

R60-33

Chick Tissue-Storage Bioassay of Alpha-Tocopherol: Chemical Analytical Techniques, and Relative Biopotencies of Natural and Synthetic Alpha-Tocopherol¹

W. J. PUDELKIEWICZ, L. D. MATTERSON, L. M. POTTER, LORNA WEBSTER AND E. P. SINGSEN
Poultry Science Department, Storrs Agricultural Experiment Station, University of Connecticut, Storrs

The prevention of fetal resorption in the rat (Mason, '42) has been the most generally used bioassay for vitamin E. By this type of bioassay, Harris et al. ('44), contrary to the belief at that time, found that the natural tocopherols had a greater biological potency than the synthetic. Later, Harris and Ludwig ('49a), who conducted rat pregnancy studies, reported that natural *d*, α -tocopherol was 1.36 times more potent than the synthetic *dl*, α -tocopherol. Using a different criterion of measurement in the rat, the dialuric acid hemolysis method, Friedman et al. ('58) reported that the natural form was 1.33 times more potent than the synthetic. Hove and Harris ('47), by feeding tocopherol to cure muscular dystrophy in rabbits, observed that the natural α -tocopherol was 1.22 times more effective than the synthetic.

Agreement concerning the relative potencies between the free and acetate esters of the natural and synthetic α -tocopherols, however, is not good. Rat pregnancy studies by Harris and Ludwig ('49b) showed that the esters of *d*, and *dl*, α -tocopherol were 1.62 times more effective than the respective free tocopherols. The dialuric acid hemolysis method, as used by Friedman et al. ('58), showed equal utilization between the free and esterified *dl* forms of α -tocopherol. Week et al. ('52), using oral doses of synthetic tocopherols in humans and measuring the increase in plasma tocopherol, reported that the free form was 35% more effective than the ester.

Thus, bioassay methods for the tocopherols have been used for three species

of animals, with 4 criteria of measurement. Based upon the extensive work of Harris and Ludwig, the 10th revision of the National Formulary stated that *d*, α -tocopherol or its acetate ester is 1.36 times more potent in anti-sterility effect on rats than *dl*, α -tocopherol or its acetate ester.

By using improved chemical methods for the determination of tocopherol in animal tissue, Bunnell ('57) showed that the tocopherol stores in the liver of the chick varied linearly with the intake of α -tocopherol.

The growing importance of vitamin E in nutrition has prompted the workers in this laboratory to use a chick bioassay to evaluate the relative potencies of the *d* and *dl* forms of α -tocopherol and their acetate esters.

EXPERIMENTAL

Day-old White Plymouth Rock male chicks were placed in electrically heated, wire batteries and fed a tocopherol-low diet (Singsen et al., '54) for 13 days. This tocopherol-low diet is presented in table 1. The smallest and largest chicks, approximately 40% of the total, were then removed, and the remaining chicks were distributed into experimental groups of 8 chicks of approximately equal weight. The basal diet, supplemented with 8 and 16 mg of the appropriate tocopherol per pound of diet, was fed on a restricted basis for

Received for publication December 23, 1960.

¹ Supported in part by grants from the Yantic Grain and Products Company, Norwich, Connecticut, and Hoffmann-LaRoche, Inc., Nutley, New Jersey.

TABLE 1
Composition of the vitamin E-low basal diet

Ingredient	Amount
White corn meal ¹	68.32
Soybean oil meal (50% protein)	10.00
Crude casein	15.00
Fish meal (60% protein)	2.50
Dicalcium phosphate	2.00
Ground limestone	1.50
Sodium chloride	0.50
Manganese sulfate	0.0125
Vitamin supplement in grams per 100 pounds of diet	
Vitamin A ² (325,000 I.U./gm)	1.7461
Vitamin D ₃ (3,000 I.C.U./gm)	22.7
Choline chloride	40.0
Biotin	0.0045
Niacin	1.500
Riboflavin	0.227
Ca pantothenate	0.635
Pyridoxine·HCl	0.227
p-Aminobenzoic acid	0.227
Inositol	0.227
Thiamine·HCl	0.090
Folic acid (3%)	0.605
Vitamin B ₁₂ (1 mg/gm)	0.600
Vitamin K (menadione)	0.045

¹ A de-germed, de-branned product manufactured by the Quaker Oats Co., Chicago.

² Dry vitamin A acetate beadlets manufactured by Hoffmann-LaRoche, Inc., Nutley, New Jersey.

