Tocopherol as an Activator of Cytochrome C Reductase

Alvin Nason and I. R. Lehman

Since its discovery more than 30 years ago as a fat-soluble factor necessary for reproduction in the rat, vitamin E(α-, β-, γ-, and δ-tocopherols) has been associated with a wide variety of biological processes. A number of recent reviews (*1–3*) have summarized the current status of our knowledge of the vitamin. Its chemical nature and synthesis have been established, and its wide distribution among plants and animals is well recognized. Detailed studies have been made of the effects of vitamin-E deficiency in various animals as well as of the effects of its addition *in vivo* and *in vitro* to various tissues and cell-free systems. Nevertheless, its primary mechanism of action in the living organism is still unknown. In addition to its role as an antisterility factor for the laboratory rat, vitamin E has also been shown to be necessary for the structural and functional maintenance of skeletal muscle, cardiac muscle, smooth muscle, and the peripheral vascular system in a number of animals. The muscular dystrophy and morphological alterations in various tissues associated with vitamin-E deficiency are accompanied by an increased oxygen consumption of the dystrophic muscle and by alterations in chemical composition and functional behavior. Many of these effects have been attributed to the action of tocopherol as an intracellular antioxidant, namely, its protective action in inhibiting the oxidation of unsaturated fats and other oxygen-sensitive substances, such as vitamins A and C, during storage. It is generally believed, however, that the nonspecific action of tocopherol as a physiological antioxidant represents a secondary role; and that the vitamin acts primarily and specifically through some enzyme system.

The role of tocopherol as an inhibitor of cytochrome *c* reduction in the metabolism of skeletal muscle has been postulated by Houchin (*4*), who reported an increased rate of succinic acid oxidation by hamster dystrophic muscle (*4, 5*). Basinski and Hummel (*6*) found, however, that under comparable conditions the succinic dehydrogenase system is apparently unaffected. Although general disturbances in phosphorylation mechanisms have been noted in dystrophic muscle, including depression of the creatine level (*5, 7*) and the diminution of adenosine triphosphatase (*8*), no decrease has been observed in oxidative phosphorylation in preparations of heart tissues of dystrophic rabbits (*9*). A decrease has been reported in the choline esterase content of tissues deficient in vitamin E (*10, 11*).

The action of tocopherol and its derivatives, especially the esters, on isolated enzyme systems has received some attention. D-α Tocopheryl phosphate has been especially used for this purpose because of its greater solubility in water and its implied involvement in energy transformations by virtue of its phosphate group. However, it has been shown that the phosphate ester, when it is added *in vitro,* markedly inhibits practically every enzyme on which it has been tested. The inhibition of the succinic oxidase system by D-α-tocopheryl phosphate apparently consists of both a specific action (*12*) and a nonspecific secondary mechanism involving calcium removal (*13*). The latter effect inhibits diphosphopyridine nucleotidase, with the result that the formation of appreciable oxaloacetate (in the DPN-malate system) occurs. (The abbreviation DPN is used to represent diphosphopyridine nucleotide.) This in turn inhibits succinate oxidation (*14*). Some of the other enzymes reported to be inhibited by α-tocopheryl phosphate are trypsin (*15*), liver acid phosphatase (*16*), hyaluronidase (*17*), liver esterase (*11*), and lipoxidase (*18*). Rabinovitz and Boyer (*19*) concluded that the observed effects of α-tocopheryl phosphate on enzyme systems are the result of its properties as an anion with a large nonpolar group and that its effects are not necessarily related to its action as a vitamin.

The hypothesis that tocopherol may possibly be acting as a carrier in biological oxidation-reduction reactions receives support from chemical studies that demonstrate reversible oxidation products of the vitamin (*20, 21*). Boyer (*21*) isolated and tentatively characterized an intermediate, biologically active, reversible oxidation product of D-α-tocopherol prepared by ferric chloride oxidation in the presence of 2,2'-bipyridine. The product, designated as D-α-tocopheroxide, is readily reduced to D-α-tocopherol by ascorbic acid or converted irreversibly to D-α-tocopheryl quinone upon exposure to dilute acid. The latter can be reduced to the corresponding hydroquinone. The tocopherol free radical of Michaelis and Wollman (*20*) is probably an intermediate in the reversible transformation between tocopherol and tocopheroxide.

