Studies on Vitamin E

6. THE DISTRIBUTION OF VITAMIN E IN THE RAT AND THE EFFECT OF α -TOCOPHEROL AND DIETARY SELENIUM ON UBIQUINONE AND UBICHROMENOL IN TISSUES*

By E. E. EDWIN, A. T. DIPLOCK, J. BUNYAN AND J. GREEN

Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

(Received 16 August 1960)

Although vitamin E deficiency and the phenomena associated with it in the rat have been studied continuously for many years, the nature of the role of vitamin E still remains obscure. Some workers consider that its role in animals may be simply defined by equating α -tocopherol with an intracellular antioxidant whose presence is necessary to prevent the accumulation of substances that would otherwise lead to peroxidation of tissue lipids and thus would interfere with normal metabolism. Other workers believe that the vitamin may play a more specific role, perhaps in connexion with electron transport or intracellular phosphorylation. It is difficult, indeed, to separate the first function (that of antioxidant) from any other that may exist.

One of the more interesting features of vitamin E deficiency is the variety of symptoms that it produces in different animals, and sometimes, by a slight change in experimental conditions, in the same animal. It seemed, at the outset of this work, that more information than was hitherto available about the distribution of tocopherol in the tissues of animals was desirable. Even our knowledge about the distribution of vitamin E, under varying nutritional conditions, in such a well-studied animal as the rat is limited. For example, examination of the literature failed to reveal any information about the vitamin E content of such a significant organ as normal rat uterus, and practically nothing appeared to be known about the actual concentrations of tocopherol in vitamin E-deficient tissues. Methods for the analysis of such tissues are now available (Edwin, Diplock, Bunyan & Green, 1960; Diplock, Green, Edwin & Bunyan, 1960b) and have extended the range of determination to encompass all the types of biological material so far encountered.

If a single thread can be said to run through the skein of observations on the effects of vitamin E deficiency, it would seem to be that there is some relation between the vitamin and cellular oxidation processes. The recent discovery of the role of ubiquinone (coenzyme Q) in electron transport

(Crane, Hatefi, Lester & Widmer, 1957) and the identification of ubichromenol, the cyclized isomer of ubiquinone, by Laidman, Morton, Paterson & Pennock (1960) have provided a new starting point for a consideration of this problem. Fortunately both these substances could be studied in tissues by the same methods as used for tocopherols and, in the present work, the relationships that exist between these groups of lipids in the rat have been investigated in some detail. In view of the interactions between vitamins A and E (the nature of which became clearer as the work progressed), vitamin A analyses were carried out on the extracts wherever possible. Because of the role of selenium in vitamin E-deficiency conditions (Schwarz & Foltz, 1957), some preliminary investigations were made into the influence of dietary selenium on the concentrations of ubiquinone and ubichromenol.

EXPERIMENTAL

Animals

Rats of the Norwegian hooded strain, of both sexes, were used. They were removed from the stock colony at about 13 days of age and placed on the appropriate test diet.

Diets

Stock diet (SD1). This was used in all tests in which a nutritionally adequate control diet was required: wheat germ (15%), meat and bone meal (5%), dried milk (10%), grass meal (5%), soya-bean meal (2%), dried brewer's yeast (3%), salt (0.53%), arachis oil (5%) and maize meal to 100%. The diet was supplemented with a stabilized vitamin A and D preparation to provide 1000 i.u. of vitamin A and 100 i.u. of vitamin D/lb.

Vitamin E-deficient basal diet (A40). This contained casein (Genatosan Ltd., 'low vitamin content'; 25%), sucrose (50%), dried yeast (Marmite Food Extract Co.; 10%), lard (Wiltshire United Dairies; 10%) and McCollum's salt mixture (5%) to provide (g./kg. of diet) the following: NaCl, 2.3; $\text{CaH}_4(\text{PO}_4)_2$, 7.3; ferric citrate, 1.57; NaH_2PO_4 , 4.7; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.6; K_2HPO_4 , 12.9; calcium lactate, 17.5; KI, 0.06; NaF, 0.015; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.06; Cu_2I_2 , 0.005; K_2SO_4 , $\text{Al}_2(\text{SO}_4)_3 \cdot \text{H}_2\text{O}$, 0.005. The diet was supplemented with vitamin A palmitate (40 i.u./g.) and vitamin D₃ (2.5 i.u./g.).

* Part 5: Bunyan, Green, Edwin & Diplock (1960).

Vitamin E-supplemented basal diet (A40E). This was identical with A40, with the addition of DL- α -tocopheryl acetate (0.1 mg./g.).

Necrogenic diet. This was that described by Green, Edwin, Bunyan & Diplock (1960).

Plan of animal experiments

Vitamin E deficiency. Rats were divided into groups of 10 or 12. Males and females were used in separate but replicate groups, there being 11 groups in all. The four groups of males were put on the test diets thus: groups A and B on diet A40, C on A40E and D on the stock diet SD1. The animals in group B were litter-mate controls for those in group A. The groups were maintained on these diets until they were 6 months old. At 72 hr. before the end of the experiment, and again at 48 hr., the rats in group B were given an oral dose of 5 mg. of DL- α -tocopheryl acetate. After 48 hr., all the animals were killed. A similar experiment was carried out with four groups of female rats (E, F, G and H, the first two groups being randomized from litter mates). In addition, three groups of females were reared to 13 months, thus: groups J and K, of randomized litter mates, on diet A40; group L on diet SD1. Groups F and K were dosed orally with α -tocopheryl acetate 72 and 48 hr. before death, as in the experiment on the males. Animals on the deficient diet A40 showed typical symptoms of vitamin E deficiency. These included depigmentation of the incisors, testicular degeneration in males and brown uterus and failure of gestation in females.

Selenium deficiency. Three experiments were carried out. In the first, weanling rats of both sexes were divided into two groups, M and N. Group M consisted of six males and eight females, which were maintained on the necrogenic diet for 7 weeks; group N, five males and four females, were on the same diet supplemented with selenium (0.05 p.p.m. as sodium selenite). In the second experiment, four groups of 12 rats of mixed sexes were given the following diets respectively: group O, the necrogenic diet; group P, the same diet with added selenium (0.05 p.p.m.); group Q, the diet with added α -tocopheryl acetate (100 p.p.m.); group R, the diet with both additions. These rats were maintained on test for 6 weeks, during which time five of the group O rats died. In the third experiment, two groups of four rats (two of each sex) aged about 3 months were given a modified vitamin E-deficient diet (similar to A40, but with only one-quarter of the vitamin A). Group T were given supplementary selenium (0.1 p.p.m.) for 2 weeks, group S acting as controls.

Analytical methods

At the end of each experiment, the rats were anaesthetized with ether, samples of blood taken from the hearts and the rats killed by cardiac excision. The animals were then dissected at once. The following tissues were taken: heart, liver, kidney, skeletal muscle (hind leg), spleen, brain, adrenal gland, lung, sciatic nerve, uterus or testis and a sample of body fat removed from below the kidneys. The organs were weighed and then the tissues from each group were pooled and stored in polythene bottles at -20° until they could be analysed (usually within a few days, occasionally as long as 4 weeks). Previous studies (Diplock *et al.* 1960*b*) showed that no significant losses of ubiquinone, ubichromenol or vitamin E occurred during storage at low temperatures; nevertheless, to minimize variation, it was

always the practice to analyse each tissue in parallel with its various controls on the same day. The adrenals were deep-frozen immediately after removal from the animal. Blood was separated into clot and serum and these were analysed separately.

The analyses of tissues and of diets were by the two-dimensional chromatographic procedure of Diplock *et al.* (1960*b*), vitamin A being measured on the same extracts, as previously described. For analysis, 10–20 g. of tissue was taken. If this amount was not available, the total available quantity was used. For analyses of the diets and dietary ingredients, 50–250 g. of each was extracted exhaustively with ether and the analysis carried out on the ether extract.

