either in the terms of a general salt effect, or in terms of specific interaction between the ions in solutions and the charged groups in the protein, with subsequent suppression of this effect.

SUMMARY

1. The equilibrium constant for the ionization of (horse) acid metmyoglobin which may be represented as $\text{Fe}^+(\text{H}_2\text{O}) = \text{FeOH} + \text{H}^+$ has been measured over a range of pH, ionic strength and temperature.

2. The effect of salts on this equilibrium is marked. Thus addition of sodium chloride favours the ionization up to an ionic strength of about 0.01 but beyond this the effect is reversed. Extrapolation to zero ionic strength gives $\text{p}K'_0 = 9.038$

± 0.030 at 20° and 8.803 ± 0.025 at 37° . From the equilibrium constant at two temperatures the ionization is found to be endothermic to the extent of 5.75 ± 0.67 kg. cal./mole. $\Delta S^\circ_{20} = -21.7 \pm 2.4$ cal./mole deg.

3. It is only possible to measure this ionization constant within the pH range 7.8–9.2. In more alkaline or acidic solutions significant interaction takes place between this equilibrium and, presumably, other ionizations in the molecule, and the value of the ionization constant falls steadily with increasing pH. This effect is suppressed at high ionic strengths which suggests specific interaction between electrolyte ions in solution and the charged groups on the protein.

REFERENCES

- Adair, G. S. & Adair, M. E. (1934). *Biochem. J.* **28**, 1230.
 Austin, J. H. & Drabkin, D. L. (1935). *J. biol. Chem.* **112**, 67.
 Bates, R. G. (1948). *Chem. Rev.* **42**, 1.
 Cannan, R. K. (1938). *Cold Spr. Harb. Symp. quant. Biol.* **6**, 1.
 Cannan, R. K., Palmer, A. H. & Kibrick, A. C. (1942). *J. biol. Chem.* **142**, 803.
 Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino-Acids and Peptides*, p. 445. New York: Reinhold.
 Cohn, E. J., Green, A. A. & Blanchard, M. H. (1937). *J. Amer. chem. Soc.* **59**, 509.
 Cohn, E. J. & Prentiss, A. M. (1927). *J. gen. Physiol.* **8**, 619.
 Coryell, C. D., Stitt, F. & Pauling, L. (1937). *J. Amer. chem. Soc.* **59**, 633.
 Duve, C. de (1948). *Acta chem. scand.* **2**, 264.
 Edsall, J. T. (1943). *Proteins, Amino-acids and Peptides*, p. 83. New York: Reinhold.
 Eley, D. D. (1943). *Trans. Faraday Soc.* **39**, 172.
 George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
 Green, A. (1932). *J. biol. Chem.* **95**, 47.
 Grönwall, A. (1942). *C.R. Lab. Carlsberg (Ser. chim.)*, **24**, 185.
 Guggenheim, E. A. (1930). *J. phys. Chem.* **34**, 1758.
 Halban, H. von & Eisenbrand, J. (1928). *Z. phys. Chem.* **132**, 401.
 Halban, H. von & Kortum, G. (1934). *Z. phys. Chem. A*, **170**, 351.
 Haurowitz, F. (1949). *Haemoglobin*, p. 53. London: Butterworth.
 Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.
 Linderstrøm-Lang, K. (1924). *C.R. Lab. Carlsberg*, **15**, no. 7.
 Roughton, F. J. W. (1949). *Haemoglobin*, p. 83. London: Butterworth.
 Scatchard, G. & Kirkwood, J. G. (1932). *Phys. Z.* **33**, 297.
 Sidwell, A. E. jun., Munch, R. H., Barron, E. S. G. & Hogness, T. R. (1938). *J. biol. Chem.* **123**, 335.
 Sørensen, S. P. L., Linderstrøm-Lang, K. & Lund, E. (1926). *C.R. Lab. Carlsberg*, **16**, no. 5.
 Theorell, H. (1932). *Biochem. Z.* **252**, 1.
 Theorell, H. (1934). *Biochem. Z.* **268**, 64.
 Tristram, G. R. (1949). *Haemoglobin*, p. 109. London: Butterworth.
 Wyman, J. jun. (1948). *Advanc. Protein Chem.* **4**, 439.