In the work reported in this article (*22*), the possibility suggested itself that if tocopherol is involved in biological oxidation-reductions, it might conceivably act as a carrier in the oxidation of reduced pyridine nucleotides. In our experiments, it has been possible to demonstrate with purified particulate or solubilized preparations from rat skeletal muscle that tocopherol can specifically function as an activator in the enzymatic reduction of cytochrome *c* by reduced diphosphopyridine nucleotide (DPNH). The reduction of cytochrome *c* by succinate in the presence of a particulate preparation from the same source has also been shown to have a tocopherol requirement.

Preparation of the enzyme and its stimulation by tocopherol. In preliminary studies using a 15- to 25-fold purified particulate fraction from rat skeletal muscle, it has been possible to show a marked enhancing effect of D-α-tocopherol on the rate of DPNH oxidation in air. Fresh muscle was homogenized with phosphate buffer (*p*H 7.5) in a Ten Broeck tissue grinder. The supernatant solution resulting from centrifugation at 2000 *g* was dialyzed for 1 to 3 hr and recentrifuged to remove a heavy gelatinous protein precipitate. Approximately 90 percent of the activity of this supernatant solution was then collected by centrifugation for 30 min at 140,000 *g*; the pellet was suspended in phosphate buffer to yield the final preparation. The addition to this system of D-α-tocopherol or its tocopheroxide as an ethanol-albumin or ethanol-γ-globulin suspension (*23*) stimulated the rate of DPNH oxidation as much as six-fold over a control without tocopherol. The tocopherol effect could be doubled by the addition of cytochrome *c*. This fact, as well as the presence of cytochrome *c* reductase and cytochrome *c* oxidase in the system, shows that these components are on the main pathway of electron transport between DPNH and oxygen in the enzyme preparation. That tocopherol acts at some point between DPNH and cyto-

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chrome *c* was shown by the fact that it stimulated cytochrome *c* reductase activity but not cytochrome *c* oxidase activity.

Isooctane extraction of the enzyme. To demonstrate the tocopherol enhancement effect with each new enzyme preparation it was necessary to store the enzyme fraction at −15°C for 2 to 10 days with occasional testing. The assumption that the enzyme system is accompanied by a tocopherol-like component that partially dissociates under the aforementioned conditions prompted an attempt to remove the presumed "tocopherol cofactor" by extraction with various nonpolar solvents. Figure 1 shows the effect on DPN-cytochrome *c* reductase activity of extracting the enzyme by shaking with isooctane (2,2,4-trimethylpentane). This was by far the most effective of some 25 organic solvents tested. Three to five extractions of the particulate enzyme fraction with isooctane resulted in about a 75-percent decrease in cytochrome *c* reductase activity. The

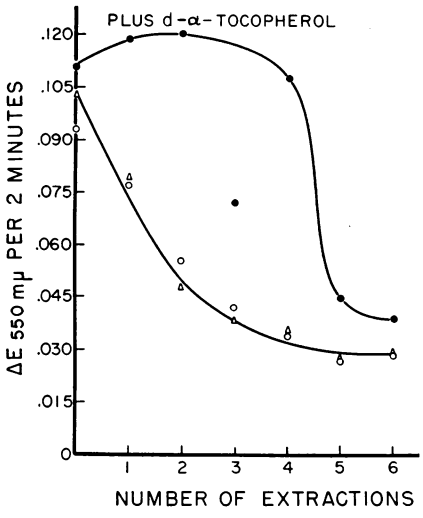


Fig. 1. Effect of isooctane extraction and subsequent reactivation by tocopherol on DPN-cytochrome *c* reductase activity in a particulate fraction from rat skeletal muscle. The reaction mixture contained 0.05 ml of enzyme (185 μg of protein), 0.10 ml of 1-percent aqueous cytochrome *c*, 0.3 ml of $10^{-2}M$ KCN, 0.1 ml of DPNH (6.1 μmole/ml) and $10^{-1}M$ phosphate buffer (*p*H 7.5) to give a final volume of 3.0 ml; 0.03 ml of 15-percent ethanol-2-percent bovine serum albumin, or 0.03 ml of D-α-tocopherol (about 4/μmole/ml) in a 15-percent ethanol-2-percent bovine serum albumin suspension was added to indicated reaction mixtures to give a final volume of 3.0 ml. Control, ○——○; Ethanol-albumin added, △——△; D-α-tocopherol added ●——●. Each isooctane extraction was performed by shaking the enzyme with an equal volume of cold isooctane (2,2,4-trimethylpentane) for 1 min. The isooctane layer was then discarded.

