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Studies on Vitamin E

1. THE DETERMINATION OF TOCOPHEROLS IN ANIMAL TISSUES

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(Received 13 July 1959)

In connexion with a programme designed to study the vitamin E status of animals under different nutritional conditions, a rapid and accurate method of determining tocopherols in animal tissues was required. Two problems exist: the extraction, without loss, of the material sensitive to oxidation and its separation from large quantities of other reducing substances before determination.

The extraction problem was of most immediate interest and has received considerable attention from previous workers in the field. Apart from blood and milk, which cause no special extraction difficulties, some kind of comminution or homogenization of the tissue is usually necessary, and a number of different methods have been used by previous workers. Karrer, Jaeger & Keller (1940) chopped organs as finely as possible and extracted them with ethanol-benzene (1:1). Devlin & Matthill (1942) homogenized rat muscle in the Waring Blendor with the foaming solvent mixture (Skellysolve B-ethanol; 8.5:10.0) of Moore & Ely (1941), three separate extractions being used to ensure complete extraction. Hines & Matthill (1943), in the first systematic study of the vitamin E content of animal tissues, used the same procedure. Emmerie & Engel (1943) first introduced the use of boiling ethanol as solvent. Kaunitz & Beaver (1946) adopted a lengthy procedure, emulsifying muscle first with large quantities of acetone in the Waring Blendor and then with several mixtures of acetone and Skellysolve B. Even after this procedure, reducing substances could still be extracted from the tissue. Kringstad

& Folkvord (1949), studying cod roes, homogenized the tissue and extracted with ether. Wanntorp & Nordlung (1947) and more recently Benze (1954) treated the problem by directly saponifying the whole tissue, but recoveries from this procedure are not given. Quaife & Dju (1949), in one of the best studies on vitamin E analysis, criticized both saponification techniques and the use of the Waring Blendor, and they adopted a dry homogenization technique, which they considered essential for the analysis of animal tissues. The tissues were first diced and then frozen in liquid nitrogen, ground in an iron mortar at -70° , sieved and finally extracted with hot ethanol vapour. A 24 hr. extraction period was necessary to remove reducing substances completely. Bratzler, Loosli, Krukowsky & Maynard (1950), studying tocopherol levels in the pig, freeze-dried the tissues and then ground the organs in a Wiley mill and the muscle in a Waring Blendor with solid carbon dioxide, finally extracting with ethanol vapour for 24 hr. Swick & Baumann (1952) used a similar technique, but homogenized smaller samples in ethanol before extraction in a Soxhlet apparatus for 18 hr. Draper & Csallany (1958) comminuted samples of rat and rabbit tissue by grinding with acid-washed sand.

The problems connected with the removal of the extensive reducing impurities from the tocopherol extract were attacked by earlier workers in many different ways and have led to extremely variable estimates for the vitamin E contents of animal tissues. The various analytical modifications that

have been used may be consulted in the *Annotated Bibliography of Vitamin E* (1940–57). For example, Feldheim (1956) has listed 40 references to methods for the analysis of vitamin E in human serum and points out that the variation in the literature figures (0.08–2.00 mg./100 ml.) reflects differences in the purification methods (no extraction difficulties occur with serum and almost all workers have used the same ferric chloride–dipyridyl method for the determination of tocopherol). A novel method of analysis was introduced recently by Nair & Magar (1954), who introduced the phosphomolybdic acid reagent, claimed to be fairly specific for tocopherols (and presumably necessitating less preliminary purification of extracts). The same authors (1956) used this reagent to determine vitamin E in blood, which method was also used by Garton, Duncan, Madsen, Shanks & Beattie (1958) in a study of serum tocopherols in the pig. Rosenkrantz (1957) used the phosphomolybdic acid method in a microprocedure for the analysis of vitamin E in human serum and rabbit tissues.

