CCXXIX. STUDIES ON VITAMIN E (TOCOPHEROL)

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AN important stage in the study of vitamin E was reached when Evans *et al.* [1936] described the isolation from the unsaponifiable fraction of wheat germ oil of two allophanates one of which was definitely crystalline and both of which on hydrolysis yielded biologically active materials. The properties of the two parent compounds, which were suspected of being isomerides and which were accordingly named α - and β -tocopherol, were as follows:

	α -Tocopherol	β -Tocopherol
m.p. of allophanate	158–160°	138°
Formula of allophanate	$C_{31}H_{52}N_2O_4$ or $C_{32}H_{54}N_2O_4$	$C_{31}H_{52}N_2O_4$
Microscopic appearance	Granules, isotropic	Long needles
Formula	$C_{29}H_{50}O_2$	$C_{29}H_{50}O_2$
u.v. absorption	$E_{1\text{ cm.}}^{1\%}$ 2980 Å. $ca. 90 \pm 10$	Stated to be similar to α -compound
Curative rat dose	2.5 mg.	Stated to be less potent than α

In another publication [Emerson *et al.* 1936] they described the isolation of the same compounds from cotton-seed oil concentrates, thus linking their work up with the observations of Olcott [1934] and Drummond *et al.* [1935, 2], that biologically active concentrates can be made from this oil as conveniently as from wheat germ oil. By a fractionation method based on selective adsorption and distillation in high vacuum Drummond *et al.* [1935, 2] were able to prepare concentrates showing $E_{1\text{ cm.}}^{1\%}$ 2940 Å. = 59 and a high persistence. Restoration of fertility was observed after administration of single doses of 0.5–1 mg. Our material gave on elementary analysis figures which were consistent with the formula $C_{29}H_{50}O_2$, but we had little confidence that our methods had led to the isolation of a pure compound. The evidence at that time indicated that if our material was a mixture then its components were substances of chemically similar character, of which one, the vitamin, might represent either the greater part or alternatively a very small fraction. When the paper of Evans *et al.* [1936] appeared we were faced with the more perplexing difficulty that they had isolated a compound showing a considerably higher degree of selective absorption at 2940–2980 Å. than any preparations we had had, but one which appeared to be a good deal less potent than ours in biological tests.

This raised once again in rather an acute form the question of the relation, if any, between selective absorption at 2940–2980 Å. and biological activity, which has already been the subject of no little discussion [cf. Olcott, 1935]. According to our spectroscopic measurements on the cleanest preparations we have made the maximum, at any rate for β -tocopherol, is at 2960 Å. with a minimum at 2590 Å. (see Fig. 1). Unfortunately our re-investigation of the subject was gravely hampered in its early stages by financial restrictions which forced us to sacrifice our animal colony. The only biological tests we can report

are those which were carried out for us by Dr A. J. P. Martin at Cambridge and by Mr A. L. Bacharach of the Glaxo Laboratories. We are deeply grateful for their willing assistance in a serious emergency.

Fig. 1.

EXPERIMENTAL

The preliminary stages of the fractionation originally employed by Evans & Burr [1927] consisted of treatment with and partition between various solvents. It is stated that after removal of the bulk of sterols from cold pentane and methyl alcohol solutions a partition between these solvents gives a fraction soluble in the former solvent which is active for rats in a dose of 10 mg. According to their tests on α -tocopherol this fraction should consist of about 25 % of the active substance. This appeared at first sight to be a more economical method of concentration than that based on adsorption which we had been using. A comparative experiment was therefore made.

The unsaponifiable fraction from 5.9 kg. wheat germ oil was treated in cold methyl alcohol to remove as much sterol as possible. A sample of 90 g. of the soluble fraction, a deep red-coloured wax, was dissolved in 900 ml. of light petroleum and six times extracted with an equal volume of dry methyl alcohol previously saturated with light petroleum. The weights of material extracted by the alcohol were as follows:

Fraction	Wt. g.	$E_{1\text{ cm.}}^{1\%}$ 2960 Å.
1	17.6	39 (inflexion only)
2	27.3	"
3	10.2	37 (inflexion only)
4	7.1	—
5	3.9	—
6	2.9	General absorption
Total 69.0		