The value for $\Delta E_{1\text{cm}}^{1\%}$ at 274.5 m μ in the borohydride assay of the quinones was taken as 152 throughout, this figure being that calculated for ubiquinone 45, the main component. Although some tissues may have contained a greater proportion of ubiquinone 50 than others, only slight changes in the results were produced by modifying the ΔE factor to take account of fluctuations in the isoprenologue ratios, and it was not considered that the analytical results would be significantly improved by so doing. Similarly, the same spectrophotometric-conversion factor was used throughout for the determination of ubichromenol, whatever the apparent changes in isoprenologue pattern. The factor for ubichromenol 45 (see Diplock *et al.* 1960*b*) was checked by determining a nearly pure specimen of ubichromenol 50 (kindly given by Professor R. A. Morton and Dr J. F. Pennock), then allowing for the difference in molecular weight. The best value we could obtain was taken as 300 for ubichromenol 50, which gave a factor of 270 for ubichromenol 45, in good agreement with that found previously. In all probability the true factor is somewhat lower than this, but its more accurate assessment must await a further supply of the pure substance.

RESULTS

Analysis of the diets and some dietary ingredients

To compare the intake of vitamin E on the different nutritional regimens, the two key diets, SD1 and A40, were analysed (Table 1). In addition, the ingredients of A40 likely to introduce tocopherol into the diet were analysed individually.

Table 1. *Analysis of diets and dietary components*

Material	n.d., Not detected; tr., trace.		
	Ubiquinone ($\mu\text{g./g.}$)	α -Toco- pherol ($\mu\text{g./g.}$)	β -Toco- pherol ($\mu\text{g./g.}$)
Stock diet	1.2	9.5	2.7
Diet A40	0.8	2.4	n.d.
Lard*	n.d.	7.9	n.d.
Casein†	tr.	0.7	0.1
Yeast (dried)‡	9.9	tr.	n.d.
Yeast (dried)§	1.1	0.3	n.d.

* Wilts. United Dairies.

† Genatosan Ltd. 'low vitamin content'.

‡ Marmite Food Extract Co.

§ Distillers Co. Ltd.

It will be observed that the vitamin E-deficient diet contains about a quarter of the tocopherol of the adequate stock diet and that most of this comes from the lard. The total amount of tocopherol in the whole diet A40 was rather more than could be accounted for by summation of the ingredients, but there is a good deal of uncertainty in the determination of these small quantities: large amounts of material must be extracted and the final measurements are small. The presence of α -tocopherol in the yeast will be discussed elsewhere.

Tissue analyses, analytical precision and the effect of added α -tocopherol on the recovery of ubiquinone and ubichromenol

The results of the various experiments on the distribution of vitamin E in the rat and its effect on other lipid constituents are presented in Tables 2-4, grouped to illustrate sex and age differences. The only tocopherol measurements recorded are for α -tocopherol. Occasionally small amounts of β -tocopherol were observed in the rats on diet SD1 and these were, in fact, measured; but, since the quantities found did not contribute significantly to the total vitamin E, they have not been included in the tables. Rats on the other diets contained only α -tocopherol. The figures for ubiquinone and ubichromenol refer to the total isoprenologue content in each case. Chromatographic separation (Diplock *et al.* 1960b) showed that the tissue extracts of most groups of animals contained mainly ubiquinones 45 and 50. However, the tissues from group L all contained ubiquinones 45, 40 and 35. The ubichromenol pattern corresponded to the quinone pattern (Diplock, Edwin, Green, Bunyan & Marcinkiewicz, 1960a).

As already shown by Diplock *et al.* (1960b) and confirmed at several points during these experiments, recoveries of ubiquinone, ubichromenol and tocopherol appeared to be essentially quantitative. In Table 5 are recorded the results of four experiments designed to determine the precision of the analytical methods. In view of our findings on the influence of vitamin E on concentrations of ubiquinone and ubichromenol in the tissues, it was essential to examine whether the addition of α -tocopherol to tissues could itself affect the recovery of either ubiquinone or ubichromenol. This was done by adding α -tocopherol to some samples of homogenized tissue (three samples of liver and one of heart) immediately before extraction and analysis. As is seen from Table 5, the addition of α -tocopherol did not materially affect the recoveries of the other lipid constituents. Statistical analysis of Table 5 shows that the standard deviation of the results amounts to about 3, 10 and 6% of the mean for ubiquinone, ubichromenol and α -tocopherol respectively.

Statistical analysis of the results from the experiments on vitamin E

The data in Tables 2-4 illuminate an intricate series of relationships between the concentrations of the four lipid substances under varying conditions of age and nutrition, and the patterns exposed can obviously only be partially interpreted. Whilst the original design of the experiments was based on the requirements for an investigation into vitamin E, it soon became clear that the influence of this vitamin on the concentrations of ubiquinone and ubichromenol was of major interest and it was important to discover whether the changes were significant and to what degree. It was apparent, however, that the application of standard statistical designs to a study of such a complex nature as had been originally envisaged, which included tissue by tissue analysis of rats of both sexes at different ages and under varying nutritional regimens, would have involved a very large number of analyses, in addition to the several hundred already represented, so great in fact as to be virtually prohibitive. It seemed reasonable therefore to conduct a separate small-scale experiment, whose results would provide an estimate of variance that could be used as a first approximation to that probably occurring in the main investigation. Table 6 records the results of such an experiment, designed to test the variability, in respect of the quantities of the four lipid substances investigated, between groups of rats treated alike. The randomized groups were killed and the hearts and livers from each group were pooled and analysed.

The variance of each set of results for both sexes combined (organ weight, ubiquinone concentration etc.) was analysed in the usual way (Snedecor, 1946a) and a coefficient of variation (C) was calculated from the error mean square:

$$C = 100 \sqrt{\frac{\text{error mean square}}{\text{mean}}}$$

C was used to give an approximate 'least significant difference', in terms of percentages, between two groups of rats ($\pm Ct/\sqrt{2}$), two pairs of groups ($\pm Ct$) and between two sets of three groups ($\pm Ct\sqrt{2}/\sqrt{3}$). Table 7 shows the data obtained in this way. Such estimates can be used only as a guide since variation in tissues other than heart and liver and in the tissues of older rats was not studied. Slightly less variability would be expected in the groups of 10-12 animals used in the main investigation.

Another approach to the problem of statistical significance in the main investigation is to treat some of the differences found as paired comparisons (Snedecor, 1946b); for example, the raised concentrations of ubiquinone found in hearts of all three

Table 2. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubiquinone in 6-month-old male rats*

Analyses of depot fat are based on extracted lipid, not the weight of the crude tissue. Group A was on the vitamin E-deficient diet A40; group B was dosed with tocopherol before death; group C was on the supplemented diet A40E; group D was on the adequate diet SD1. n.d., Not detected; tr., trace.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
Heart	A	1.20	41	12	2.3	0.9
	B	1.17	132	7	4.2	2.1
	C	1.22	168	31	34.1	11.0
	D	1.00	133	29	25.8	3.8
Liver	A	11.62	83	10	3.0	148
	B	11.20	62	13	9.6	160
	C	11.65	79	20	21.6	750
	D	8.06	107	42	14.1	177
Kidney	A	2.40	45	4	0.8	1.1
	B	2.41	72	6	6.0	3.9
	C	2.40	194	11	14.4	13.8
	D	1.60	78	11	8.9	3.6
Muscle (hind leg)	A	—	15	13	4.3	1.5
	B	—	37	3	8.3	3.0
	C	—	44	16	14.6	8.8
	D	—	40	18	12.6	—
Spleen	A	1.20	14	11	5.4	4.3
	B	1.09	21	13	23.4	5.7
	C	1.08	22	13	43.9	27.1
	D	1.55	17	16	14.2	—
Brain	A	1.89	14	6	3.7	9.5
	B	1.85	22	3	4.3	13.5
	C	1.96	19	4	6.3	6.6
	D	1.86	16	n.d.	6.5	—
Adrenal gland	A	0.056	89	92	68.4	71.4
	B	0.060	143	37	118	80.1
	C	0.060	132	67	218	104
	D	0.050	71	63	47.0	79.4
Lung	A	2.57	11	tr.	8.7	—
	B	2.43	16	3	14.2	—
	C	1.79	12	2	31.3	13.3
	D	1.78	6	5	13.5	4.2
Nerve	A	0.106	n.d.	n.d.	5.7	21.0
	B	0.114	22	n.d.	11.8	27.5
	C	0.103	21	8	25.7	7.2
	D	0.099	56	15	39.1	—
Testis	A	2.06	15	3	1.1	6.3
	B	1.77	25	3	4.8	11.2
	C	3.08	14	1	14.5	8.0
	D	2.78	7	n.d.	17.5	—
Fat	A	5.50	n.d.	n.d.	17.2	—
	B	5.89	n.d.	n.d.	16.5	—
	C	4.71	n.d.	n.d.	54.2	1.5
	D	2.70	tr.	25	108	—
Clot	A	—	2	5	2.1	0.2
	B	—	4	n.d.	4.5	—
	C	—	n.d.	3	2.9	0.2
	D	—	28	14	11.8	—
Serum	A	—	n.d.	n.d.	5.0	n.d.
	B	—	n.d.	n.d.	5.5	2.8
	C	—	n.d.	n.d.	1.8	—
	D	—	n.d.	n.d.	4.6	1.1