In the present work, a new method of homogenization and extraction has been used. The tissue is frozen by immersion in acetone at -70° and then ground at this temperature with a mixture of sodium sulphate and acetone. There is rapid conversion into a powder, which can be extracted easily with more acetone in a Soxhlet apparatus. Separation of the tocopherols from each other and from reducing impurities follows previously described methods.

METHODS

Homogenization and extraction

The tissue (20 g., or less if this quantity is not available) is cut into 1 cm. pieces and these are sliced through repeatedly on both sides of the tissue with a sharp scalpel. The scored pieces are then plunged into a bath containing 30 ml. of acetone and solid carbon dioxide (freshly cracked to prevent too much water accumulating). After about 10 min. at -70° the frozen pieces are removed with a scoop and broken into smaller fragments with a pestle and mortar. Then 20–40 g. of anhydrous sodium sulphate is added and the mixture ground under enough acetone to give a fairly fluid paste. The acetone from the freezing bath is then added and grinding continued until a fine paste is obtained. The time taken for the whole grinding operation is normally about 10–15 min., but is less for soft tissues, whereas for tough old tissues it may take 25 min. The paste is transferred to a 43 mm. Soxhlet thimble, the excess of acetone is filtered through and the residue extracted for 3 hr. with 150 ml. of fresh acetone. The residue, after extraction, is a free-flowing powder.

General procedure for tocopherol analysis

The extracts were analysed by methods based on the two-dimensional paper-chromatographic procedure (previously used for plant materials) of Green, Marcinkiewicz &

Watt (1955), more fully described in the Report of the Vitamin E Panel of the S.A.C. (1959). The following important modifications were made.

Saponification. Saturated potassium hydroxide (1 ml.) was used for the saponification of the total acetone extract, provided that it weighed less than 1 g. If the weight of lipid was greater (as with crude body fat), the quantities of potassium hydroxide for saponification were increased accordingly.

Removal of sterols. This separation, by freezing in methanol at -12° , was necessary for all animal tissues.

Floridin-earth chromatography. This was by the method described in Appendix II to the Report of the Vitamin E Panel. The tocopherol was adsorbed from light petroleum and eluted with 25 ml. of benzene. This modification, which is essential for the analysis of animal tissues, removes much paraffin material that would normally be eluted in the benzene fraction and would interfere with paper chromatography.

Paper chromatography. Because the amount of tocopherol in animal tissues is usually very small (see below) it is desirable to chromatograph as large a quantity as possible, but in certain cases this is not always possible as the addition of too much material to the paper may affect the chromatographic separation. Normally, starting with 20 g. of a tissue such as muscle or liver, the eluate from the floridin-earth column is evaporated and dissolved in 1 ml. of benzene. We have successfully chromatographed half this total extract on one paper when dealing with vitamin E-deficient tissues; the other half is always chromatographed in a duplicate paper and may be sprayed with the Emmerie–Engel reagent, for spot-visualization. With tissues containing more adequate amounts of tocopherol, less may be chromatographed. The two-dimensional separation closely follows the procedure of the Report of the Vitamin E Panel, except that, with extracts from animal tissues, the substitution of 80% (v/v) ethanol for 75% ethanol as mobile phase during the second-dimensional (partition) run gives superior results. This is due to the fact that the chief impurity remaining in the tocopherol extracts has an R value in the first (zinc carbonate-adsorption) dimension very close to that of α -tocopherol. This impurity is easily visible under ultraviolet light (especially if the papers have been rendered fluorescent with sodium fluorescein) as a quenching band. It can be extracted from the paper with ethanol, is only slightly reducing and appears to consist largely of ubiquinone ($\lambda_{\max}$ 272 m μ) and certain related substances. The material is quite insoluble in the mobile phase and remains unmoved during partition chromatography. The α -tocopherol, which is nearly always present in a much smaller amount, may become trapped in the impurity if 75% ethanol is used as mobile phase. If 80% ethanol is used, however, the increase in the partition ensures good separation. In certain cases, where α -tocopherol is the only tocopherol of interest, and where exceptionally heavy amounts of ubiquinone are present, 90–95% (v/v) ethanol is even more satisfactory as mobile phase. In this system α -tocopherol has R_f 0.70.