In view of the poor spectroscopic quality of extracts the fractions were reunited, dissolved again in light petroleum and extracted, as in the procedure of the American workers, with 92 % methyl alcohol. Fifteen extractions were made in all. They removed 23 g. of material showing only a suggestion of

selective absorption. From the light petroleum were recovered 45 g. of a red-coloured wax exhibiting an inflexion at 2960 Å. corresponding to $E_{1\text{ cm.}}^{1\%}$ 40. The rather laborious procedure had given us, therefore, a 50 % yield of material still rather unsatisfactory from the spectroscopic examination because of the difficulty of assessing the true absorption at 2960 Å. owing to the presence of substances showing a strong general absorption. By further treatment of this fraction by dissolving in hot methyl alcohol and cooling with CO₂ snow 21 g. of insoluble material showing no selective absorption were separated but even then the residual 24 g. remaining in solution still showed only an inflexion corresponding to $E_{1\text{ cm.}}^{1\%}$ 39. In a previous paper [Drummond *et al.* 1935, 2] we emphasized the importance of measuring not only absorption at the maximum but also at the minimum, then thought to be at 2670 Å., so as to determine the persistence. Because of its poor spectroscopic quality this yield, representing approximately 25 % of the original fraction, was disappointing, compared with those we obtained by using the chromatographic method of selective adsorption as a preliminary step. Direct comparison of the two methods made this clear. 90 g. of the same original fraction were dissolved in light petroleum and poured through columns of aluminium oxide, as described in our previous papers [Drummond *et al.* 1935, 1, 2]. The following fractions were obtained:

Fraction	Position in column	Wt. g.	$E_{1\text{ cm.}}^{1\%}$	Persistence
A	Unabsorbed	11.0	—	—
B	—	16.0	—	—
C	Bottom	12.0	General absorption	
D	Intermediate	7.4	Marked inflexion 2960 Å.	
E	Top	43.0		

Fractions D and E were united, a small amount of sterol removed from methyl alcohol solution and the residue readsorbed from light petroleum.

Fraction	Position in column	Wt. g.	$E_{1\text{ cm.}}^{1\%}$	Persistence
DE 4	Bottom	7.60	33	1.9
DE 3	—	25.86	Strong inflexion at 2960 Å.	
DE 2	—	7.33		
DE 1	Top	1.2	General absorption	

Fractions DE 2 and 3 were later readsorbed to yield further material showing a definite band.

It was then decided to try whether adsorption would separate the substances responsible for general absorption from the 24 g. of concentrate prepared by the solvent process of Evans *et al.* The chances were improved by removal of sterols precipitable under the ordinary conditions by digitonin. This yielded 14 g. of residue which in light petroleum solution were passed through an aluminium oxide column in the usual manner.

Fraction	Position in column	Wt. g.	$E_{1\text{ cm.}}^{1\%}$ 2960 Å.	Persistence
1	Top	2.8	Inflexion	—
2	—	1.1	—	—
3	—	8.25	28	1.0
4	Bottom	1.8	32	2.0

In our hands, therefore, a two-stage adsorption process gave more than four times as great a yield of material with $E_{1\text{ cm.}}^{1\%}$ 2960 Å. about 30 and a persistence of about 2 as was obtained by supplementing the solvent extraction process by a single adsorption. The solvent extraction gave very disappointing results. In all subsequent work, therefore, we used the adsorption process.

Removal of "sterols" from vitamin fractions

There is perplexing variety of "sterol-like" substances closely associated with the vitamin E fraction obtained by the adsorption technique. We are, of course, not dealing here with the main bulk of "sterols" which are removed by cooling the methyl alcohol solution in the early stages of fractionation, or with those found in the upper part of the adsorption column. We are considering only those closely associated with the vitamin fraction.

One that can sometimes be separated in part by freezing the methyl alcohol solution of the fractions or can also be obtained from the digitonide precipitate has m.p. 156° and $[\alpha]_D^{20}$ (alcohol) -18° . The relatively small quantities we have handled can be recrystallized many times from methyl and ethyl alcohols without change of m.p. or rotation, but fractionation of the 3:5-dinitrobenzoate showed that it was not homogeneous. Two nitrobenzoates were obtained:

(a) *Less soluble*. m.p. $202-203^{\circ}$. $[\alpha]_D^{20}$ chloroform -7.2° . (Found: C, 70.9; H, 8.5; N, 4.87%. Required for $C_{36}H_{50}O_6N_2$: C, 70.9; H, 8.25; N, 4.62%.)