Table 3. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubiquinone in 6-month-old female rats*

Analysis of depot fat are based on extracted lipid, not on the weight of crude tissue. Group E was on the vitamin E-deficient diet A 40; group F was dosed with tocopherol before death; group G was on the supplemented diet A 40 E; group H was on the adequate diet SD1. n.d., Not detected; tr., trace.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone ($\mu\text{g./g.}$)	Ubichromenol ($\mu\text{g./g.}$)	α -Tocopherol ($\mu\text{g./g.}$)	Vitamin A (i.u./g.)
Heart	E	0.82	87	18	7.0	2.2
	F	0.77	159	15	12.6	3.9
	G	0.90	98	10	31.1	—
	H	0.78	158	20	34.0	5.3
Liver	E	7.42	90	53	4.3	323
	F	7.03	114	23	24.2	323
	G	7.29	88	39	36.2	500
	H	6.3	106	59	24.6	—
Kidney	E	1.46	97	6	4.8	4.1
	F	1.39	124	5	11.9	5.1
	G	1.47	49	11	18.3	18.4
	H	1.22	78	16	12.9	5.1
Muscle (hind leg)	E	—	31	5	5.4	1.7
	F	—	46	3	7.5	2.3
	G	—	46	22	27.1	—
	H	—	31	22	12.4	—
Spleen	E	0.80	18	11	10.1	1.3
	F	0.76	26	12	34.9	2.8
	G	0.70	50	18	33.8	—
	H	0.97	6	16	18.7	—
Brain	E	1.74	21	6	2.7	0.8
	F	1.72	28	4	4.2	1.1
	G	1.78	24	12	15.3	1.2
	H	1.75	21	n.d.	9.7	—
Adrenal gland	E	0.059	81	73	63.8	66.0
	F	0.061	130	44	176	86.0
	G	0.063	134	72	300	113
	H	0.050	139	81	285	77.5
Lung	E	1.56	6	3	4.9	13.5
	F	1.77	15	2	15.6	18.5
	G	1.61	13	11	33.6	12.1
	H	1.60	11	5	25.6	—
Nerve	E	0.097	10	tr.	28.5	10.1
	F	0.082	61	tr.	66.9	13.1
	G	0.077	95	24	104.3	30.2
	H	0.110	48	15	46.5	—
Uterus	E	0.71	17	9	8.2	3.1
	F	0.78	67	6	46.9	3.3
	G	0.67	18	7	68.6	4.3
	H	0.90	32	21	33.8	—
Fat	E	—	13	9	4.8	10.5
	F	—	n.d.	n.d.	6.9	2.9
	G	—	15	4	118	1.3
	H	—	tr.	22	161	—
Clot	E	—	n.d.	n.d.	n.d.	0.5
	F	—	tr.	tr.	4.2	1.0
	G	—	tr.	tr.	5.6	1.3
	H	—	27	15	15.4	—
Serum	E	—	n.d.	n.d.	0.6	—
	F	—	n.d.	n.d.	3.2	—
	G	—	n.d.	n.d.	1.6	—
	H	—	n.d.	n.d.	5.0	—

Table 4. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubichromenol in 13-month-old female rats*

Analyses of depot fat are based on extracted lipid, not on the weight of crude tissue. Group J was on the vitamin E-deficient diet A 40; group K, also on A 40, was dosed with tocopherol before death; group L was on diet SD1. n.d., Not detected.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
Heart	J	1.00	41	23	—	—
	K	1.00	106	14	5.7	0.6
	L	1.02	106	14	28.8	1.3
Liver	J	7.68	96	46	7.5	104
	K	7.92	114	71	20.7	102
	L	8.68	133	50	29.9	—
Kidney	J	1.60	86	14	1.2	1.7
	K	1.59	112	7	6.8	1.7
	L	1.58	90	8	25.9	—
Muscle (hind leg)	J	—	22	4	2.1	0.5
	K	—	30	2	6.3	0.6
	L	—	31	5	21.4	—
Spleen	J	0.94	21	8	3.0	2.0
	K	1.03	25	10	44.8	2.0
	L	1.09	31	46	30.6	—
Brain	J	1.74	5	5	1.0	n.d.
	K	1.77	14	6	3.6	n.d.
	L	1.90	29	5	11.1	—
Adrenal gland	J	0.067	122	178	13.9	104
	K	0.073	191	90	211	138
	L	0.069	128	84	264	102
Lung	J	1.92	7	4	8.1	4.2
	K	1.99	11	2	10.3	5.3
	L	1.93	6	4	20.4	5.2
Nerve	J	0.126	28	35	11.4	54.5
	K	0.098	65	18	27.6	9.0
	L	0.113	—	—	94.0	10.9
Uterus	J	1.43	6	4	n.d.	2.7
	K	1.18	22	3	24.6	3.6
	L	1.11	6	2	71.6	1.4
Fat	J	1.71	21	13	2.9	11.5
	K	1.29	78	8	28.1	—
	L	2.51	n.d.	n.d.	6.5	6.6
Clot	J	—	n.d.	3	3.1	n.d.
	K	—	4	1	5.8	0.2
	L	—	5	7	5.7	0.5
Serum	J	—	n.d.	n.d.	2.2	0.38
	K	—	n.d.	n.d.	7.3	—
	L	—	n.d.	n.d.	2.0	—

Table 5. *Precision of analyses and the effect of adding α-tocopherol to rat tissues*

Portions (5–10 g.) of each tissue were analysed. When tocopherol was added, it was added in an acetone solution directly to the acetone used for the initial homogenization and extraction.

Tissue	α-Tocopherol added (μg.)	Replicate analyses (μg./g.)		
		Ubiquinone	Ubichromenol	Tocopherol
Liver A	—	63.8	13.0	8.8
	—	61.7	11.0	9.6
Liver B	—	188	15.0	—
	250	181	15.8	—
Liver C	—	72.5	20.6	—
	250	73.7	23.6	—
Heart	—	204	15.2	16.3
	—	214	12.7	17.5

Table 6. *Measurement of variability between groups of 4-month-old vitamin E-deficient rats*

Expt. 1: Four groups of eight male rats, each group consisting of fully randomized litter-mates. Expt. 2: Four groups of eight male rats selected at random from a batch of 24 born within the same fortnight. Organs from each group were pooled and analysed. Figures given below represent the mean of each set of four results $\pm$ s.e.

Expt.	Organ	Organ wt. (g.)	Ubiquinone (μ g./g.)	Ubichromenol (μ g./g.)	α -Tocopherol (μ g./g.)	Vitamin A (i.u./g.)
1	Heart	0.8 $\pm$ 0.031	211 $\pm$ 3.5	10.2 $\pm$ 0.25	4.2 $\pm$ 0.033	2.3 $\pm$ 0.05
1	Liver	13.0 $\pm$ 0.58	58 $\pm$ 2.0	20.7 $\pm$ 0.67	2.6 $\pm$ 0.07	—
2	Heart	0.74 $\pm$ 0.034	153 $\pm$ 2.1	14.1 $\pm$ 0.12	4.8 $\pm$ 0.11	3.5 $\pm$ 0.14
2	Liver	7.6 $\pm$ 0.71	105 $\pm$ 1.4	38.3 $\pm$ 0.30	3.5 $\pm$ 0.08	226 $\pm$ 2.2

Table 7. *Least significant percentage differences* calculated from the data in Table 6*

Least significant percentage differences (<i>P</i> 0.05) between	Organ wt.	Ubiquinone	Ubichromenol	α -Tocopherol	Vitamin A
Two lots of one group	51	11	10	13	11
Two lots of two groups	36	8	7	9	7
Two lots of three groups	30	6	6	7	6

* Calculated from the pooled data on heart and liver.