Determination. After location under ultraviolet light the tocopherol spots were cut out and determined as previously described. Since the net extinctions were considerably lower than those usually met with in the analysis of plant extracts, the preparation of the blank was of prime importance. The instructions for its preparation in the Report

of the Vitamin E Panel should be closely followed. The presence and identity of a tocopherol spot was always confirmed by spraying a second paper with the Emmerie-Engel reagent, and, if necessary, by cochromatography with a specimen of the pure tocopherol.

EXPERIMENTAL AND RESULTS

Variation in the homogenization procedure

Several other systems were investigated for their efficiency in homogenization. These included mixtures of sodium chloride and acetone, methanol and light petroleum (b.p. 40–60°), and similar mixtures with sodium sulphate. The efficiency of breakdown was studied on young pig skeletal muscle and was judged on (a) ease of grinding, (b) adequacy of subsequent extraction, and (c) appearance of the residue after extraction. Poor homogenates were obtained with mixtures containing methanol. Mixtures of tissue with sodium sulphate and light petroleum could not be ground, but treatment with sodium chloride–light petroleum gave good results. Acetone was the best solvent investigated and, as indicated in the description of the final method, sodium sulphate–acetone mixtures gave uniformly good results with all tissues.

Study of extraction procedures

In view of the variety of solvent systems used by previous workers, several extraction methods were investigated. In the first series of experiments, young pig skeletal muscle was used. The tissue (20 g.) was removed from the deep-freeze (in which it had been stored for several days), it was homogenized by the sodium sulphate–acetone method and extracted in a Soxhlet apparatus, three times for 1 hr., with acetone, 150 ml. of fresh solvent being used for each extraction. The experiment was repeated with another 20 g. of tissue, which was extracted five times with hot acetone vapour in an apparatus similar to that of Quaife & Dju (1949). Each extract was evaporated, the residue was saponified and total reducing substances were measured. In Table 1 the results are expressed as net extinctions at 520 m μ . In a further experiment, not included in Table 1, 20 g. of tissue was homogenized and then extracted, first with three successive portions of acetone in a Soxhlet apparatus, and then five times, for 1 hr. each time, with hot acetone vapour. The last-named extracts all contained much reducing material, but when the extracts were purified on flordin earth and chromatographed on paper, they contained no tocopherol. In a second series of experiments, 20 g. samples of skeletal muscle from an adult pig were homogenized and extracted for 2 hr. with acetone, ether or ethanol. Extractions by hot vapour and in the Soxhlet apparatus were compared (Table 2).

α -Tocopherol was determined in each extract by the chromatographic method.

These two series show that acetone is a good solvent for tocopherol extraction and that, from homogenates prepared from normal tissues, extraction is probably complete in 2 hr. Ethanol appears to be a poor solvent in a Soxhlet-type extractor (probably related to boiling point and heat of evaporation), but is satisfactory in a vapour extractor. The use of increased extraction times and the use of hot solvent vapours, such as have been used by several other workers, appear to produce undesirable non-tocopherol reducing substances from animal tissues.

In a third series of experiments, the efficiency of extraction from tissues high in tocopherol content was studied. A male and a female rat from the stock colony were each given an oral dose of 5 mg. of α -tocopherol acetate every day for 30 days. They were then killed and samples of their skeletal muscle removed. A portion (20 g.) from each rat was homogenized and extracted twice with acetone in a Soxhlet apparatus for an hour each time, and then for a further hour with ethanol vapour. Each fraction was separately analysed for tocopherol by the full chromatographic procedure. The results (Table 3) confirm that extraction, even from these tissues, is substantially complete in 2 hr.

Table 1. *Effect of successive extractions of pig muscle with acetone*

Pig muscle (20 g.) was extracted successively for 1 hr. periods with 150 ml. volumes of acetone.