On hydrolysis this gave a crystalline sterol, m.p. $141-142^{\circ}$. Analyses of the free sterol also correspond with $C_{29}H_{46}O$. Its colour reactions and general behaviour indicated that it was one of the sitosterols.

(b) *More soluble*. 3:5-Dinitrobenzoate melted at $180-182^{\circ}$, showed $[\alpha]_D^{20}$ chloroform $+15.7$. (Found: C, 70.93; H, 8.32; N, 4.94%. Required for $C_{36}H_{50}O_6N_2$: C, 70.9; H, 8.25; N, 4.62%.)

On hydrolysis it gave a "sterol", $C_{29}H_{48(44)}O$, m.p. 156° , but the amount was insufficient for satisfactory purification. The i.v. was 206, corresponding with three ethylenic linkages. The colour reactions were similar to those of sitosterol.

"Sterols" from digitonide precipitation

After removal of as much of the "sterols" as is possible by cooling alcoholic solutions another fraction can be separated by precipitation with digitonin. When carried out in fairly concentrated solution this procedure gives a digitonide which on resolution yields a soft waxy material, usually melting between 90 and 100° , and showing $[\alpha]_D^{20}$ (alcohol) between $+10$ and 25° . On fractionation from methyl alcohol solution this is fairly readily separated into two main fractions. The less soluble melts at 156° ; $[\alpha]_D^{20} +9.7^{\circ}$. It may be the compound described by Fernholz and Wallis [1936] as α_2 -sitosterol. The more soluble fraction yields the "new sterol" we described previously [Drummond *et al.* 1935, 2]. It is characterized by the curious behaviour on crystallization to which we then drew attention. It seems highly probable that it is the compound described recently by Karrer & Salomon [1937] to which they gave the name α -tritisterol. The properties are compared below:

	Our "new sterol" (1936)	α -Tritisterol (1937)
m.p.	$105-106^{\circ}$	$114-115^{\circ}$
$[\alpha]_D$	$+58.5$ (benzene)	$+54.3$ (alcohol)
m.p. acetate	$98-100^{\circ}$	$107-108^{\circ}$
$[\alpha]_D$	$+65.7$ (benzene)	$+70.4$ (chloroform)

The colour reactions of both compounds are similar and recall those of lanosterol. The probability is that our material was an impure preparation of α -tritisterol—we had very small amounts to handle—although there are two rather serious discrepancies. Karrer & Salomon found the analytical data of α -tritisterol to agree with $C_{30}H_{50}O$; we believed our compound to be $C_{29}H_{48}O_2$,

or, according to later analyses, $C_{30}H_{50}O_2$. Another disagreement is the M.P. of the 3:5-dinitrobenzoate which Karrer & Salomon find to be 182° and which we recorded as $156\text{--}158^\circ$. It certainly has only one reactive hydroxyl group. The description of Karrer & Salomon's β -tritisterol arouses suspicions that it may be an impure preparation of the α -compound. We found our "new sterol" to be inactive as a source of vitamin E.

Kimm's crystalline naphthoate of vitamin E

Kimm [1935] described the isolation from rice germ oil concentrates of four crystalline α -naphthoates. Two of these on hydrolysis gave materials having "strong activity" in vitamin E tests. By closely following his description we were able to prepare the compound, M.P. 158° . Its analysis agrees with the figures published by the Japanese authors. (Found: C, 84.92; H, 9.77%. $C_{40}H_{54}O_2$ requires C, 84.8; H, 9.54%.)

The "sterol" from the naphthoate, probably $C_{29}H_{48}O$ or perhaps $C_{30}H_{50}O$, showed no selective absorption in the ultraviolet and did not prove curative in doses up to 3.5 mg. Kimm reported that a single dose of 0.5 mg. restored fertility in 5 rats. We did not investigate this "sterol" further, but its colour reactions do not suggest that it is α -tritisterol.

