alkaline with ammonia, and the alkaloids were extracted with ether. The dried ether solution was evaporated. The alkaloids were dissolved in a few drops of methanol and spotted on sheets of Whatman no. 1 filter paper buffered in 2% ammonium sulfate solution. The solvent system employed was a mixture of isobutanol (100), acetic acid (20), and water (*ca.* 40 parts to saturation) in terms of volume (Nadeau *et al.*, 1958). The R_F value for isothebaine in this system is 0.69, that for thebaine 0.79, and for oripavine 0.94. Dragendorff's reagent was used to detect the spots. The spots were eluted onto planchets with formic acid solution to prepare the alkaloids for radioactivity assays.

Identification of Oripavine.—Twenty grams of seeds were extracted, and the alkaloids were separated as above. Only the spots with R_F 0.69 and 0.94 were obtained on chromatograms. The faster-running spot was eluted with acetic acid solution, the acid solution was made alkaline with ammonia, and the alkaloid was extracted with ether. The dried ethereal solution was treated with a small amount of diazomethane. After evaporation of the solvent, the residue was chromatographed on paper as described above. A spot at R_F 0.79 was obtained which was presumably thebaine.

Amounts of Alkaloids Involved.—Although the extraction cycles were not devised for quantitative recovery, it is possible to ascertain the approximate amounts of the different alkaloids involved. Ultraviolet absorption spectra of chromatogram eluates were used as a basis for this assay, and the amounts of individual alkaloids were estimated with the help of extinction coefficients established for the purpose.

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REFERENCES

Battersby, A. R. (1961), *Quart. Rev. 15*, 2501.
Battersby, A. R., and Harper, B. J. T. (1958), *Chem. and Ind.*, 364.
Battersby, A. R., and Harper, B. J. T. (1960), *Tetrahedron Letters No. 27*, 21.
Bentley, K. W., and Dyke, S. F. (1957), *J. Org. Chem. 22*, 429.
Konovalova, R. A., and Kiselev, V. V. (1948), *J. Gen. Chem. 18*, 142.
Leete, E. (1958), *Chem. and Ind.*, 977.
Leete, E. (1959), *J. Am. Chem. Soc. 81*, 3948.
Nadeau, G., Sobolewski, G., and Fiset, L. (1958), *J. Chromatog. 1*, 327.
Stermitz, F. R., and Rapoport, H. (1961), *J. Am. Chem. Soc. 83*, 4045.

Biological Activity of an *l*-Epimer of *d*-α-Tocopheryl Acetate*

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2*l*,4′D,8′D-α-Tocopheryl acetate, an *l*-epimer of natural *d*-α-tocopherol, was 21% as active as *d*-α-tocopheryl acetate in the resorption-gestation bioassay using vitamin E-deficient female rats. 2*d*,4′D,8′D-α-Tocopheryl acetate had essentially the same biological activity as *d*-α-tocopheryl acetate derived from natural sources. 2*dl*,4′D,8′D-α-Tocopheryl acetate synthesized from natural phytol and 2*dl*,4′DL,8′DL-α-tocopheryl acetate synthesized from racemic isophytol both had about 60% the activity of *d*-α-tocopheryl acetate. The epimeric configuration at the 2-position of α-tocopheryl acetate is apparently dominant in determining biological activity.

α-Tocopherol, the most active form of vitamin E, has optically active centers at the 2, 4′, and 8′ carbon atoms (see Fig. 1). Robeson and Nelan (1962) recently reported the isolation of a *d*-epimer (2*d*,4′D,8′D) and an *l*-epimer (2*l*,4′D,8′D) by fractionation of 2*dl*,-4′D,8′D-α-tocopherol *via* a piperazine complex. The *d*-epimer had practically the same physicochemical properties as natural *d*-α-tocopherol.

Bioassays of these two preparations are of special interest, since knowledge of the biological activity of α-tocopherol has heretofore been limited to the naturally occurring *d*-α-tocopherol and to synthetic preparations, racemic at the 2-position and, in some cases, racemic also at the 4′- and 8′-positions. To add to the pertinent biological data, we are also reporting the bioassays of the partially separated mixtures that were examined during the progress of the fractionation studies.