The Estimation of Vitamin E

2. QUANTITATIVE ANALYSIS OF TOCOPHEROL MIXTURES BY PAPER CHROMATOGRAPHY

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The chemical methods originally used for estimating the vitamin E potency of natural products, notably those of Emmerie & Engel (1938) and of Furter & Meyer (1939), did not distinguish between the different tocopherols. Since α -tocopherol is the only member of the group which possesses marked vitamin E activity, and since the tocopherol mixtures present in natural products differ widely in

composition, failure to differentiate between the individual tocopherols is a considerable disadvantage. The fact that γ - and δ -tocopherols couple with diazonium salts, whereas the α and β compounds do not, has, however, been used to achieve a partial analysis of the individual tocopherols (Quaife, 1944; Weisler, Robeson & Baxter, 1947). More recently, Quaife (1948) has described a method

for the quantitative determination of the individual tocopherols in which the nitroso derivatives of the β , γ and δ compounds are separated by chromatography on zinc carbonate-Celite. The concentration of α -tocopherol in the mixture is then determined by difference following summation of the values obtained for the β -, γ - and δ -tocopherols. In a new approach to the problem, Emmerie (1949) showed that α - and γ -tocopherols may be separated from one another by chromatography on alumina or Floridin Earth.

The advantages of using paper chromatography for the precise, qualitative analysis of tocopherol mixtures occurring in natural products have already been described (Brown, 1952). Application of this method to the quantitative estimation of synthetic and naturally occurring mixtures of tocopherols forms the subject of the present paper.

EXPERIMENTAL

Preparation of solutions for chromatography. Since lipids, sterols and carotenoids interfered with the running of the tocopherols on the chromatograms, it was necessary to remove them from extracts of natural products. Lipids were readily removed by saponification (Tošić & Moore, 1945) and the vitamin A and carotenoids were separated from the tocopherols by adsorption on Floridin Earth (Emmerie & Engel, 1939; Glavind, Kjølhed & Prange, 1942). The removal of sterols presented a more difficult problem, and the method finally selected was to crystallize the sterols from methanol solution at -10° . After centrifugation and removal of the mother liquor the crystals were redissolved in warm methanol and the solution again cooled to -10° . This process was repeated until the supernatant liquors no longer contained tocopherols and the mother liquors were then combined. Although this method is tedious and does not remove the sterols completely, sufficient material is eliminated to avoid interference on the chromatogram.

Standard solutions. These were prepared by dissolving the synthetic ($\pm$)-tocopherols in absolute ethanol. The solutions were stored in amber-coloured bottles at 2° and the reducing activity tested periodically by the Emmerie-Engel method. Solutions containing more than one tocopherol (e.g. α plus β solution for use in examining wheat products) were most conveniently prepared from the stock solutions.

When solutions of tocopherols which had been stored for considerable periods (about 6 months) were chromatographed (see below) faint pink spots were obtained on the starting line on spraying with 2:2'-dipyridyl and FeCl_3 . In addition, α -tocopherol solutions which had been stored for considerable periods gave a distinct spot with an R_f value of 0.16 in 75% (v/v) ethanol as well as an authentic α -tocopherol spot. This value corresponds closely with the R_f value of α -tocopherylquinone under the same conditions (Blaxter & Brown, 1952). When these non-tocopherol spots were obtained on chromatographing the standard solutions fresh standards were prepared.

Chromatography. Whatman no. 1 filter paper was coated with Vaseline according to the method previously described (Brown, 1952), and 75% (v/v) ethanol was used as the mobile

phase. The ethanol was distilled from KOH and KMnO_4 before use. Sheets of impregnated paper measuring 30×12 cm. were used so that four spots, each 3 cm. from its neighbour, could be accommodated on a starting line drawn parallel to the shorter edge of the paper. Two spots of the unknown solution and two spots of a standard solution, containing approximately the same amounts of individual tocopherols as the unknowns, were used for a single determination. The unknown spots were run in positions 1 and 2, while the standard spots occupied positions 3 and 4. The maximum quantity of any individual tocopherol applied to the paper was restricted to $30 \mu\text{g}$. since amounts in excess of this gave elongated spots on the developed chromatograms.

After developing the chromatogram for about 16 hr., using the ascending method of Williams & Kirby (1948), the sheet was dried for 15 min. at room temperature and then cut longitudinally into four strips, each containing the tocopherols from one original spot on the starting line. The alternate strips 2 and 4, representing the standard and the unknown, were sprayed with 2:2'-dipyridyl solution (0.25%, w/v, in ethanol) and then with FeCl_3 solution (0.1%, w/v, in ethanol). This gave a clear definition of the positions occupied by the individual tocopherols. The sprayed strips were then placed alongside the unsprayed strips and the appropriate 'tocopherol zones' of the latter cut out, extracted with 4 ml. hot ethanol, and 1 ml. benzene added in order to clear the cooled solutions. Blank zones, equal in size to the tocopherol zones, were cut from the strips and treated in the same way. Samples of the filtered ethanol-benzene solutions were then tested with 2:2'-dipyridyl and FeCl_3 (Emmerie & Engel, 1938) the colour being measured after 2 min. (Baxter *et al.* 1947).