Table 8. *Changes in concentrations of ubiquinone and ubichromenol, where these occurred in the same direction in young male, young female and older female rats after oral dosage with vitamin E*

Changes in organs other than those listed did not occur in the same direction in all groups of rats.

Tissue	Mean increase* in ubiquinone (%)	<i>P</i>	
		From least significant difference (see Table 7)	From paired comparisons
Heart	57	<0.05	0.02
Kidney	26	<0.05	0.001
Muscle	40	<0.05	0.1
Spleen	26	<0.05	0.05
Brain	38	<0.05	0.01
Adrenal glands	37	<0.05	0.02
Lung	43	<0.05	0.1
Nerve	74	<0.05	0.05

Tissue	Mean increase* in ubichromenol (%)	<i>P</i>	
		From least significant difference (see Table 7)	From paired comparisons
Heart	-33	<0.05	0.1
Muscle	-64	<0.05	<0.25
Spleen	14	<0.05	0.05
Adrenal glands	-50	<0.05	0.1

* Related to the higher figure in each case.

groups of rats when dosed with α -tocopherol may be treated thus, and the increment over the concentrations in the controls proves to be significant at *P* 0.02. A number of such changes in ubiquinone and ubichromenol (these being the changes of prime interest), occurring consistently in the tissues of the three groups of rats after oral dosage with vitamin E, are set out in Table 8 and their significance is assessed by both methods. In several cases, the paired-comparison method gave a lower

degree of significance than the other method, possibly due to the larger value of *t* (4.3) for the former, compared with *t* 2.26 for the latter. A comparison between the effects of diets A40 and A40E was made only with the younger rats, and the changes in the concentrations of ubiquinone and ubichromenol are recorded in Table 9. Differences of 8 and 7% respectively would probably be significant (see Table 7). The changes in these two substances have been dealt with in more detail because of the biochemical implications. The significance of variation in concentrations of vitamins E and A may be judged by reference to the least significant differences set out in Table 7.

Distribution of vitamin E in the rat

Although several figures already exist in the literature for the vitamin E content of rat tissues (Quaife, Swanson, Dju & Harris, 1949; Swick & Baumann, 1952), certain tissues recorded in the present Tables do not appear to have been studied before. These are erythrocytes, nerve and (surprisingly) uterus. This also seems to be the first study of tissues from the vitamin E-deficient rat, where levels are generally below the range amenable to study by earlier workers.

Younger rats on stock diet SD1. The particular interest of this group of analyses is that they may be taken as representing those found in the normal healthy adult rat. It is at once apparent that there is a considerable sex difference with respect to vitamin E. There was a greater concentration of tocopherol in every tissue taken from females than in the corresponding tissue from the males. Even when the results are recalculated on a 'whole-organ' basis, so as to compensate for the greater weights of the organs in the males (see Tables 2-4), it is still clear that the female rat contains much

Table 9. *Magnitude and significance of changes in concentration of ubiquinone and ubichromenol in tissues of 6-month-old male and female rats on supplementing the deficient diet with vitamin E*

A change of 7-8% is probably significant (see Table 7).

Tissue	Mean increases (%) [*] (A 40 E compared with A 40)	
	Ubiquinone	Ubichromenol
Heart	52	†
Liver	-3	†
Kidney	†	55
Muscle	49	53
Spleen	55	29
Brain	19	†
Adrenal glands	36	-16
Lung	32	77
Nerve	91	100

^{*} Related to the higher figure in each case.

† Change different in different sexes.

more vitamin E than the male. The tissues themselves show considerable variation in tocopherol content. High concentrations occurred particularly in adrenal gland, nerve, heart and uterus. As already shown by Quaife *et al.* (1949), the rat adrenal gland is extraordinarily rich in α -tocopherol. In the present series of experiments the female adrenal gland (whether from stock-colony, vitamin E-deficient or vitamin E-supplemented animals) was found to contain several times more vitamin E than any other tissue.

Younger rats on deficiency diet A 40. The tissues from 6-month-old rats on the vitamin E-deficient diet showed certain differences from those of stock animals. The organs depleted at different rates, resulting in a partial redistribution of vitamin E in the body. The vitamin E-deficient male adrenal glands, for instance, contained more tocopherol than those from animals on diet SD 1. It seemed that female rats depleted more readily than males; this is shown by the fact that five tissues (including the depot fat) in the deficient males contained somewhat more vitamin E than those in the females. No tocopherol could be detected in blood clot from the deficient females, although some was still present in the serum. Major decreases in tocopherol occurred in male testis and kidney during depletion.

Younger rats on supplemented diet A 40 E. The tissue analyses from the 6-month-old animals on this diet also present some interesting features. The diet provided about ten times as much vitamin E as the stock diet SD 1, but uptake by the tissues was variable and, to some degree, selective. In the female, large increases of vitamin E were observed in uterus (compared, for instance, with skeletal muscle) and in nerve and adrenal gland. In general,

most tissues from females on A 40 E contained more tocopherol than those from females on diet SD 1. However, the difference in the composition of the two diets obviously had a profound effect on the uptake of vitamin E: in spite of the high concentrations of tocopherol in A 40 E and in many organs of the rat fed on this diet, the tissues concerned with transport and storage of the vitamin, i.e. serum, fat and, possibly, erythrocytes, contained concentrations of tocopherol that were quite low compared with those in tissues from stock rats. In the male rats on A 40 E, only the adrenal gland showed an unusually high tocopherol level, although spleen and lung contained amounts in excess of normal. Males did not show the big increase in the sciatic nerve, and, in general, the male tissues (including fat and testis) had a lower concentration of vitamin E than did rats on the stock diet. Male serum, however, contained more tocopherol than female serum.

Administration of vitamin E to deficient animals. Dosage of male and female rats with α -tocopherol 48 hr. before death increased the concentrations in nearly all tissues (fat, as might be expected, being hardly affected). The major increases in the male were in spleen and adrenal: heart, brain and liver showed only small changes. Female tissues, in general, showed bigger increases than male tissues. Exceptionally big increases were again found in female adrenal gland, nerve and uterus.

Older rats. The tissues of 13-month-old female rats showed considerable differences compared with those from the younger animals. Comparing, first, animals on the stock diet, although several tissues in the older rats contained less tocopherol than those from the younger ones, the only really big change was in fat, in which a dramatic drop in the vitamin E reserves was apparent. Muscle, spleen and, in particular, nerve and uterus showed big increases on aging. When the two sets of deficient animals are compared (on diet A 40), it will be seen that, in most cases, the tissues of the older rats contained considerably less vitamin E than the younger ones. Uterus at 13 months contained no detectable tocopherol. Administration of 10 mg. of α -tocopheryl acetate to the older animals produced effects similar to those in the younger ones, the biggest increases being in adrenal gland and uterus.

Relationships between vitamins A and E in experimental vitamin E deficiency

It is well known that vitamin A levels in the rat are affected by the amounts of vitamin E in the diet. Since the studies of Morton and co-workers (see, for example, Morton & Phillips, 1959a) have indicated that concentrations of ubiquinone and ubichromenol in rat liver are influenced by vitamin A,

throughout most of the present series of experiments vitamin A was measured in the tissues whenever this was possible, and the results are recorded in Tables 2-4. As was to be expected from the findings of earlier workers, for example Moore (1939), the vitamin A of most tissues fell in concentration during vitamin E deficiency (although in adrenal gland and lung little change was observed). Administration of tocopherol before death caused appreciable increases of vitamin A in nearly all the tissues with the exception of fat and liver, the effects being similar in both sexes.

Ubiquinone and ubichromenol in the stock-colony rat

Although a few isolated reports have already appeared dealing with concentrations of ubiquinone in the rat, these have so far been restricted to a few tissues. Moore & Sharman (1959) and Moore (1960) reported figures for heart and liver, but the values are much higher than any found in our experiments: Moore did not use either chromatographic purification or borohydride assay, however. Morton & Phillips (1959*a, b*) examined liver, kidney, heart and testes and the concentrations reported by them for stock animals are of the same order as found in our series.