14 days. The same tocopherol samples were used in all replications of the experiment. The natural (*d*, α -tocopherol, *d*, α -tocopheryl acetate²) and synthetic (*dl*, α -tocopherol, *dl*, α -tocopheryl acetate) tocopherols were used in the form of fine gelatin sugar beadlets and added directly to their respective diets on an equimolar basis equivalent to tocopheryl acetate.³ Feed was restricted severely for the first day, approximately 7 gm per chick, then regulated to the amount of diet consumed by the group of chicks with the least appetite. Feed consumption on the last day of the experimental period was approximately 38 gm per chick. At the end of the supplementation period, blood samples were taken by heart puncture; 3 ml were removed from each chick and all 8 samples from each group pooled. The birds were then decapitated to insure more complete elimination of blood from the liver. The pooled livers from each group were removed, homogenized in a high speed blender, sampled into extrac-

tion thimbles and frozen at -20°C in tightly stoppered plastic tubes.

METHODS

Since the method used for tissue tocopherol analysis in this laboratory consists of parts of a number of methods which are scattered throughout the literature, it was felt desirable to compile these various processes, along with some modifications in technique, and incorporate them into one publication. Using this method, recoveries of 95% or better were obtained when tocopherol standards were added to liver samples.

Reagents

Purified Skellysolve B. Approximately 1500 ml of crude Skellysolve B were shaken vigorously in a two-liter separatory funnel with three to 4 100-ml portions of concentrated sulfuric acid, or until the acid was colorless after shaking. The acid was drawn off, the Skellysolve was washed once with water, then shaken vigorously with 100 ml of saturated potassium permanganate to which 1 to 2 ml of concentrated sulfuric acid had been added. (CONCENTRATED SULFURIC ACID SHOULD NOT BE ALLOWED TO LAYER OVER OR STAND IN CONTACT WITH POTASSIUM PERMANGANATE CRYSTALS.) All the permanganate was washed out with water, and the solvent was dried over Drierite for 24 hours. It was then distilled, the first and last 10% of the portions being discarded.

Absolute ethanol. Ethanol was distilled over potassium hydroxide pellets and potassium permanganate in an all glass apparatus, and the first and last 20% of the portions were discarded.

2,2' Bipyridine solution. One-half (0.50) gm of 2,2' bipyridine was weighed into a glass-stoppered 100-ml low-actinic volumetric flask and made up to volume with purified absolute ethanol. This solution

² The natural form after being esterified for increased stability.

³ The authors are indebted to the Floridin Company, Tallahassee, Florida, for the Florex XXS; to Distillation Products Industries, Rochester, New York, for the natural tocopherols; to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for the synthetic tocopherols and for processing the various tocopherols into beadlets.

was stored in a refrigerator but brought to room temperature prior to use.

Ferric chloride solution. Two-tenths (0.20) gm of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, reagent grade, was weighed into a 100-ml glass-stoppered low-actinic volumetric flask and made up to volume with purified absolute ethanol. This solution was stored in a refrigerator but brought to room temperature prior to use.

Potassium hydroxide solution. Forty gm of potassium hydroxide, reagent grade, were dissolved in 27 ml of distilled water.

Benzene. Baker's analyzed.

Sodium sulfate. Anhydrous, reagent, granular.

Stannous chloride. Reagent grade.

Florex XXS. 60 to 90 mesh.

Extraction

The method of extraction used was basically that described by Quaife et al. ('48, '49). An extraction thimble, containing the sample, was placed into a siphon extraction cup which was suspended by means of nichrome wire from the inner hooks of a Standard Taper 55/50 inner joint of a reflux condenser. A boiling chip and 75 ml of absolute ethanol were placed into a 500-ml Erlenmeyer flask equipped with a Standard Taper 55/50 outer joint. The flask was placed on a multiple unit extraction hot plate at high heat. After extracting for 20 hours in complete darkness or subdued light, the flask was cooled rapidly to room temperature, and approximately 2 gm of anhydrous sodium sulfate, 65 to 70 ml of water, and exactly 40 ml of Skellysolve B were added. The flask was stoppered with a Standard Taper 55/50 inner stopper (the joint was first wetted with water) and shaken in a mechanical shaker for 20 minutes. When the layers separated after a few minutes of standing, the aqueous layer was aspirated off and the remaining Skellysolve extract was quickly transferred to a glass stoppered centrifuge tube and centrifuged for 5 minutes. The above and subsequent operations were performed under subdued light.