Method of extraction	No. of extraction	Total reducing substance (<i>E</i> at 520 m μ)
1. Acetone in Soxhlet apparatus	1	3.30
	2	0.45
	3	0.23
	4	Negligible
2. Hot acetone vapour	1	3.23
	2	1.34
	3	0.45
	4	0.73
	5	0.31; incomplete at 5 hr.

Table 2. *Different methods of extraction of α -tocopherol from samples of pig muscle*

Pig-muscle samples (20 g.) were extracted under various conditions for 2 hr. and the α -tocopherol was determined in each extract by paper chromatography.

Method of extraction	α -Tocopherol (μ g./g. of tissue)
1. Acetone, Soxhlet apparatus	1.28
2. Acetone vapour	1.20
3. Ether, Soxhlet apparatus	1.18
4. Ethanol, Soxhlet apparatus	0.64
5. Ethanol vapour	1.26

Table 3. *Completeness of tocopherol extraction from rat muscle*

See text for details.

Extract	Tocopherol ($\mu\text{g./g.}$ of muscle)			
	α -Tocopherol		β - + γ -Tocopherol	
	Male rat	Female rat	Male rat	Female rat
1. First acetone (1 hr.)	1126	1430	132	119
2. Second acetone (1 hr.)	137	128	<2	<2
3. Ethanol vapour (1 hr.)	28	17	<2	<2

Table 4. *Recovery of α -tocopherol after boiling in solvents*

See text for details.

Tocopherol added ($\mu\text{g.}$)	Solvent	Time of heating (hr.)	Antioxidant added	Tocopherol recovery (%)
250	Acetone	1	None	98.5
50	Acetone	1	None	98.6
250	Acetone	1	Ascorbic acid	100.6
1000	Ethanol	6	Pyrogallol	100.0*
1000	Ethanol	6	None	99.5*

* In the light.

Quantitative studies on grinding and extraction

In Table 4 are recorded the results of some experiments in which small quantities of D- α -tocopherol were heated under reflux in acetone (150 ml.) for periods of 1 hr. In one experiment ascorbic acid was added as an antioxidant. Each solution was evaporated, the residue was put through the saponification procedure and tocopherol then determined. Recoveries of tocopherol were quantitative with and without the addition of ascorbic acid. Boumann & Slater (1957), referring to some findings of Eggitt & Ward (1953), have stated that if small quantities of α -tocopherol are heated in ethanol in the presence of artificial light and absence of an antioxidant, up to 80% is destroyed in 6 hr. They found, further, that the addition of 100 mg. of pyrogallol/mg. of tocopherol did not prevent the destruction of nearly 50% of the tocopherol, and darkness and the presence of an antioxidant were both necessary to obtain quantitative recovery. Since these results conflicted with ours and since we were unable to find a statement in Eggitt & Ward's paper corroborating the losses, we repeated the experiments of Boumann & Slater. α -Tocopherol (1 mg.) was dissolved in ethanol (60 ml.), with and without the addition of 100 mg. of pyrogallol, and the solutions were heated under reflux for 6 hr. in daylight and with an additional 100w incandescent lamp 20 cm. from the boiling solutions. As shown in Table 4, quantitative recovery was obtained from both experiments. We suggest that the results of Boumann & Slater were due to a failure to extract their tocopherol effectively with light petroleum. [This danger is pointed out

Table 5. *Recovery of α -tocopherol added to tissues and subjected to the complete analytical procedure*

Weight of each tissue was 20 g.

Tissue	α -Tocopherol ($\mu\text{g./g.}$)	Added α -tocopherol ($\mu\text{g.}$)	Recovery of added tocopherol (%)
Pig muscle	1.28	5	90
Pig muscle	1.28	100	92
Rat liver	8.3	20	98

by Lehman (1955) and we have confirmed it on many occasions; only ether is satisfactory, unless the immiscible solvent is added before dilution with water.] The differences they found in experiments in which pyrogallol was either present or not present would probably be caused by additional losses on subsequent saponification without an antioxidant. In our experience, the only normal analytical procedure in which it is necessary to protect tocopherol by an antioxidant is saponification; during extraction the addition of pyrogallol is not only unnecessary but, in fact, deleterious, as paper-chromatographic analysis shows that non-saponifiable reducing impurities are formed by the prolonged heating of such phenols.