To compare the quantitative nature of our results with those of Morton & Phillips, we have compared our extraction and saponification procedure with that of these workers, from which it differs in several respects. A quantity of pooled rat livers was minced and divided into several 10 g. portions. These were saponified and extracted by several methods as described in Table 10, which records the concentrations of ubiquinone, ubichromenol

and tocopherol determined by each method. The method of Mervyn & Morton (1959), in which the total tissue is saponified, gave good agreement with our standard method, in which the lipid extract only is treated for 5 min. under reflux with ethanolic potassium hydroxide. When the ethanol used in the method of Mervyn & Morton was replaced by Grignard-purified methanol, the recovery of ubiquinone was excellent, but the ubichromenol figure was lower. Saponification of the tissue with aqueous alkali gave a poor recovery of all three substances. Methanolic potassium hydroxide cannot be used successfully on the extracted lipid. Major differences in the recovery of ubiquinone and ubichromenol (but not of α -tocopherol) were found when ordinary analytical reagent-grade methanol was substituted for the specially purified methanol and some caution is obviously necessary when this reagent is used. Comparison of the ubiquinone fractions obtained from these experiments indicated that the compound previously tentatively identified by us as a dihydroubiquinone (Diplock *et al.* 1960*a*) may have been a small amount of the ethoxy compound produced during the saponification process (Linn, Trenner, Shunk & Folkers, 1959).

As shown in Tables 2-4, the highest amounts of ubiquinone in animals on diet SD 1 were found in heart at 6 months of age; in the older females the amount in heart had decreased considerably, more than was compensated for by the increase in weight of the organ. High concentrations of ubiquinone were observed in liver, and also in adrenal, nerve and kidney. Uterus contained considerably more ubiquinone than testis at 6 months, and at 13 months, the uterus was heavily depleted of ubi-

Table 10. *Effect of various methods of saponification of vitamin E-deficient rat liver on the recovery of ubiquinone, ubichromenol and α -tocopherol*

Portions (10 g.) of a pooled batch of liver were used, and in Expts. 1-5 they were saponified directly according to Mervyn & Morton (1959); in Expts. 6-9 they were extracted and then saponified with the reagents described in the Table. Each non-saponifiable extract was separately analysed, after removal of sterols and treatment on Decalco, by two-dimensional paper chromatography.

Expt. no.	Conditions	Saponification time (min.)	Ubiquinone (μ g./g.)	Ubichromenol (μ g./g.)	α -Tocopherol (μ g./g.)
1	Ethanol (4 ml.)-KOH (1 ml.)	3	33.9	26.9	2.2
2	Ethanol (4 ml.)-KOH (2 ml.)	5	35.2	26.9	2.4
3	Purified methanol* (4 ml.)-KOH (2 ml.)	5	23.1	26.9	2.1
4	Methanol† (4 ml.)-KOH (2 ml.)	5	7.7	3.3	3.6†
5	Water (4 ml.)-KOH (2 ml.)	10	8.2	8.7	1.2
6	Total tissue with ethanol	60	36.2	25.7	2.2
7	Total tissue with methanol†	60	34.2	10.2	3.7†
8	Total tissue with purified methanol*	60	26.0	27.3	1.9
9	Total tissue with water	60	11.7	10.0	2.4

* Methanol (A.R.) purified by treatment with methyl magnesium iodide, followed by fractional distillation.

† Methanol (A.R.) from drum.

‡ Colours increased continually during Emmerie-Engel determination. This was often noticed after other saponifications with methanolic KOH.

quinone although, it should be noted, the tocopherol content had increased. As with vitamin E, the female tissues generally contained more ubiquinone than did the male tissues, the main exception being found in spleen. Ubiquinone was found in blood clot in considerable quantities, but none was found in serum and only traces could be detected in body fat. More recent work, however, has indicated that washed erythrocytes do not contain appreciable amounts of ubiquinone, and, in all probability, the amounts recorded in blood clot were derived from leucocytes.

Ubichromenol was found in all tissues except serum. Concentrations were usually lower than corresponding concentrations of ubiquinone in the same tissue and were highest in adrenal gland and liver in both sexes. Fat from both sexes contained appreciable quantities of ubichromenol, although very little ubiquinone could be detected: this was the only tissue in which this occurred.

Effect of dietary vitamin E on ubiquinone and ubichromenol

With a few exceptions, concentrations of ubiquinone were found to be significantly lower in the vitamin E-deficient animals on diet A 40, compared with either stock-colony animals or those on the supplemented diet A 40E (see Tables 7 and 8). In the younger rats, vitamin E deficiency produced substantial falls in the ubiquinone level in sciatic nerve, spleen and adrenal gland. Some tissues showed little change, whereas female kidney (6 months) and male adrenal gland and testis showed small increases. With testis, changes in organ weight under the influence of vitamin E tend to obscure the interpretation of the ubiquinone

concentrations. Ubichromenol concentrations were also decreased in most tissues of the vitamin E-deficient 6-month-old rat, the only exceptions being brain in both sexes and adrenal gland and testis in the male. In the old females, higher concentrations of ubichromenol were found in some of the vitamin E-deficient tissues when these were compared with stock-colony animals.

Effect of vitamin E administration before death

Administration of 10 mg. of DL- α -tocopheryl acetate 72–48 hr. before death increased the concentrations of vitamin E in all tissues of the three groups investigated. Particularly big increases occurred in uterus, adrenal glands, spleen and, in the younger females, sciatic nerve. Vitamin E administration produced significantly increased concentrations of ubiquinone in most tissues studied (see Table 8); the chief exceptions found were in fat from 6-month-old females and in liver from the males. The largest increases were in uterus, heart and nerve: the concentration changes in liver were relatively small.

The increase in ubiquinone was in most tissues accompanied by a decrease in ubichromenol (see Table 8) but this did not occur consistently in all three experiments. A few exceptions were observed: spleen showed a significant increase in ubichromenol, and male liver, almost the only tissue in which ubiquinone decreased, also showed an increase in ubichromenol.

Effect of dietary selenium on ubiquinone and ubichromenol

The role of dietary selenium in its relation to vitamin E-deficiency conditions is not understood;

Table 11. *Effect of dietary selenite on ubiquinone and ubichromenol in the tissues of the rat*

Groups M and O were fed on the necrogenic diet. Groups N and P were fed on the necrogenic diet + selenite. Group Q was fed on the necrogenic diet + α -tocopherol. Group R was fed on the necrogenic diet + α -tocopherol and selenite. Group S was fed on diet A 40. Group T was fed on diet A 40 + selenite.

Expt. no.	Tissue	Group	Ubiquinone (μ g./g.)	Ubichromenol (μ g./g.)	α -Tocopherol (μ g./g.)
1	Heart	M	99.7	*	2.6
		N	157	*	1.9
	Kidney	M	45.9	*	0.3
		N	62.9	*	0.5
2	Heart	O	84.6	9.5	6.9
		P	136	22.2	5.5
		Q	92.9	7.1	18.7
		R	128	9.2	20.2
	Liver	O	35.6	8.9	2.6
		P	57.1	18.7	3.4
		R	71.6	16.4	16.4
3	Heart	S	207	10.2	7.5
		T	205	13.6	7.3
	Liver	S	101	17.7	2.3
		T	233	29.2	4.0

* Present but not measured.

but, since the identification of Factor 3, which prevents liver necrosis in rats, with natural selenium compounds (Schwarz & Foltz, 1957), a close and growing correspondence between selenium and α -tocopherol in preventing nutritional deficiencies has been recognized. It was therefore of interest to study the influence of selenium on concentrations of ubiquinone and ubichromenol.

Table 11 records the results of the three experiments carried out, details of which have been given in an earlier section. It was found that the addition of selenium to the necrogenic diet significantly increased the amount of ubiquinone in the two tissues examined (heart and kidney) in the first experiment. In the second experiment, selenium added to the necrogenic diet significantly increased the amount of ubiquinone in heart and liver. In the third experiment, selenium added to a vitamin E-deficient diet (non-necrogenic) increased ubiquinone in liver, but not in heart.