latter could then be completely restored by addition of D-α-tocopherol. Further extractions resulted in a progressive loss of activity that could be only partially reversed by subsequent addition of the vitamin. The oxidation of DPNH exhibited essentially the same behavior as is shown in Table 1; whereas cytochrome *c* oxidase (*24*) showed a progressive loss of activity that could not be restored by subsequent addition of tocopherol. The succinate-cytochrome *c* reductase system from the same source also showed a similar striking tocopherol requirement after isooctane treatment. Experiments that involved combining the unextracted and isooctane-extracted enzymes ruled out the presence of an inhibitor in the latter.

Treatment of the particulate system with an aqueous digitonin solution, followed by centrifugation at 140,000 *g* for 30 min, resulted in a clear supernatant solution containing more than 50 percent of the DPN-cytochrome *c* reductase. This activity could then be decreased to as little as 10 percent by a single isooctane extraction step and then completely restored by the addition of D-α-tocopherol.

Specificity of tocopherol stimulation. Figure 2 summarizes the effects of different concentrations of the various tocopherols and derivatives, as well as other fat-soluble compounds, in restoring the activity of isooctane-extracted DPN-cytochrome *c* reductase (*25*). Although the tocopherols and D-α-tocopheroxide showed some differences in their affinities and saturation levels for the enzyme, they were all quite effective. On the other hand the succinate, acetate, and polyethylene glycol 1000 succinate esters showed little or no effect, as is typified by the data for the disodium salt of D-α-tocopheryl phosphate (Fig. 2). The vitamin-E nucleus (2,2,5,7,8-pentamethyl, 6-hydroxychroman), vitamin D_2, lipoic acid, menadione, cystine, oleic acid, and cholesterol (in final concentrations ranging from 10^{-4} to $10^{-5}M$) showed little or no activity, as is typified by the vitamin K_1 results (Fig. 2). The antioxidants nordihydroguaiaretic acid, santoflex B, propylparasept, dibutyl *p*-cresol, diphenyl *p*-phenylene diamine, and santoquin (*25*) were completely inactive at final concentrations ranging from 10^{-4} to $10^{-5}M$. The latter two compounds have been reported to be effective substitutes for vitamin E in protecting against dietary necrotic liver degeneration (*26*). These results emphasize the specific requirement for the tocopherols by cytochrome *c* reductase. The comparable activities of tocopherylquinone (Fig. 2) and its hydroquinone are of interest, especially in view of their biopotency in the rabbit but not in the rat (*2*). The failure of

Table 1. Effect of isooctane extraction (enzyme extracted with isooctane as described in Fig. 1) on DPNH oxidase activity in a particulate fraction from rat skeletal muscle and subsequent reactivation by addition of tocopherol. The control reaction mixture contained 0.05 ml of one-fifth-diluted enzyme (43 μg of protein), 0.05 ml of 2-percent aqueous cytochrome *c*, 0.10 ml DPNH (1.15 μmole/ml), and $10^{-1}M$ tris(hydroxymethyl) aminomethane buffer (*p*H 7.9) to give a final volume of 1.0 ml. The albumin mixture contained 0.03 ml of 15-percent ethanol-2-percent bovine serum albumin suspension added to reaction mixtures indicated to give a final volume of 1 ml. The tocopherol mixture contained 0.03 ml D-α-tocopherol (1.1 μmole/ml) in a 15-percent ethanol-2-percent bovine serum albumin suspension added to reaction mixtures indicated to give a final volume of 1.0 ml.

No. of extractions	DPNH oxidase activity ($-\Delta E_{340}$ per 4 min × 10^3)		
	Control	Albumin added	D-α-Tocopherol added
0	121	153	232
1	169	158	239
2	95	73	250
3	49	44	236
4	42	37	199
5	41	36	175
6	18	31	158

the tocopheryl esters to act is perhaps indicative of the need for the free hydroxyl or carbonyl group in the 6-position for activity. The activities of the different tocopherols in the cytochrome *c* reductase system show no correlation with their biological potencies as reported in the literature (*1–3*).