Three full recovery experiments were carried out. Two 20 g. samples of adult pig muscle and one 20 g. sample of male rat liver (animal from the stock colony) were homogenized with added α -tocopherol in acetone. The samples were analysed by the full chromatographic procedure. Table 5 shows that the method gives recoveries of over 90%.

Comparison with other methods

We have compared the results obtained by the present method on a number of tissues with results obtained on the same samples by using previously described methods. Two samples of liver, one (A) from an adult rat that had been 14 months on a vitamin E-free diet and another (B) from a stock-colony rat, and a sample of adult pig muscle were analysed by the method of Swick & Baumann (1952). The same two livers and also the kidneys from vitamin E-deficient rats (14 months on diet) were analysed by the method of Rosenkrantz (1957). Two samples of cow serum were analysed by the phosphomolybdic acid method of Nair & Magar (1956) and, in addition, by the two methods described by Rosenkrantz (1957). This author apparently omits the cold-saponification step of Nair & Magar and measures vitamin E by a modification of the phosphomolybdic acid method and also by the normal Emmerie-Engel method. Each extract obtained by the various methods was then analysed by two-dimensional paper chromatography, and, finally, each tissue was analysed by the present method. Thus at least three results were obtained from each tissue: (a) the apparent tocopherol content found by following the quoted method, (b) the true tocopherol in the same extract, determined after paper-chromatographic analysis, and (c) the tocopherol content found by the present method.

The results, grouped in Table 6, show that the Swick & Baumann extraction procedure leads to some loss of tocopherol, but the losses are not great. Nevertheless, the determination by this method of tocopherol in tissues from rats fed on a stock diet gave figures that were 300–600% too high, whereas on the liver (A), which actually contained no tocopherol determinable by paper chromatography (thus less than $0.1 \mu\text{g./g.}$), the Swick & Baumann method indicated $22.7 \mu\text{g./g.}$ This experiment illustrates well the extent of non-tocopherol reducing impurities that may still be present after classical purification processes. Rosenkrantz's

procedure extracts only about half the tocopherol from the liver: in spite of this, the 'vitamin E' figures obtained by the method are about ten times too high for the normal liver, and the method gives anomalous results for the deficient liver (A) and kidney.

The serum analyses by Rosenkrantz's methods were respectively about twenty times and four times too high. The analytical procedure of Nair & Magar, on the other hand, destroyed over 90% of the existing tocopherols in the serum, but still gave 'vitamin E' figures about five times too high.

A study of the phosphomolybdic acid method

The reagent introduced by Nair & Magar (1954) gives a yellow-green colour with tocopherols and this is measured at $725 \text{ m}\mu$. Contrary to the original findings of Nair & Magar, Rosenkrantz (1957) found that the reagent reacted to a considerable extent with vitamin A, β -carotene and some other substances. Both groups of workers nevertheless

Fig. 1. Inhibition of phosphomolybdic acid-tocopherol reaction by cholesterol and fat. Weight of tocopherol used was $20 \mu\text{g.}$; colour was measured at $725 \text{ m}\mu$. ●, Effect of pure lard; ○, effect of cholesterol.

Table 6. Comparison of different analytical methods

Tocopherols were determined by the following methods: 1, Swick & Baumann (1952); 2, Rosenkrantz (1957), with (a) phosphomolybdic acid and (b) ferric chloride-dipyridyl; 3, Nair & Magar (1954, 1956); 4a, b and c, paper-chromatographic analysis on methods 1, 2 and 3 respectively; 5, present method.