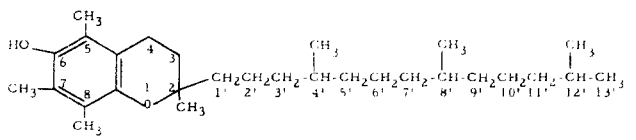

FIGURE 1

EXPERIMENTAL

Synthetic Tocopherols.—Preparations of 2*dl*,4′D,8′D-α-tocopherol[1] were synthesized by condensing trimethylhydroquinone with natural phytol[2] by the procedure of Smith and Ungnade.[3] The reaction products

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[1] Natural phytol contains two centers of optical activity with D-configurations (Burrell *et al.*, 1959; Crabbe *et al.*, 1959). Thus, the configurations at the 4′ and 8′ carbon atoms of α-tocopherol synthesized from natural phytol can be assigned a D-configuration.

[2] Isophytol and natural phytol were obtained from Charles Bowman and Company, N. Y.

[3] U. S. 2,411,942, Dec. 3, 1946.

TABLE I

RELATIVE VITAMIN E POTENCIES OF EPIMERS OF 2*dl*,4′D,8′D-α-TOCOPHERYL ACETATE

Preparation	$[\alpha]_D^{25}$ of Acetate Ester	$[\alpha]_D^{25}$ $K_3Fe(CN)_6$ Oxidation Product	% 2*l*,4′D,8′D Epimer	Median Fertility Dose mg α-Tocopherol ± S.E.		Relative Potency
				d-α-Tocopheryl Acetate (Reference Standard)	Test Preparation	
B-1439	+2.8°	+26°	0	0.46 ± 0.04	0.46 ± 0.08	1.00
B-1560	—	+22°	8	0.42 ± 0.04	0.46 ± 0.04	0.91
B-1562	—	+14°	23	0.42 ± 0.04	0.59 ± 0.03	0.71
B-1561	—	+ 2.8°	45	0.42 ± 0.04	0.64 ± 0.05	0.66
B-1328[a]	+0.5°	+ 0.5°	50	0.46 ± 0.04	0.79 ± 0.12	0.58
B-1328[a]	+0.5°	+ 0.5°	50	0.45 ± 0.03	0.80 ± 0.09	0.56
B-1310[a]	+0.5°	+ 0.5°	50	0.48 ± 0.05	0.79 ± 0.09	0.61
B-1438	−1.10°	−13°	77	0.46 ± 0.04	1.33 ± 0.06	0.35
B-1518	—	−18.5°	88	0.39 ± 0.03	1.03 ± 0.11	0.38
B-1519	—	−22°	95	0.39 ± 0.03	1.45 ± 0.15	0.27
B-1465	−1.9°	−24°	97	0.39 ± 0.03	1.77 ± 0.19	0.22
B-1465	−1.9°	−24°	97	0.42 ± 0.03	1.72 ± 0.17	0.24
B-1602	−2.1°	−25°	100	0.29 ± 0.03	1.29 ± 0.04	0.22
B-1327[b]	0	—	50	0.45 ± 0.03	0.78 ± 0.08	0.58
B-1311[b]	0	—	50	0.48 ± 0.05	0.72 ± 0.06	0.67

[a] 2*dl*,4′D,8′D-α-tocopheryl acetate synthesized from natural phytol. [b] 2*dl*,4′DL,8′DL-α-tocopheryl acetate synthesized from isophytol.

($E_{1cm}^{1\%}$ (292 mμ) = 54, 58, 59 for three preparations) were repeatedly chromatographed on Florisil adsorbant (Floridin Co.) to give fractions of purified α-tocopherol having $E_{1cm}^{1\%}$ (292 mμ) = 70 or above.

Preparations of racemic 2*dl*,4′DL,8′DL-α-tocopherol were synthesized from trimethylhydroquinone and synthetic isophytol[2] by the same procedure. After purification, two samples had $E_{1cm}^{1\%}$ (292 mμ) = 67 and 74, respectively.

Fractionation of Epimers.—Samples of the purified 2*dl*,4′D,8′D-α-tocopherol were fractionated by crystallization of the piperazine complex (Robeson and Nelan, 1962) to give a series of mixtures of the *d*- and *l*-epimers, as well as products characterized as pure 2*d*,4′D,8′D-α-tocopherol and pure 2*l*,4′D-8′D-α-tocopherol.

Estimation of Isomer Composition.—The ratio of the *d*- and *l*-epimers in the fractionated products was estimated from measurements of the optical rotation of samples after oxidation (Nelan and Robeson, 1962) with potassium ferricyanide. This method of estimation was based on the finding (Robeson and Nelan, 1962) that the oxidation products from the epimers showed sufficient difference in specific optical rotations to be easily measured.