In the absence of added tocopherol, complete restoration of enzyme activity has been obtained by adding to the extracted cytochrome *c* reductase the residue remaining after vacuum distillation of the isooctane extract of boiled enzyme. Preliminary examination of such active isooctane-extraction residues has failed to reveal, in almost all cases, the presence of any free tocopherol as measured by a paper-chromatography procedure (*27*) with a demonstrated ability to detect 5 μg. Furthermore, spectrophotometric examination of the residue before and after treatment with ascorbic acid, the latter to reduce any possible tocopheroxide present (*21*), failed to show the absorption in the region of 297 mμ that is characteristic of tocopherol. These results are suggestive of the existence of a lipid cofactor replaceable specifically by tocopherol. However, the possibility still exists that vitamin E is unrelated to such a cofactor.

Antimycin inhibition and indophenol reduction. The striking inhibition of the solubilized, unextracted cytochrome *c* reductase by antimycin A (Fig. 3) is suggestive of a factor between DPN and cytochrome *c* heretofore not reported for a soluble system. At concentrations of antimycin A causing 40 to 70 percent inhibition, partial reversal of the inhibitory effect was obtained by adding D-α-tocopherol but not by adding solutions of crystalline bovine serum albumin or human serum γ-globulin, both of which have served as vehicles for the α-tocopherol. At higher concentrations of antimycin A, no reversal by tocopherol was attained. Further experiments showed a competitive inhibition of antimycin A with tocopherol in the cytochrome *c* reductase system as demonstrated by a Lineweaver-Burk plot (*28*). When 2,6-dichloroindophenol was used as the electron acceptor in place of added cytochrome *c*, there was no inhibition by antimycin A (0.001 to 0.2 μg/ml of reaction mixture) of dye reduction. Extraction with isooctane caused no change, as compared with the unextracted enzyme, in the rate of dye reduction with or without added D-α-tocopherol;

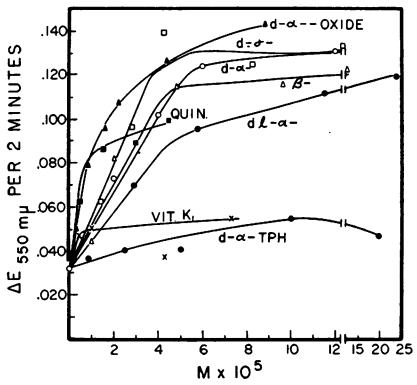
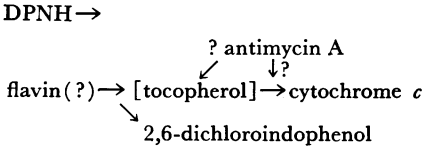


Fig. 2. Effect of the tocopherols and other fat-soluble compounds in reactivating a soluble preparation of isooctane-extracted DPN-cytochrome *c* reductase from rat skeletal muscle. The reaction mixture contained 0.05 ml of enzyme (66 μg of protein), 0.10 ml of 2-percent aqueous cytochrome *c*, 0.3 ml of $10^{-2}M$ KCN, 0.1 ml of DPNH (1.15 μmole/ml), the indicated fat-soluble compounds suspended in 15-percent ethanol-2-percent bovine serum albumin, and $10^{-1}M$ tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.9) to give a final volume of 3.0 ml. The disodium salt of D-α-tocopheryl phosphate and vitamin K_1 were added as aqueous and alcohol solutions, respectively. D-α-Tocopherol, ○——○; D-γ-tocopherol, □——□; β-tocopherol, △——△; DL-α-tocopherol, ●——●; D-α-tocopheroxide, ▲——▲; D-α-tocopheryl quinone, ■——■; D-α-tocopheryl phosphate, disodium salt, ⊕——⊕; vitamin K_1, ×——×. The enzyme was extracted once with isooctane, as is described in Fig. 1.

whereas the rate of cytochrome *c* reduction was more than 50 percent lower if tocopherol was not added.