DISCUSSION

The vitamin E-deficient diet (A40) used in these experiments was found to contain $2.4 \mu\text{g.}$ of α -tocopherol/g. As far as we are aware, this is the first direct determination to be made of a tocopherol-deficient rat diet, and the figure is surprisingly high, if it is compared with that obtained on the nutritionally adequate diet, SD 1 ($9.5 \mu\text{g./g.}$). Most of the tocopherol in the deficient diet evidently comes from the lard. Lest it be thought that the type of lard used contained unduly large amounts of tocopherol, we have in fact examined several 'pure' lards and found them to be similar in tocopherol content. The extracted casein also contained tocopherol. The discovery of these hitherto unsuspected amounts of tocopherol in constituents believed to be practically vitamin E-free is a direct consequence of the substitution of Decalso for activated floridin earth in the purification stage of vitamin E analysis. Although floridin-earth purification is suitable for the analysis of vegetable oils (Report of the Vitamin E Panel, S.A.C., 1959) and normal animal tissues (Edwin *et al.* 1960), there seems no doubt that it must now be regarded as unsuitable for the determination of tocopherol in exceptionally low concentrations, such as occur in vitamin E-deficient animal tissues and purified diets and fats. As a further example of this, we have analysed the 'stripped' lard, especially prepared by Distillation Products Industries (U.S.A.) for use in vitamin E-free diets. Bieri, Briggs, Pollard & Fox (1960) reported less than $0.5 \mu\text{g.}$ of tocopherol/g. in this material by the use of a method essentially similar to ours, but using activated Florex for purification. When we determined the α -tocopherol on the un-

saponifiable fraction from 20 g. of this lard, with Decalso followed by paper chromatography, we found $3.3 \mu\text{g./g.}$ (as such, it should be pointed out, it was the most tocopherol-free lard we encountered and fell well within the manufacturers' declaration).

In spite of the amount of tocopherol in diet A40, this diet produced all the usual symptoms of vitamin E deficiency in our rats. Presumably it contained less vitamin E than the standard bio-assay diet recommended by Mason & Harris (1942), which contained up to 10% of olive oil (a rich source of tocopherol by these standards).

These findings confirm what is already recognized, that the requirements for vitamin E are dependent on both the quantitative and qualitative aspects of dietary composition, particularly, but probably not only, with respect to fat. This is generally reinforced by the results of the present series of experiments, which show that deposition of tocopherol in the adipose tissue of the rat is profoundly affected by the type of diet, more so than by the amount of tocopherol ingested. It follows from these experiments that vitamin E status in the rat cannot be simply defined. It must certainly be based on actual tissue analyses, and it would seem, further, that those most usually carried out (serum or fat analyses) might not, at least in the rat, be the most helpful. A better guide to vitamin E status would appear to be the analysis of heart muscle.

The rat is able to take up large amounts of α -tocopherol from its diet (or from an administered dose) and incorporate them into its tissues. This ability is not so unremarkable as it might seem: in succeeding papers it will be shown that it is a characteristic not found in all species. The data presented in Tables 2-4 show that the tissues of the rat vary considerably in tocopherol uptake, and this is so in both sexes. Adrenal gland and nerve, particularly the former, accumulate exceptionally large concentrations of tocopherol, and it may be noted that these concentrations tend to persist into extreme vitamin E deficiency (which, in the rat, is marked by virtual exhaustion of the tocopherol stored in the fat). If these high concentrations of tocopherol in the adrenal gland are considered in conjunction with an earlier finding of Quaife *et al.* (1949), who showed that the concentration of tocopherol in rat pituitary was three times as great as that in the adrenal gland, it is tempting to speculate that there is a specific physiological need for the vitamin connected with high metabolic activity of these two organs. If vitamin E is simply acting as an intracellular antioxidant, highest concentrations might well be expected in those tissues where high rates of metabolic turnover would conceivably give rise to other labile intermediates whose removal

from the cell was essential. However, such a simple approach is certainly not encouraged by the finding that both liver and kidney take up only moderate amounts of tocopherol and deplete very readily; organs of low metabolic activity such as uterus and nerve build up high concentrations of tocopherol.

In view of our present findings, it is essential to examine the 'antioxidant' theory of vitamin E more extensively. Several workers, especially Tappel & Zalkin (1959*a, b*) and Zalkin & Tappel (1960), have suggested that the biological role of vitamin E is limited to that of an intracellular antioxidant. Two functions are usually considered: (a) the protection of other essential but sensitive substances, such as vitamin A and unsaturated fats, and (b) the removal of free-radical intermediates produced by the oxidation of unsaturated lipids, which might react with and damage other cellular components, such as members of the electron-transport chain. With regard to the first type of function, although it is clear from our results that vitamin E has a sparing effect on the vitamin A in the tissues, there does not appear to be any regular correlation in the concentrations of these two substances. It is true that adrenal gland contains much vitamin A, but liver contains much more, and there is no evidence of high vitamin E concentrations in those organs that contain exceptional amounts of vitamin A, such as lung or kidney. There are more serious difficulties about accepting the conclusions of Tappel & Zalkin for the second function. First, the formation of lipid 'peroxides' (as measured by the thiobarbituric acid reagent) in various tissues cannot, it seems to us, be related to the vitamin E content of these tissues, nor can the somewhat limited effect of tocopherol in preventing peroxidation be simply related to the amount or type of unsaturated lipid in the tissues. Secondly, the specific effects of vitamin E deficiency in animals, which vary according to the species under study, cannot be related to the action of tocopherol in preventing tissue peroxidation. Thus in the rabbit enhanced peroxidation has been demonstrated by Zalkin & Tappel in vitamin E-deficient liver and kidney, but not in muscle, which in this animal is the main tissue affected by vitamin E deficiency (see, however, Carpenter, Kitabchi, McCay & Caputto, 1959). Similarly, Carpenter *et al.* have shown that although rat liver is subject to peroxidation and that this can be prevented by tocopherol, rat testis, the organ in the male chiefly affected by vitamin E deficiency, is readily peroxidized and this cannot be prevented by vitamin E. Pritchard & Singh (1960) related the extent of peroxidation of several rat tissues to the polyunsaturated fatty acid content of the tissues. They showed (a) that adrenal gland was extraordinarily sensitive to

peroxidation and (b) that neither the peroxidation nor its prevention by vitamin E could be simply related to the polyunsaturated fatty acid composition of the tissue. It would appear therefore that the organ most susceptible to lipid peroxidation in the rat is the one that, according to our findings, contains more vitamin E than any other organ in the body, whether from deficient or supplemented animals. Pritchard & Singh also showed that vitamin E-deficient liver was the tissue least susceptible to peroxidation, and, again, if our findings are relevant, this is a tissue that contains one of the lowest tocopherol concentrations in the body. Pritchard & Singh found that brain peroxidation was very high and could not be prevented by administration of vitamin E. Our results show that, although brain contains relatively small amounts of vitamin E, tocopherol passes the blood-brain barrier [compare Bieri (1960), who has suggested that in chicks this might not be so, and Abderhalden & Elsaesser (1946), who found no tocopherol in human cerebrospinal fluid]. Unpublished work in this laboratory has confirmed that high levels of peroxidation are normal in the brains of adequately fed rats and chickens, indicating that the 'peroxidation' measured by the thiobarbituric acid reagent, at any rate, is not necessarily either an abnormal or deleterious phenomenon in tissues. In this connexion, although Zalkin & Tappel consider that the cellular damage produced by vitamin E deficiency takes place in the mitochondria, where most of the unsaturated lipid is, Pritchard & Singh found that most of the peroxidation in rat brain and heart took place in the microsomes and supernatant fraction (see also Carpenter *et al.* 1959). It would be interesting to compare the peroxidation of nerve tissue with brain in the rat, in view of the completely different amounts of vitamin E in these two tissues.