Molecular distillation

A description of the pot still and its critical dimensions were reported by Quaife et al. ('46, '48). An aliquot of the Skellysolve B extract from the extraction procedure was pipetted into an aluminum sample-holder and evaporated with the aid of heat and a stream of nitrogen. The sample was placed into the pot of the still and degassed at a pressure of 10 μ of mercury or less for 5 minutes. The vacuum was released and the condenser and aluminum liner, which contained ethanol or acetone, were inserted. After the vacuum pump was operating, dry ice was added to the solvent in the aluminum liner. The oil bath, which contained Dow Corning 550 silicone oil, was raised so that the surface of the oil was even with or slightly above the bottom of the condenser, and a pressure of 10 μ or less was maintained. The distillation was allowed to proceed for 30 minutes at a temperature between 220 to 225°C. At the end of the distillation period, the oil bath was lowered. While the glass pot was cooling, 25 ml of benzene were pipetted into a 100-ml beaker and covered with a watch glass. The aluminum liner, which contained the dry ice and solvent, was removed from the condenser, and warm water (about 50°C) was added to prevent the condensation of moisture on the cold distillate when the vacuum was released. The vacuum was then released and the distillate removed completely by carefully swirling the condenser in the benzene. This benzene solution was poured into a 50-ml Erlenmeyer flask and stoppered. An aliquot of this solution was taken for the subsequent chromatographic procedure. Extreme care was exercised to prevent the solvent from coming in contact with rubber or cork, since reducing substances would be extracted which would interfere with the analysis.

Chromatography

The chromatographic method used was essentially the procedure described by Brown ('52) and Swick and Baumann ('52). Sixteen gm of Florex XXS and 2.0 gm of SnCl_2 were weighed into a 250-ml Erlenmeyer flask, then 60 ml of 10 N

HCl were added. This was sufficient Florex to make 4 columns. This was boiled for approximately one minute. The hot solution was poured into the 4 chromatography tubes (body of tube—14 mm inside diameter, 125 mm long) into which plugs of glass wool had been previously placed. The adsorbent and the sides of each tube were washed down, first with 5 ml and then 3 ml of absolute ethanol. This was followed with 5 washes of 5 ml of benzene. After the third addition of the benzene, the adsorbent was stirred up with a thin stirring rod and then packed down firmly with the flattened end of a glass rod. After the last portion of benzene had passed through, an aliquot of the sample from the molecular distillation procedure was introduced and eluted with 5-ml portions of benzene until a 25-ml volumetric flask was brought to volume. A 5- or 10-ml aliquot of the sample was added directly; however, if more than this amount of aliquot was used, it was placed into a 50-ml Erlenmeyer flask and evaporated to a volume of approximately 5 ml by the use of heat and a stream of nitrogen prior to transfer to the Florex column. The Erlenmeyer flask was then rinsed with 5-ml portions of benzene which were transferred to the chromatographic column.

Color formation

An aliquot of benzene from the chromatographic step was placed into a 50-ml Erlenmeyer flask and evaporated to dryness with the aid of heat from a water bath and a stream of nitrogen. The last milliliter was evaporated without heat. The residue was dissolved completely with exactly 10 ml of absolute ethanol and exactly 8 ml were transferred to an Evelyn cuvette. One milliliter of the bipyridine solution was added by use of a serological pipette and mixed by swirling. The Evelyn photoelectric colorimeter was set to read 100% transmission. With another serological pipette, 1 ml of the FeCl_3 solution was added, quickly swirled until well mixed, and the percentage transmission read, at 515 or 520 $\text{m}\mu$, 15 seconds after addition of the FeCl_3 solution. A standard curve was made by preparing standard solutions of *d* or *dl*, α -tocopherol in absolute

ethanol, ranging between 5 and 75 μg of tocopherol per 8 ml of solution. The density of a blank, read 15 seconds after addition of the FeCl_3 solution, was subtracted from the density of each solution that contained tocopherol. A blank was required each time tocopherol determinations were made. The 15-second color development was considered sufficient by Quaife and Harris ('44) for α -tocopherol to react completely.

Plasma tocopherol

The method used to determine plasma tocopherol was described by Dicks et al. ('57), using the hydrogenation procedure.

Beadlets

Beadlets were dissolved in acidified warm water and extracted three times in a separatory funnel with petroleum ether. The combined extracts were dried by passing through anhydrous sodium sulfate into a round bottom boiling flask and evaporated to dryness with the aid of nitrogen and heat. The acid hydrolysis and ceric sulfate titration procedure was then followed as reported by Lehman ('57).