In view of these results it appears that in the stepwise reduction of cytochrome *c* by DPNH, mediated by the enzyme system from rat skeletal muscle, vitamin E, perhaps in a conjugated form, is involved as an activator or cofactor according to the following scheme:



Whether tocopherol (or an unknown lipid cofactor) serves in some indirect fashion or undergoes alternate oxidation and reduction during the course of electron transport—possibly to the free radical (*20*) or to the tocopheroxide (*21*)—has not been established. Thus far it has not been possible to show either that D-α-tocopherol will serve as an electron donor in place of DPNH for cytochrome *c* reduction, or that D-α-tocopheroxide will serve as an electron acceptor for DPNH-oxidation in this system. A function of tocopherol or a lipid cofactor in this system in coupling phosphorylation with electron transport between DPNH or succinate and cytochrome *c* may not be unlikely.

By use of the isooctane extraction procedure, it has been possible to demonstrate a D-α-tocopherol stimulation of cytochrome *c* reductases in rat liver, yeast, neurospora, soybean leaves, hamster skeletal muscle, and beef heart fractions. On the contrary, purified soluble beef heart DPN-cytochrome *c* reductase (*29*), kindly provided by H. R. Mahler, did not show any tocopherol effect by the extraction method; nor did purified pig liver TPN-cytochrome *c* reductase (*30*), kindly furnished by B. L. Horecker, respond to the same procedure. A marked enhancement of both succinate- and DPN-cytochrome *c* reductase by tocopherol in ammonium sulfate fractions of human pectoral muscle after isooctane treatment has also been demonstrated; this constitutes tentative evidence for a functional requirement of tocopherol in man.

Mattill (*2*) has very aptly stated that "the search for some unifying principle or correlating ideas as to the manner of action [of vitamin E] has not been rewarding." Whether the presently indicated function of the tocopherols, perhaps in a conjugated form, represents its primary role remains to be proved. The relationship between a possible natural lipid cofactor and the tocopherols, their possible implication in oxidative phosphorylation, and the behavior of the

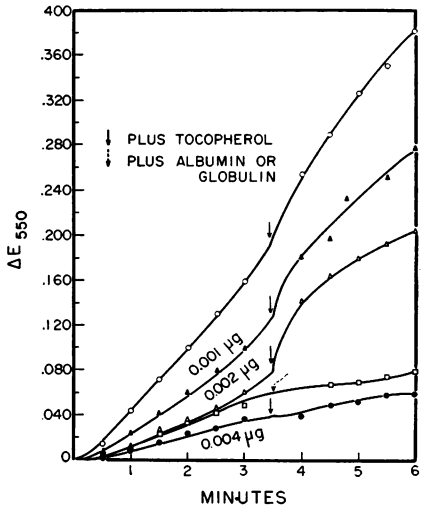


Fig. 3. Antimycin-A inhibition of soluble DPN-cytochrome *c* reductase from rat skeletal muscle and its partial reversal by tocopherol. The control reaction mixture contained 0.1 ml of digitonin-solubilized enzyme (120 μg of protein), 0.04 ml of 2-percent aqueous cytochrome *c*, 0.1 ml of $10^{-2}M$ KCN, 0.04 ml of DPNH (1.15 μmole/ml), and $10^{-1}M$ tris(hydroxymethyl)aminomethane buffer (*p*H 7.9) to give a final volume of 1.0 ml. As indicated, 0.02 ml of absolute ethanol containing various concentrations of antimycin A were added at zero time; control, ○——○; 0.001 μg, antimycin A, ▲——▲; 0.002 μg antimycin A, △——△ and □——□; .004 μg antimycin A, ●——●. Three minutes after the reactions had been started the following additions were made as indicated: 0.02 ml D-α-tocopherol (4.3 μmole/ml) in a 15-percent ethanol-2-percent human γ-globulin suspension; 15-percent ethanol-2-percent human γ-globulin; 15-percent ethanol-2-percent bovine serum albumin.

cytochrome *c* reductase of dystrophic muscle in both human and vitamin-E-deficient organisms are now under investigation.