In this work, the polarimeter readings were made on isooctane solutions of the total oxidation products. Values for $[\alpha]_D^{25}$ of +25.8° and −24.6°, respectively, for the oxidation products from the *d*- and *l*-epimers were used as standards in the estimation of epimer composition of the various fractionated products. The standard of purity of the oxidation product was taken to be $E_{1cm}^{1\%}$ (300 mμ) = 45, the average from several oxidations of the *d*-epimer. When the *E*-values for the oxidized materials differed from 45, the optical rotations were adjusted proportionately so that the values of [α] would depend only on the ratio of concentration of the two epimers.

Acetates.—The above α-tocopherol preparations were converted to the acetate esters by acetylation with acetic anhydride. $E_{1cm}^{1\%}$ (284 mμ) values for the acetate esters ranged from 40.7 to 43.5.

d-α-Tocopheryl acetate, used as the bioassay standard, was prepared from natural sources.[4]

[4] *d*-α-Tocopheryl acetate, Type 6-100, Distillation Products Industries.

Bioassay Procedure.—The vitamin E activity was measured by the resorption-gestation bioassay of Mason and Harris (1947) employing vitamin E–deficient sexually mature female rats (Holtzman Rat Co.). The female rats were maintained on vitamin E–free test diet 301, described by Harris and Ludwig (1949). Four to six dose levels were used for both the test preparation and the reference standard, with 9 to 12 rats per dose level. Supplements of the standard and test preparations were orally administered by calibrated dropper in five equivalent doses on the 4th to 8th days of the gestation period.

The biological response for each level of each material was expressed as the ratio of the number of females with viable fetuses to the total number of females mated (litter efficiency). Dose response curves were obtained as the regression of the probit of the percentage litter efficiency on the logarithm of the dose in milligrams. From this regression, the median fertility dose and its standard error were computed. The relative biopotency was calculated as the ratio of the median fertility dose of the *d*-α-tocopheryl acetate to that of the test preparation.

RESULTS

The results of bioassays of the 2*l*,4′D,8′D-, the 2*d*,-4′D,8′D- the 2*dl*,4′D,8′D- and the 2*dl*,4′DL,8′DL-α-tocopheryl acetates are summarized in Table I. The % *l*-epimer and the relative biopotency show an inverse linear relationship, which is not surprising since the materials tested are pure mixtures of only two substances.

The regression of % relative biopotency on % *l*-epimer was found to be:

$$\%\ \text{relative biopotency} = 95.8 - 0.74\ (\%\ l\text{-epimer})$$

with $s_{y \cdot x} = 4.03$

Based on this formula, the relative biopotency of 100% *l*-epimer is 22.2% (s = 1.7). The relative biopotency of 100% *d*-epimer from this regression is 95.8% (s = 2.3). This value is not statistically different from 100%, indicating that the *d*-epimer and the reference standard (*d*-α-tocopherol from natural sources) have the same biological activity. This fact adds to the physicochemical evidence (Robeson and Nelan, 1962)

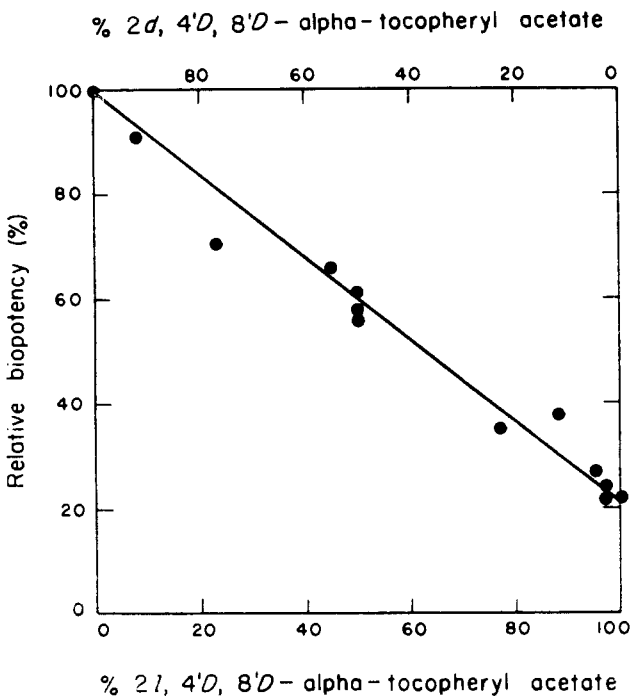

FIG. 2.—Biopotency of *l*- and *d*-epimers of α-tocopheryl acetate relative to *d*-α-tocopheryl acetate

that the *d*-epimer is, indeed, identical with *d*-α-tocopherol.