Method ...	Total tocopherols ($\mu\text{g./g.}$)							
	1	2a	2b	3	4a	4b	4c	5
Tissue								
Pig muscle	7.7	—	—	—	0.81	—	—	1.23
Rat liver A	22.7	120.4	57.2	—	0	0	—	0
Rat liver B	29.8	123.6	—	—	9.3	4.1	—	11.1
Rat kidney	—	43.2	20.8	—	—	0	—	0
Cow serum A	—	48.8	20.1	12.0	—	—	0.21	2.39
Cow serum B	—	37.6	12.2	15.6	—	0.47	0	1.82

used the method for determinations of vitamin E in serum. After obtaining the results described above with the phosphomolybdic acid method we carried out a study of the effect of other substances on the tocopherol-phosphomolybdic acid reaction. Contrary to the findings of Nair & Magar (1954), who stated that the reaction was free from interference from calciferol, carotenoids and cholesterol, it was found (Fig. 1) that small quantities of cholesterol (British Drug Houses Ltd.: Laboratory Reagent) markedly inhibited the reaction. The colour development was also inhibited, to much the same extent, by fat. Contrary, also, to Rosenkrantz we found the reagent unsuitable for the detection of tocopherols on paper chromatograms. Although it is true that the reagent will detect small amounts of tocopherols when these are merely added, as spots, to the surface of ordinary filter paper, the colour reaction is suppressed by all types of impregnated paper (whether of the absorption or partition variety) known to us to be useful for the chromatography of tocopherols.

DISCUSSION

The method described for the homogenization and extraction of animal tissues preparatory to the analysis of vitamin E would appear to have several advantages over previously described methods. Tissue breakdown is exceptionally efficient and simple, and the extraction with hot acetone is rapid and, moreover, gives extracts that are free from interfering substances than those usually obtained by earlier methods. The procedure has been applied, without difficulty, to many types of tissue and may well prove to be suitable for the extraction of other labile, fat-soluble substances.

The full analytical procedure, from extraction to paper-chromatography separation, gives recoveries of added α -tocopherol of over 90%. Comparison of the results obtained by the method with the results obtained by other methods of analysis were illuminating. The values found by the Swick & Baumann (1952) procedure may be taken as typical of the results of a great number of similar methods, based on the original Emmerie-Engel analysis, and with an activated earth used to remove most of the impurities. Swick & Baumann's careful study is doubly interesting because these authors compared their results with another classical method, that of Quaife & Dju (1949), which includes two unusual methods of purification: hydrogenation over a palladium catalyst and micromolecular distillation. Swick & Baumann showed that their own method, when tested on seven types of rat tissue, gave results consistently lower than did the method of Quaife & Dju (in some cases the latter method gave results several times higher than those of the

former). Therefore the present finding that the true tocopherol contents of tissues may be several times lower than the figures found by Swick & Baumann leads to the conclusions that many (and probably all) the literature values for vitamin E contents in animal tissues must be seriously in error.

Studies of the phosphomolybdic acid method of analysis lead to the conclusion that the results that have been obtained by its use, up till now, must be regarded as bearing little relation to real vitamin E figures. The errors consequent on its use may well account, for example, for the results of Garton *et al.* (1958), who found that the addition of tocopherol to the diet of pigs did not apparently increase serum-tocopherol levels. It cannot be stressed too strongly that 'vitamin E' figures obtained without the use of a paper-chromatographic (preferably two-dimensional) separation may be grossly misleading, for non-tocopherol reducing impurities are usually present in amounts much greater than the tocopherol itself, even in the purest extracts obtained by classical methods. Draper & Csallany (1958), for example, have recently found, by a non-chromatographic method, that the 'tocopherol' levels of rabbit muscle and rat liver were apparently not affected by dietary depletion.