The above method permitted the quantitative separation of the α and δ compounds. Since the β and γ isomers have the same R_f value under these conditions, the following method was used for their estimation. If no reaction was obtained when the spot in the β plus γ position was sprayed with diazo-*o*-dianisidine, the value obtained by the Emmerie-Engel procedure was taken to be β -tocopherol. If spraying with the diazo solution revealed γ to be present, it was estimated in a duplicate chromatogram by the method of Weisler *et al.* (1947). The difference between the value obtained by the Emmerie-Engel procedure for β - plus γ -tocopherols and the value obtained for the γ isomer was taken to represent β . So far, however, β - and γ -tocopherols have not been found to occur together in the limited material examined.

RESULTS

Recovery of tocopherols from the chromatograms. The recovery of tocopherols from the developed chromatograms was not quantitative, but depended on the length of time of development (see Fig. 1). When the chromatograms were run for 16 hr. the recoveries were about 80%. By reducing the time of development to 6 hr. the recoveries could be increased to about 90% but the shorter time of development did not allow a clear-cut separation of the γ and δ compounds.

The variation in the recovery with alteration in the time of development suggested that the atmosphere in the chromatogram chamber was a possible cause of destruction of the tocopherols.

Filling the chamber with nitrogen gas after closing the chamber did not, however, decrease the losses. In addition, tocopherols spotted on to the paper above the greatest height reached by the solvent could be recovered quantitatively after 24 hr. exposure to chamber atmosphere. It appeared likely, therefore, that the destruction was caused by a non-volatile constituent of the solvent, but attempts to eliminate the contaminant by distillation were unsuccessful. Use of alternative solvents such as aqueous methanol or acetic acid likewise

standards alongside each unknown solution, as described in the Experimental section. As a further check, the recovery of tocopherols in an unknown solution was calculated from the potency of the solution applied to the starting line compared with the sum of the potencies of the individual spots (tocopherols plus artifacts) on the chromatogram. Recoveries of tocopherols from unknown and standard solutions agreed to $\pm 5\%$ when chromatographed under identical conditions on the same sheet, as for example in *a*, *b* and *c* in Table 1.

Examination of vegetable oils. The tocopherol content of vegetable oils has been examined quite extensively in the past, mainly because they are relatively rich sources of these compounds. It was thought that their examination offered the best means of comparing the present method with those previously published (e.g. Quaife, 1948). In Table 2 the results obtained in the present investigation are compared with the values of other workers. It can be seen that the results are in good agreement, considering that entirely different samples of oil were used by the different workers. In cases where non-tocopherol spots were obtained (e.g. wheat-germ oil) the reducing power of the artifact (calculated as tocopherol) is indicated. The artifact occurring in the wheat-germ oil examined in the present work contributed 9% to the reducing power of the extract applied to the chromatogram. The nature of the artifact was not ascertained, but it was not carotenoid in nature since repeated passage through Floridin Earth columns did not remove it. A similar observation was made with a sample of shark-liver oil; in this case the artifact contributed 43% of the total reducing power of the extract applied to the chromatogram. The potential error which is present in the estimation of the vitamin E potency of natural products is thus clearly demonstrated.

Fig. 1. The effect of time of development on the recovery of tocopherols from the chromatograms.

gave non-quantitative recoveries. In fact, with 90% (v/v) acetic acid prepared from different batches of Analar glacial acetic acid the recoveries ranged from 30 to 80% and were not altered by distillation.

With natural products failure to obtain quantitative recovery of the tocopherols from the developed chromatograms was allowed for by running

Table 1. *Recovery of tocopherols from paper chromatograms*

Test	Solution	Reducing power applied (calc. as $\mu\text{g.}$ tocopherol)	Recovery (%)
(a)	α -Tocopherol	18.8	78
	β -Tocopherol	10.8	79
	Wheat-germ oil*	36.0	77
(b)	α -Tocopherol	18.8	84
	γ -Tocopherol	11.8	75
	Cottonseed oil†	24.4	78
(c)	α -Tocopherol	17.2	81
	γ -Tocopherol	21.6	77
	δ -Tocopherol	12.8	85
	Soya-bean oil‡	59.2	81

* The wheat-germ oil contained a mixture of α - and β -tocopherols and artifact.