If we accept this identity, a more precise estimate of the relative biopotency of the *l*-epimer can be calculated from the regression of relative biopotency on % *l*-epimer in which the line is fitted through the point, 100% relative biopotency, 0% *l*-epimer. This new regression line, shown in Figure 2, is:

$$\%\ \text{relative biopotency} = 100.0 - 0.79\ (\%\ l\text{-epimer})$$

with $s_{y \cdot x} = 4.42$

On the basis of this formula the relative biopotency of the *l*-epimer is 21.1% ($s = 1.9$).

From this last regression, the relative biopotency of a mixture of 50% each of 2*d*,4'D,8'D- and 2*l*,4'D,8'D-α-tocopheryl acetate is calculated to be 60.5% ($s = 1.3$). This value is in good agreement with a mean relative biopotency of 58.3% for the three bioassays of 2*dl*,-4'D,8'D-α-tocopheryl acetate. It is also quite close to the relative biopotencies of 58% and 67% found for 2*dl*,4'DL,8'DL-α-tocopheryl acetate synthesized from isophytol (see Table I). No significant differences were observed in the relative biopotencies of the 2*dl*,4'D,8'D- and 2*dl*,4'DL,8'DL-α-tocopheryl acetates. Thus the mean relative biopotency of α-tocopheryl acetates racemic at the 2-position is 60.0% ($s = 1.9$) based on these five bioassays.

DISCUSSION

The configuration at the 2-position of α-tocopherol is most important in determining biological activity. Thus, in our bioassays of the acetates, the *d*-epimer (2*d*,4'D,8'D) was about five times as active as the *l*-epimer (2*l*,4'D,8'D), with the unresolved *dl* mixture (2*dl*,4'D,8'D) intermediate. On the other hand, the 2*dl*,4'D,8'D- and 2*dl*,4'DL,8'DL-α-tocopheryl acetates were equally active. Thus, the configuration at the 4'- and 8'-positions appears to have little effect, if any, on the biological potency. If the biological activity of an α-tocopherol preparation is dependent solely on the optical configuration at the 2-position, then the activity of mixtures of *d*- and *l*-epimers can be estimated chemically by the potassium ferricyanide oxidation procedure of Nelan and Robeson (1962).

On the basis of optical rotation data and the mixed melting points of the acetate and acid succinate esters, Robeson and Nelan (1962) concluded that pure 2*d*,-4'D,8'D-α-tocopherol was identical with natural *d*-α-tocopherol. A single bioassay of pure *d*-epimer showed it to be equal in biological activity to natural *d*-α-tocopherol. The calculated regression line relating relative biopotency and *l*-epimer shows that the relative potency of the *d*-epimer in admixture with *l*-epimer was not significantly different from *d*-α-tocopheryl acetate from natural sources. We therefore conclude that *d*-α-tocopherol derived from natural sources is in fact 2*d*,4'D,8'D-α-tocopherol.

In contrast, 2*l*,4'D,8'D-α-tocopheryl acetate has only 21% of the biological activity of the *d*-epimer. Racemic 2*dl*,4'DL,8'DL-α-tocopheryl acetate synthesized from isophytol was biologically indistinguishable from 2*dl*,4'D,8'D-α-tocopheryl acetate. The racemic mixture contains only 12.5% each of the two epimers studied in this investigation, but of course it contains a total of 50% of epimers with a *d*-configuration at the 2-position and 50% of epimers with an *l*-configuration at the 2-position. One can infer that the three epimers with an *l*-configuration at the 2-position have about 21% the biological activity of the three epimers with a *d*-configuration at the 2-position. However, we do not yet know the exact biological potency for six of the eight possible optical isomers in completely racemic α-tocopherol.

This present study confirms the biological superiority of *d*-α-tocopherol over the *dl* forms and presents evidence that much, if not all, of the superiority is due to the configuration at the 2-position in the chromane ring.

REFERENCES

Burrell, J. W. K., Jackman, L. M., and Weedon, B. C. L. (1959), *Proc. Chem. Soc.* 263.

Crabbe, P., Djerassi, C., Eisenbraun, E. J., and Liu, S. (1959), *Proc. Chem. Soc.* 264.

Harris, P. L., and Ludwig, M. I. (1949), *J. Biol. Chem. 179*, 1111.

Mason, K. E., and Harris, P. L. (1947), *Biol. Symp. 12*, 459.

Nelan, D. R., and Robeson, C. D. (1962), *Nature 193*, 477.

Robeson, C. D., and Nelan, D. R. (1962), *J. Am. Chem. Soc. 84*, 3196.