A further point of interest that emerges from the data on vitamin E distribution is the significant response of uterus both to depletion of and to administration of vitamin E. In view of the nature of this organ, whose tissue has relatively little metabolic or synthetic activity, it would appear reasonable to associate this property with its potential function, that is, with the requirements of foetal development. It is known that vitamin E can be transferred across the rat placenta only with difficulty (Mason & Bryan, 1938). It is possible therefore that the uterine reserves of tocopherol in the rat may be needed for transfer to the foetus in the early stages of development. The significance of this concept is that such a deficiency might be not only relative but local, in the sense that it could arise as a unique manifestation of the deficiency state as a result of an abnormally high tocopherol

requirement of the young foetal tissue, and whilst other tissues in the body still contained sufficient tocopherol for their needs. This possibility that foetal rather than solely uterine requirements for tocopherol are involved in the phenomenon of gestation-resorption in the rat receives some support from observations made by various workers in the classical studies of the condition. Among these may be mentioned the fact that tocopherol can prevent resorption if given during the first week of pregnancy (Mason, 1942), the absence of an influence of uterine pigmentation or other uterine changes on subsequent pregnancy, provided that vitamin E is given (Evans & Emerson, 1943), and the observed anatomical and histological phenomena associated with foetal death, which include diminished haemopoiesis and asphyxia due to failure of the maternal-foetal vascular relationship (Evans & Burr, 1927; Urner, 1931; compare the histological manifestations of encephalomalacia in the chick, which also involves vascular degeneration, described by Wolf & Pappenheimer, 1931). It may be speculated whether the specific manifestations of vitamin E deficiency in other species may not similarly reflect local tissue deficiencies of tocopherol.

Concentrations of ubiquinone and ubiquinol also show large variations from tissue to tissue, and, in general, are highest in those tissues that are richest in tocopherol.

The statistical treatment of the data leads to the conclusion that deficiency of vitamin E has a marked influence on the concentrations of ubiquinone and ubiquinol in the rat. The rapid effect of oral administration of vitamin E in restoring ubiquinone and ubiquinol concentrations in the tissues suggests that a biosynthetic relationship exists between the three substances, although this may be of an indirect nature. There appears to be no obvious connexion between these findings and any auxiliary function that tocopherol may have as a physiological antioxidant. If tocopherol were 'sparing' ubiquinone in the same manner as, at a lower level of concentration, it spares vitamin A, it is difficult to see why it does not spare ubiquinol, which we have found to be even more sensitive to oxidation than the quinone.

If 'antioxidant' theories are insufficient to account for the action of vitamin E, it is necessary to look elsewhere for an explanation of its effect on ubiquinone. Gray (1959) has shown that, in rats, dietary supplementation with vitamin E increases the production of cholesterol by liver homogenates *in vitro*. A similar effect might well be involved in the synthesis of the ubiquinone side chain, which is derived, like cholesterol, from mevalonic acid [the effect of vitamin E in counteracting hypercholesterolaemia in the rabbit (Smith, Shin & Freier,

1960; Draper, 1959) and decreasing concentrations of cholesterol produced by exogenous cholesterol in the rat (Aftergood, Deuel & Alfin-Slater, 1957) is due to another mechanism, the control of unsaturated fatty acid uptake (see, in this connexion, Pritchard & Singh, 1960)]. A number of workers have tentatively implicated vitamin E with oxidative phosphorylation, and Chernick, Moe, Rodnan & Schwarz (1955) suggested that its effect may be on adenosine triphosphate (ATP) synthesis. The recent demonstration by Gray, Chisholm & Lee Peng (1960) that dietary supplementation with α -tocopherol significantly increases liver synthesis of ATP and coenzyme A in the rat must be carefully considered. Gray *et al.* (1960) have proposed a hypothetical scheme of synthesis for ATP, which involves tocopherol in phosphorylation reactions. It seems germane to ask, does the increased synthesis of ATP lead to the increased synthesis of substances such as cholesterol, coenzyme A and ubiquinone; or, does tocopherol have a more specific influence on ubiquinone synthesis, which, by increasing oxidative-phosphorylation processes, would in turn lead to an increase in ATP? It may be significant that, in the present experiments, the influence on ubiquinone was observed with only one-hundredth of the dietary α -tocopherol used by Gray *et al.* (1960) (but compare the difference in dietary composition). Whatever the mechanism, though, such an effect of tocopherol on ubiquinone must have other consequences. The latter substance is an essential component of the electron-transport chain, connecting the flow of electrons from substrate to the cytochromes (Hatefi, Lester, Crane & Widmer, 1959). Tocopherol therefore, by an effect on ubiquinone concentration, could affect electron transport. Such a role would make relevant and understandable many of the earlier findings that have linked vitamin E with respiration processes (for example, see Hove, Hickman & Harris, 1945; and Taylor, 1953). Subsidiary effects, for example on the synthesis of ascorbic acid (Caputto, McCay & Carpenter, 1958), might also be related.

Study of the data does not disclose any obvious connexion between concentrations of ubiquinone and vitamin A in the various tissues. Morton and his co-workers have discussed in several papers the relation between concentration of ubiquinone and vitamin A deficiency in the rat (for example, Lowe, Morton & Harrison, 1953; Heaton, Lowe & Morton, 1957), and it seems that the matter must now be more fully elucidated in terms of the complex relationship between vitamins A and E. Morton & Phillips (1959*b*) found that vitamin E had no effect on amounts of ubiquinone in rat liver, heart, kidney or testis. While we are unable to account for the difference between their results and ours,

there were not only great differences in analytical technique, but the vitamin A status of the animals was quite different in the two sets of experiments.

The effect of dietary selenium in increasing concentrations of both ubiquinone and ubichromenol is similar to the effect of dietary tocopherol. There are two additional points of interest. Whereas the effect of tocopherol is usually small in liver, that of selenium appears to be more pronounced. This may have some bearing on the role of selenium in preventing nutritional liver necrosis in rats (Schwarz & Foltz, 1957), whereas it is ineffective in preventing gestation-resorption (Harris, Ludwig & Schwarz, 1958). Secondly, dietary selenium was obviously able to enhance ubiquinone concentrations (in liver, at least), even though the basal diet must be assumed to have contained adequate amounts of Factor 3 (organic selenium compounds), because of its casein content. The observations already made, however, confirm the duality of the biochemical roles of selenium and α -tocopherol and render it unlikely that the biological role of the latter can be understood in terms of its function as a physiological antioxidant.

SUMMARY

1. The distribution of vitamin E, vitamin A, ubiquinone and ubichromenol has been studied in 14 tissues of the rat. Tissues richest in vitamin E are usually richest in ubiquinone. High concentrations of vitamin E and ubiquinone are present in adrenal gland, heart, uterus and nerve.

2. The vitamin E content of the tissues is affected by the nature of the diet.

3. Tissues lost their vitamin E at different rates during depletion. Tissues such as adrenal gland and nerve retain high concentrations of tocopherol, whereas uterus and liver readily lose their tocopherol. The susceptibility of uterus to tocopherol depletion and administration is related to the gestation-resorption syndrome in the rat.

4. The distribution of the four substances has been compared in 6-month-old and 13-month-old female rats and in males and females at 6 months.

5. Female rats contain higher amounts of the four substances than male rats of the same age.

6. Male and female vitamin E-deficient rats have lower concentrations of ubiquinone in their tissues than either the corresponding animals whose diet has been supplemented with vitamin E or animals on the stock diet. Oral administration of vitamin E to such deficient animals increases the ubiquinone concentration in their tissues. This increase in ubiquinone is often accompanied by a decrease in ubichromenol concentration, which tends to be elevated in the vitamin E-deficient rat.

7. Dietary selenium increases the concentration

of ubiquinone in the same way as does α -tocopherol. It seems that there is a biosynthetic relationship between tocopherol, selenium, ubiquinone and ubichromenol.

8. The specific syndrome of vitamin E deficiency in animals may be related to a local tissue deficiency of this vitamin.

We wish to thank Mrs W. S. Miller and Miss Jill Malein for their great help with the analyses.