† The cottonseed oil contained a mixture of α - and γ -tocopherols.

‡ The soya-bean oil contained a mixture of α -, γ - and δ -tocopherols.

Table 2. *Tocopherol content of vegetable oils*

Oil	Total tocopherol (mg./100 g.)	Individual tocopherols (% of total)					
		α -	β -	γ -	δ -	Artifact	
Corn (Maize)	86	6	0	93	0	1	Present work
	90	10	—	90	—	—	Fisher (1945)
	102	12	0	88	0	—	Quaife (1948)
	104*	6	—	—	—	—	Hove & Hove (1944)
Cottonseed	70	74	0	26	0	0	Present work
	87	69	—	31	—	—	Fisher (1945)
	95	75	—	25	—	—	Fisher (1945)
	110	69	—	31	—	—	Fisher (1945)
	87	57	0	43	0	—	Quaife (1948)
	83*	62	—	—	—	—	Hove & Hove (1944)
Soya bean	83	15	0	51	34	0	Present work
	94	21	—	79	—	—	Fisher (1945)
	99	21	—	79	—	—	Fisher (1945)
	74	13	0	56	31	—	Quaife (1948)
	92*	10	—	—	—	—	Hove & Hove (1944)
Sunflower seed	51	100	0	0	0	0	Present work
Wheat germ	262	64	27	0	0	9	Present work
	268	60	40	—	—	—	Quaife (1948)
	274*	70	—	—	—	—	Hove & Hove (1944)

* In the work of Hove & Hove (1944) only the total and the α -tocopherol were determined.

DISCUSSION

Since the original chemical methods for determining vitamin E in natural products (Emmerie & Engel, 1938; Furter & Meyer, 1939) do not distinguish between the individual tocopherols, which differ markedly in biological activity, comparison between chemical and biological methods is virtually impossible. Almost all the methods for the determination of the individual tocopherols in natural products (Quaife, 1944, 1948; Weisler *et al.* 1947) have the disadvantage that the concentration of α -tocopherol, the most biologically active member of the vitamin E group, is estimated by difference.

The present method allows each of the individual tocopherols to be determined separately and simultaneously, and has the additional advantage that it is applicable to microgram quantities of tocopherols. This is an important consideration in the examination of animal tissues, which frequently contain only small amounts of tocopherols. In addition, the

presence of non-tocopherol reducing substances in the extracts of natural products is readily demonstrated by this method. On the other hand, small losses of the tocopherols occur in paper chromatography, but this disadvantage can be overcome by running standards alongside each unknown solution.

SUMMARY

A method involving paper chromatography has been developed for the quantitative estimation of the individual tocopherols (α -, β -, γ - and δ -). Results obtained by this method for certain vegetable oils have been compared with those obtained for the same oils by other workers.

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REFERENCES

- Baxter, J. G., Lehman, R. W., Hove, E. L., Quaife, M. L., Weisler, L. & Stern, M. H. (1947). *Biol. Symp.* **12**, 484.
 Blaxter, K. L. & Brown, F. (1952). To be published.
 Brown, F. (1952). *Biochem. J.* **51**, 237.
 Emmerie, A. (1949). *Ann. N.Y. Acad. Sci.* **52**, 309.
 Emmerie, A. & Engel, C. (1938). *Rec. Trav. chim. Pays-Bas*, **57**, 1351.
 Emmerie, A. & Engel, C. (1939). *Rec. Trav. chim. Pays-Bas*, **58**, 283.
 Fisher, G. S. (1945). *Industr. Engng Chem. (Anal. ed.)*, **17**, 224.
 Furter, M. & Meyer, R. E. (1939). *Helv. chim. Acta*, **22**, 240.
 Glavind, J., Kjølhed, K. Th. & Prange, I. (1942). *Kem. Mannedsblad*, **23**, 43.
 Hove, E. L. & Hove, Z. (1944). *J. biol. Chem.* **156**, 601.
 Quaife, M. L. (1944). *J. Amer. chem. Soc.* **66**, 308.
 Quaife, M. L. (1948). *J. biol. Chem.*, **175**, 605.
 Tošić, J. & Moore, T. (1945). *Biochem. J.* **39**, 498.
 Weisler, L., Robeson, C. D. & Baxter, J. G. (1947). *Anal. Chem.* **19**, 906.
 Williams, R. J. & Kirby, H. M. (1948). *Science*, **107**, 481.