SUMMARY

1. A method is given for the analysis of tocopherols in animal tissues. The tissues are first comminuted by grinding with solid carbon dioxide and acetone at -70° , and the material is then extracted with acetone in a Soxhlet apparatus. The resulting extract is analysed for tocopherols by a procedure that includes saponification, removal of sterols, chromatography on florisil earth and two-dimensional paper chromatography.

2. Comparisons of results with those obtained on the same tissues by other methods have shown that paper-chromatographic separation is essential for tocopherol determinations on such tissues, which contain very large amounts of non-tocopherol reducing substances, normally included in the Emmerie-Engel colour determination. It is concluded that many literature figures for vitamin E contents of animal tissues must be seriously in error.

3. The phosphomolybdic acid method has been found unsuitable for the analysis of tocopherols in unpurified extracts of animal tissue, and the dangers involved in its use, particularly for estimations of tocopherols in serum, have been stressed.

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Studies on Vitamin E

2. REVERSAL OF RESPIRATORY DECLINE IN NUTRITIONAL LIVER NECROSIS BY INTRAPORTAL ADMINISTRATION OF TOCOPHEROLS AND OTHER SUBSTANCES

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(Received 13 July 1959)

The onset of overt symptoms in dietary liver necrosis is preceded by a metabolic lesion that impairs the ability of the liver to maintain its respiratory rate (Chernick, Moe, Rodnan & Schwarz, 1955). By comparing the rates of respiration of liver slices at two different time intervals, the intensity of the defect could be assessed. Chernick *et al.* showed that dietary supplements of vitamin E or factor 3 (naturally occurring selenium compounds) prevented or delayed both the necrotic degeneration and the biochemical lesion. Later, Rodnan, Chernick & Schwarz (1956) described the effects of intraportal administrations of α -, β -, γ - and δ -tocopherol on the metabolic defect and showed that, although these substances did not markedly alter the initial rate of respiration, they arrested the respiratory decline. The four tocopherols showed considerable differences in their activity, α -tocopherol being the most active. It was of interest to extend this study to other tocopherols that have since been isolated and, in view of other work on the connexion between metal ions and vitamin E deficiency (King, Lee & Visscher, 1955; Bunyan, Edwin & Green, 1958; Bunyan, Green, Edwin & Diplock, 1960), to investigate the effect of metal ions.

MATERIALS AND METHODS

Rats. Norwegian hooded rats of both sexes were reared on a stock diet until they were about 45–60 g. in weight. They were fed on the necrogenic diet until the respiratory decline developed, usually in a further 35–55 days, when the rats weighed 60–120 g., depending on their age and weight when started on the necrogenic diet.

Necrogenic diet. This consisted of 45% of sucrose, 10% of glucose, 5% of McCollum's salt mixture, 5% of pure lard (Wilts. United Dairies Ltd.), 30% of dried baker's yeast and 5% of a vitamin B mixture in glucose, with oral doses of vitamins A, D and K to supply respectively 1400 i.u., 100 i.u. and 4 μ g./rat/week. The vitamin B mixture supplied to each kg. of diet was: 9 mg. of thiamin, 18 mg. of riboflavin, 90 mg. of nicotinic acid, 9 mg. of pyridoxine, 90 mg. of calcium pantothenate, 2 mg. of folic acid, 0.3 mg. of cobalamin, 90 mg. of inositol, 90 mg. of *p*-aminobenzoic acid and 900 mg. of choline tartrate. The salt mixture included ferric citrate, potassium iodide and potassium alum.

Tocopherols. The 5:7:8-trimethyltolcol (α -tocopherol), 5:8-dimethyltolcol (β -tocopherol), 7:8-dimethyltolcol (γ -tocopherol), 8-methyltolcol (δ -tocopherol), 7-methyltolcol (η -tocopherol), 5-methyltolcol, 5:7-dimethyltolcol and tococ were synthetic specimens. The ϵ -tocopherol was the natural substance, prepared from the unsaponifiable fraction of wheat-bran oil.

Intraportal injections. The procedure of Rodnan *et al.* (1956) was followed, with certain modifications. Rats were