12.3	6.0	6.9	88
	C	73.9	22.7	7.5	34.5	125
	D	64.5	30.1	4.0	24.3	93
Uterus	A	18.8	12.5	4.4	8.5	1.8
	B	13.9	5.6	1.5	3.0	1.1
	C	19.4	6.0	3.4	3.6	1.3
	D	14.8	7.3	1.3	7.0	1.4
Nerve	A	0.470	12.1	10.0	41.8	7.9
	B	0.426	12.9	10.8	41.5	8.1
	C	0.405	14.0	5.3	50.0	8.7
	D	0.548†	—	—	—	—
Brain	A	7.28	9.5	2.8	12.5	0.9
	B	8.51	8.3	4.3	7.6	0.6
	C	8.80	8.2	3.1	8.5	2.6
	D	7.86	10.2	3.6	8.7	0.9
Skeletal muscle (hind leg)	A	—	13.9	n.d.	4.0	0.8
	B	—	7.2	n.d.	0.9	0.5
	C	—	9.3	3.4	2.7	0.4
	D	—	12.4	n.d.	2.9	0.4
Fat	A	—	14.5	n.d.	120	7.8
	B	—	14.5	n.d.	63.8	4.0
	C	—	30.8	4.9	124	8.4
	D	—	12.2	n.d.	31.6	4.0

* In group A, small amounts of other tocopherols were occasionally observed in the tissues. These were measured, but, being small compared with the α -tocopherol found, have not been recorded here.

† Analyses lost.

from the amounts ingested in the diet are sufficient criteria on which to base judgment of vitamin E status. Much more must be discovered about the actual concentrations of tocopherol in the specific affected sites of vitamin E deficiency in animals, particularly in conditions of dietary or metabolic stress.

SUMMARY

1. Four groups of female rabbits were maintained on various diets in order to study the distribution of vitamin E in their tissues. After a test period of 15 weeks, seven tissues were analysed for tocopherol, vitamin A, ubiquinone and ubichromenol.

2. Skeletal muscle in the rabbit contained little tocopherol and became depleted to exceptionally low levels. This has been related to the muscular dystrophy that occurs in this species. In contrast with the rat uterus, the uterus in the rabbit is little affected by tocopherol. This reflects the difference in the vitamin E-deficiency syndrome in the two species.

3. Vitamin E-deficient rabbits have lower concentrations of ubiquinone in heart, liver and

skeletal muscle than animals on the same diet supplemented with α -tocopheryl acetate. In most tissues except nerve and brain, ubichromenol was also lower. Administration of single doses of vitamin E to deficient animals increased ubiquinone in all tissues examined except fat and also decreased ubichromenol. There appears to be a relationship between α -tocopherol, ubiquinone and ubichromenol in the tissues of the rabbit.

4. Rabbits kept on a vitamin E-deficient diet for 15 weeks still contain large reserves of vitamin E in their adipose tissue. Deficiency states can apparently be produced by local deprivation of tocopherol from a specific tissue.

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O-Sulphate Esters of L-Serine, L-Threonine and L-Hydroxyproline

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The hydroxy amino acids, with the exception of tyrosine, have not so far been shown to occur naturally as O-sulphate conjugates. The presence of tyrosine O-sulphate in one of the two peptides which are liberated from bovine fibrinogen by the action of thrombin was first noted by Bettelheim (1954). This ester has also been detected in similar peptides prepared from rabbit and pig fibrinogens (Blombäck, Boström & Vestermark, 1960; Blombäck & Sjöquist, 1960) and in human and horse fibrins (Bettelheim-Jevons, 1958). These findings raise the possibility that other hydroxy amino acids may occur in proteins as O-sulphate conjugates. The wide distribution of the O-phosphate conjugates of serine and threonine in phosphoproteins is well known (see Perlmann, 1955).

Attempts to detect O-sulphate esters of hydroxylated amino acids in tissues have been preceded by the study of the preparation and properties of the O-sulphate esters of L-serine, L-threonine and L-hydroxyproline which is reported here.

EXPERIMENTAL

Preparation of amino acid O-sulphates

Potassium L-serine O-sulphate. The O-sulphate esters of tyrosine and tyrosine derivatives have been prepared by Dodgson, Rose & Tudball (1959) with relatively mild conditions of sulphation. A similar method gave a poor yield of potassium L-serine O-sulphate. The method of Reitz, Ferrel, Fraenkel-Conrat & Olcott (1946) for the preparation of O-DL-serine acid sulphate, when used with L-serine, yielded a product which was contaminated with inorganic SO_4^{2-} ions and unchanged starting material. A satisfactory product was obtained by modifying this method.

L-Serine (1 g.) was added at room temperature to 5 ml. of H_2SO_4 (sp.gr. 1.84) and the mixture stirred until the material dissolved (usually 10 min.). The reaction mixture was kept *in vacuo* for the remainder of 1 hr., when it was poured into 400 ml. of dry ether at -17° . After 15 min. at this temperature the supernatant was decanted and the residue washed ($\times 3$) with 200 ml. portions of ether at -17° , the supernatant being decanted after each washing.