Feed

Basal diets and diets containing non-esterified tocopherols were analyzed by the same method as described for tissue tocopherol. Diets that contained esterified tocopherol were analyzed as above, except that 4 ml of the potassium hydroxide solution were added to the extraction flask one-half hour prior to the end of the extraction period. At the end of the one-half hour saponification period, the saponification mixture was acidified by adding 65 ml of water containing 2 ml of concentrated sulfuric acid. Sodium sulfate was not added in this procedure since the solution contained a sufficient amount from the neutralization reaction. The analysis was then continued, beginning with the addition of exactly 40 ml of Skellysolve B, as described under the section entitled *extraction*.

RESULTS

The total tocopherol content of the basal diets varied between 4.6 and 7.1 mg per

pound of diet expressed as tocopheryl acetate. Feeding this basal diet for 13 days lowered the tocopherol content of the liver tissue from approximately 150 μg to 3 μg per gm of tissue.

The data presented in table 2 represent 5 experiments in which liver was analyzed for tocopherol and 4 experiments in which plasma was analyzed for tocopherol. A direct relationship can be observed between the dietary tocopherol level and the response as measured by the tocopherol content of the liver. The relationship was found to be linear, in agreement with a similar observation reported by Bunnell ('57). Slopes of these lines were compared with the slope produced by *dl*, α -tocopheryl acetate and expressed as ratios. These data, both for liver and plasma tocopherol, were analyzed by the multiple slope ratio assay as described by Finney ('52). The model is essentially $Y = a + b_1x_1 + b_2x_2 + \dots + b_nx_n$. In solving the equations, the abbreviated Doolittle method, as given in Anderson and Bancroft ('52), was used. By solving the equations for the 5 experiments in which liver tocopherol was the response measured, the following slope ratio was obtained:

$$\frac{d,\alpha\text{-tocopheryl acetate}}{dl,\alpha\text{-tocopheryl acetate}} = 1.34$$

with 95% confidence limits between 1.26 and 1.42. The data for plasma tocopherol, analyzed by the multiple slope ratio assay, resulted in a ratio of 1.36 with 95% confidence limits between 1.23 and 1.50.

When the free *dl*, α -tocopherol was compared with its ester, using liver tocopherol as the criterion of measurement, the following ratio was obtained:

$$\frac{dl,\alpha\text{-tocopherol}}{dl,\alpha\text{-tocopheryl acetate}} = 0.99$$

with 95% confidence limits between 0.92 and 1.06. The same comparison, but measuring the tocopherol content of plasma, gave a ratio of 0.95 with 95% confidence limits between 0.84 and 1.06. The ratio comparing *d*, α -tocopherol with its acetate ester was not determined by the multiple slope ratio assay because the beadlets containing *d*, α -tocopherol were not available until the last two experiments

were run. The averages of the slope ratios in the last two experiments, when liver and plasma were used as the criteria of measurement, were 0.97 and 1.01, respectively. Confidence limits were not determined because of insufficient replication.

DISCUSSION

From the data, it would appear that the chick utilized *d*, α -tocopheryl acetate equally as well as the rat when the comparison was made with *dl*, α -tocopheryl acetate. Although plasma tocopherol could be used for measuring the relationship between the *d* and *dl* forms, a greater variation was associated with it than there was with the liver tocopherol. The acetate ester, on the other hand, was utilized equally as well as the free form; again, a greater variability was noted with plasma tocopherol. This relationship occurred whether the natural or the synthetic tocopherol was being compared with its respective acetate ester. In rat pregnancy assays, the ratio of *d*, α -tocopheryl acetate to *d*, α -tocopherol was reported to be 1.62. It was interesting to note that Friedman et al. ('58), using the same species but a different criterion of measurement, obtained equal utilization.

Although the chick bioassay, as with most bioassay methods, is not a short method, it does have an advantage in that it does not involve keeping a breeding stock of animals. Also, no specialized selection is required, for in these experiments one breed of chicks was obtained from three hatcheries. The experiments were carried out over all seasons with essentially good replication, and final values, favorably comparable with rat bioassays, were obtained.

It is believed that with some modification in experimental procedure and technique, the time required to obtain a final answer could be reduced and precision in analytical determinations could be further improved.

SUMMARY

A chick bioassay method is described for comparing the potencies of *d* and *dl*, α -tocopherol with their respective acetate esters. It was found that *d*, α -tocopheryl acetate was 1.34 times more potent than

dl, α -tocopheryl acetate as measured by liver tocopherol content, and *d* or *dl*, α -tocopherol were utilized equally as well as their respective acetate esters.

ACKNOWLEDGMENTS

The authors are grateful to Julius Tlustohowicz, Peter McManus and Mrs. Harriet Plumley for technical assistance, and to Dr. H. D. Eaton of the Animal Industries Department for assistance with the statistical analysis.

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