REFERENCES

- Abderhalden, R. & Elsaesser, K. H. (1946). *Z. Vitaminforsch.* **17**, 317.
- Aftergood, L., Deuel, H. J. & Alfin-Slater, R. B. (1957). *J. Nutr.* **62**, 129.
- Bieri, J. G. (1960). *Nature, Lond.*, **184**, 1148.
- Bieri, J. G., Briggs, G. M., Pollard, C. J. & Fox, M. R. S. (1960). *J. Nutr.* **70**, 47.
- Bunyan, J., Green, J., Edwin, E. E. & Diplock, A. T. (1960). *Biochem. J.* **77**, 47.
- Caputto, R., McCay, P. B. & Carpenter, M. P. (1958). *J. biol. Chem.* **233**, 1025.
- Carpenter, M. P., Kitabchi, A. E., McCay, P. B. & Caputto, R. (1959). *J. biol. Chem.* **234**, 2814.
- Chernick, S. S., Moe, J. G., Rodnan, G. P. & Schwarz, K. (1955). *J. biol. Chem.* **217**, 829.
- Crane, F. L., Hatefi, Y., Lester, R. L. & Widmer, C. (1957). *Biochim. biophys. Acta*, **25**, 220.
- Diplock, A. T., Edwin, E. E., Green, J., Bunyan, J. & Marcinkiewicz, S. (1960a). *Nature, Lond.*, **186**, 554.
- Diplock, A. T., Green, J., Edwin, E. E. & Bunyan, J. (1960b). *Biochem. J.* **76**, 563.
- Draper, H. H. (1959). *Proc. Soc. exp. Biol., N.Y.*, **202**, 737.
- Edwin, E. E., Diplock, A. T., Bunyan, J. & Green, J. (1960). *Biochem. J.* **75**, 450.
- Evans, H. M. & Burr, G. O. (1927). *Mem. Univ. Calif.* **8**, 1.
- Evans, H. M. & Emerson, G. A. (1943). *J. Nutr.* **26**, 555.
- Gray, D. E. (1959). *J. Vitaminol., Japan*, **5**, 19.
- Gray, D. E., Chisholm, J. & Lee Peng, C. H. (1960). *J. Vitaminol., Japan*, **6**, 62.
- Green, J., Edwin, E. E., Bunyan, J. & Diplock, A. T. (1960). *Biochem. J.* **75**, 456.
- Harris, P. L., Ludwig, M. I. & Schwarz, K. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 686.
- Hatefi, Y., Lester, R. L., Crane, F. L. & Widmer, C. (1959). *Biochim. biophys. Acta*, **31**, 490.
- Heaton, F. W., Lowe, J. S. & Morton, R. A. (1957). *Biochem. J.* **67**, 208.
- Hove, E. L., Hickman, K. C. D. & Harris, P. L. (1945). *Arch. Biochem.* **8**, 395.
- Laidman, D. L., Morton, R. A., Paterson, J. Y. F. & Pennock, J. F. (1960). *Biochem. J.* **74**, 541.
- Linn, B. O., Trenner, N. R., Shunk, C. H. & Folkers, K. (1959). *J. Amer. chem. Soc.* **81**, 1263.
- Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.
- Mason, K. E. (1942). *Yale J. Biol. Med.* **14**, 605.
- Mason, K. E. & Bryan, W. L. (1938). *Biochem. J.* **32**, 1784.
- Mason, K. E. & Harris, P. L. (1942). *Biol. Symp.* **12**, 459.
- Mervyn, L. & Morton, R. A. (1959). *Biochem. J.* **72**, 106.
- Moore, T. (1939). *Chem. & Ind.* **58**, 65.

- Moore, T. (1960). *Nature, Lond.*, **184**, 607.
- Moore, T. & Sharman, I. M. (1959). *Biochem. J.* **73**, 67.
- Morton, R. A. & Phillips, E. J. (1959a). *Biochem. J.* **73**, 416.
- Morton, R. A. & Phillips, E. J. (1959b). *Biochem. J.* **73**, 427.
- Pritchard, E. T. & Singh, H. (1960). *Biochem. biophys. Res. Commun.* **2**, 184.
- Quaife, M. L., Swanson, W. J., Dju, M. Y. & Harris, P. L. (1949). *Ann. N.Y. Acad. Sci.* **52**, 300.
- Report of the Vitamin E Panel, Society for Analytical Chemistry. Quoted in *Analyst* (1959), **84**, 356.
- Schwarz, K. & Foltz, C. M. (1957). *J. Amer. chem. Soc.* **79**, 3292.
- Smith, L. C., Shin, Y. S. & Freier, D. T. (1960). *Proc. Soc. exp. Biol., N.Y.*, **103**, 56.
- Snedecor, G. W. (1946a). *Statistical Methods*, 4th ed., 10.2. Ames, Iowa: State College Press.
- Snedecor, G. W. (1946b). *Statistical Methods*, 4th ed., 2.13. Ames, Iowa: State College Press.
- Swick, R. W. & Baumann, C. A. (1952). *Analyt. Chem.* **24**, 758.
- Tappel, A. L. & Zalkin, H. (1959a). *Arch. Biochem. Biophys.* **80**, 326.
- Tappel, A. L. & Zalkin, H. (1959b). *Arch. Biochem. Biophys.* **80**, 333.
- Taylor, D. W. (1953). *J. Physiol.* **121**, 477.
- Urner, J. A. (1931). *Anat. Rec.* **50**, 175.
- Wolf, A. & Pappenheimer, A. M. (1931). *J. exp. Med.* **54**, 399.
- Zalkin, H. & Tappel, A. L. (1960). *Arch. Biochem. Biophys.* **88**, 113.

Biochem. J. (1961) **79**, 105

Studies on Vitamin E

7. THE EFFECT OF THIAMINE, RIBOFLAVIN AND PANTOTHENIC ACID ON UBIQUINONE AND UBICHROMENOL IN THE RAT

By A. T. DIPLOCK, J. BUNYAN, J. GREEN AND E. E. EDWIN
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

(Received 16 August 1960)

In the preceding paper, Edwin, Diplock, Bunyan & Green (1961) have shown that concentrations of ubiquinone and ubichromenol in the tissues of the rat are dependent on vitamin E status. It was of interest to determine whether this effect was specific to vitamin E, or whether concentrations of ubiquinone in the rat were generally influenced by nutritional status, in particular by other vitamins. In the present work, the effect of thiamine, riboflavin and pantothenic acid on ubiquinone was studied.

EXPERIMENTAL

Animals, diets and analyses

Male and female rats of the Norwegian hooded strain were used. They were removed from the stock colony at 4-5 weeks of age and placed on the test diets. They were killed with ether.

Basal diet. This had the following composition: casein (Genatosan Ltd. 'low-vitamin content'; 25%), sucrose (50%), glucose (10%), arachis oil (10%), a vitamin mixture (0.4%) (to supply per kg. of diet: nicotinic acid, 90 mg.; pyridoxine, 9 mg.; folic acid, 2 mg.; calcium pantothenate 90 mg.; vitamin B₁₂, 0.03 mg.; inositol, 90 mg.; *p*-aminobenzoic acid, 90 mg.; choline hydrogen tartrate, 1.8 g.; menaphthone, 20 mg.) and Cox's salt mixture (5%) to provide (g./kg. of diet): NaCl, 2.7; CaH₄(PO₄)₂, 21.7; ferric citrate, 1.9; NaH₂PO₄, 5.5; MgSO₄·7H₂O, 4.3; KCl,

13.6; KI, 0.055; NaF, 0.0021; MnSO₄·4H₂O, 0.011. Vitamin A, vitamin D₃ and α -tocopheryl acetate were added to supply respectively 4000 i.u., 400 i.u. and 120 mg./kg.

Thiamine-deficient diet. This was the basal diet to which had been added riboflavin, 19 mg./kg., and calcium pantothenate, 90 mg./kg.

Riboflavin-deficient diet. This was the basal diet to which had been added thiamine, 9 mg./kg., and calcium pantothenate, 90 mg./kg.

Pantothenic acid-deficient diet. This was the basal diet to which had been added thiamine, 9 mg./kg., and riboflavin, 18 mg./kg.

Experimental plan and analyses. The experiments on thiamine and riboflavin deficiency were carried out simultaneously with one group of controls. Rats were divided into groups of eight (equal numbers of males and females in each) at 28 days of age. Group A received the basal diet supplemented with thiamine and riboflavin. Groups B and C received the thiamine-deficient diet, groups D and E the riboflavin-deficient diet. The groups were maintained on their diets for 38 days, by which time the deficient groups were showing weight losses. At 72 hr. before the end of the experiment, and again at 48 hr., group C rats were given oral doses of 0.5 mg. of thiamine and group E rats were given oral doses of 1.25 mg. of riboflavin. A similar experiment was devised for pantothenic acid deficiency. Three groups of rats were used (10 in each group, equal numbers of each sex). Group F received the deficient diet supplemented with calcium pantothenate, groups G and H the deficient diet. At the end of the experiment (28 days),