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22. Contribution No. 115 of the McCollum-Pratt Institute. A report of this work, as presented here, was given at the Federation meetings in April 1955 [A. Nason and I. R. Lehman, *Federation Proc.* 14, 259 (1955)]. We are indebted to Bella C. Averbach for her invaluable technical assistance.
23. Crystalline bovine serum albumin and human γ-globulin were generously provided by Walter L. Hughes.
24. L. Smith, *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, in press).
25. The various tocopherols and their derivatives used in this study were generously supplied by the Distillation Products Division of the Eastman Kodak Co. Some of the D-α-tocopherol used in a number of the experiments reported here, as well as vitamin K_1, menadione, and vitamin D were generously donated by Merck and Co. The antioxidants nordihydroguiaretic acid, santoflex B, propylparasept, dibutyl *p*-cresol, diphenyl *p*-phenylenediamine, and santoquin were kindly provided by Klaus Schwartz.
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A. P. Colburn, Humanitarian and Chemical Engineer

In the death of Allan Philip Colburn on 6 February 1955, chemical engineering lost one of its most distinguished men and science, a great humanitarian. In an age of narrow specialization, Colburn stood out for his breadth and depth of interests and for a remarkable combination of the best of science and culture. He was conversant with literature, philosophy, and economics, and he devoted some of his little leisure to painting. He found time for an active part in the Delaware chapter of the American Red Cross, the Delaware branch of the American Cancer Society, his church, and other community activities. When I saw him shortly before his death, he was discussing plans for assisting in a program to improve the quality of teaching in the Delaware schools. Seldom has one man encompassed so much so well.

Allan P. Colburn was born in Madison, Wisconsin, on 8 June 1904, the son of Willis Paul Colburn, high-school principal, and Jane Grimm Colburn. After 2 years at Marquette University, he transferred to the University of Wisconsin where he was awarded a B.S. degree in 1926, an M.S. degree in 1927, and a Ph.D. degree in chemical engineering in 1929. His thesis, "Studies in heat transmission," was published by the Wisconsin Engineering Experiment Station in 1930 and stands as a classic in this field and a major stimulus to the work in heat transmission and mass transfer that has followed. The achievement was the more remarkable because he had only foreign literature to guide him and had to construct most of his own equipment. From this auspicious beginning, he went on to make significant contributions and published a long list of technical papers on heat transfer, fluid flow, distillation, absorption and extracting, and other subjects.

During the years 1929–38 at the experimental station of the E. I. duPont de Nemours and Company in Wilmington, Colburn not only matured in his science but also fought a long battle with tuberculosis, which left him with only one functioning lung and a deepened sense of social responsibility and personal idealism. While he was at Saranac Lake he won the first Walker award for outstanding publications in chemical engineering and in 1948 was the first recipient of the Professional Progress award of the American Institute of Chemical Engineers. In 1951 he was selected to deliver the principal address in London, England, at a symposium on heat transmission held jointly by American and European engineering societies. He was honored with a Civilian Service award in 1954 and posthumously with a certificate of achievement for his services as chairman of the U.S. Army Chemical Corps Advisory Council.

His public service during World War II was substantial and, typically, went far beyond a wise use of his limited physical resources. Important war research was carried on by the department of chemical engineering at the University of Delaware, a department that he organized and headed. He served on the National Defense Research Committee, on the National Advisory Committee for Aeronautics, at the Office of Rubber Reserve, and as a consultant to strategic war industries. With B. F. Dodge of Yale University, he prepared the curriculum on chemical engineering for the AST program that was taught throughout the war.

His keen sense of responsibility to his profession and to science as a whole was reflected in his positions in a wide range of professional societies. His honorary societies included Phi Kappa Phi, Phi Lambda Upsilon, Tau Beta Pi, and Sigma Xi.

In his capacities as assistant to the president, acting president, and provost of the University of Delaware, Colburn demonstrated his appreciation of the importance of developing research in the social sciences and of the broadening influences of educational programs for students which fostered the understanding of human relations and international problems.

President John A. Perkins of the University of Delaware has expressed well what all of Allan Colburn's friends and associates would want to say of him: "The most wonderful quality about him was his ability to inspire others with his own infectious enthusiasm. The breadth of his interest grew not only out of his active intellect but also out of his deep human sympathy. When he heard of other people's problems and concerns, they immediately became his own, and his active mind was driven to learn more about them. This quality made him a great teacher as well as an outstanding scientist. His creative talents, balanced by his vast store of scientific information, enabled him to make significant contributions to engineering education and higher education generally."

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