Vitamin E fractions

Treatment with cyanic acid. Several attempts were made to apply to material such as is described on p. 1854 the process for the separation of the allophanates of α - and β -tocopherol, successfully used by Evans *et al.* [1936]. Although we followed this method as closely as we could the results were without exception disappointing. The crude benzene-soluble allophanates recrystallized from methyl alcohol gave two fairly satisfactory crystalline products. One, similar to that described by the American investigators, usually melted about 255° . They suspected it was the allophanate of β -amyirin, a substance which we had previously shown to be associated with the vitamin fraction [1935, 1]. Until quite recently we were inclined to doubt whether this was so, because we failed to raise the M.P. to that of the allophanate of β -amyirin, 266° [Drummond & Hoover, 1937]. This allophanate, once it is separated, proves to be curiously little soluble in most of the common solvents and, moreover, it is rather slowly hydrolysed. Finally we were able to satisfy ourselves that our preparations were mixtures consisting of β -amyirin allophanate and a relatively small proportion of another allophanate with M.P. 232° . β -Amyirin we can ignore as it is known to have no vitamin E activity. The second allophanate was constantly turning up as one of the less soluble fractions from methyl alcohol treatment of the crude preparations. It is interesting that Evans and his collaborators did not, apparently, find it for in our experience it was troublesome. On several occasions we thought we had isolated their α -tocopherol allophanate, for our crude preparations melted about 150° . When further purified the M.P. rose to 232° and no higher. On hydrolysis it yielded a "sterol" with the high M.P. of $233\text{--}234^\circ$; $[\alpha]_D + 39.8^\circ$ in chloroform solution. With Liebermann-Burchard's reaction it gave a golden brown darkening on standing and exhibiting a strong green fluorescence. Duplicate analysis of the "sterol" gave C, 84.65, 84.81%; H, 11.69, 11.80%. This corresponds with $C_{29}H_{50}O$. It was biologically inactive. Having separated these two allophanates we did not anticipate any difficulty in obtaining the less soluble of the tocopherol allophanates, that of the α -compound, M.P. $158\text{--}160^\circ$.

ERRATA

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line 2 *for* ester *read* ether

line 4 *read* “if tocopherol is an ether of duroquinol.”

Repeatedly, however, we failed to get it in a clean condition. Some of the preparations showed incipient melting at that temperature but none of them was satisfactory. As our material still contained substances showing general absorption in the ultraviolet which reduced the persistence of the 2960 Å. band we decided to attempt their removal by distillation in high vacuum. This procedure is believed to lower biological activity but it does not affect the spectroscopic quality. A typical distillation is described. 3 g. of material regenerated from crude allophanates, after removal of those melting at 255 and 232°, were found to have $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 52 and a persistence of 2.0. On distillation at 0.0001 mm. it gave four fractions and a considerable proportion of high-boiling residue.

Fraction	Temperature	Wt. g.	$E_{1\text{ cm.}}^{1\%}$, 2960 Å.	Persistence
I	150–160°	0.31	16	2
II	160–180°	0.80	55	4.7
III	180–190°	0.50	51	6.5
IV	190–215°	0.23	34	2

Fractions II and III were reconverted into allophanates. By using very small quantities of solvent and great care in recrystallization we obtained about 90 mg. of a product melting at 136–138°, which appeared to correspond with the β -tocopherol allophanate described by Evans *et al.* [1936]. Owing to the compound being so readily soluble in all the organic solvents tried the yield of purified material was very poor. On hydrolysis it gave a yellow oil showing $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 73.5 with a persistence of 8.5. Other values we have recorded are respectively 75 and 6.7. Several such preparations have been made but in no single case after distillation have we succeeded in isolating the α -tocopherol allophanate, m.p. 158–160°. Always the β -compound, m.p. 138°, was obtained. The regenerated β -tocopherol analyses in conformity with the formula $\text{C}_{29}\text{H}_{50}\text{O}_2$. (C, 80.60; H, 11.54%. Mol. wt. (camphor) 465. Required for $\text{C}_{29}\text{H}_{50}\text{O}_2$: C, 80.94; H, 11.62%. Mol. wt. 430.) All our preparations were optically inactive. Evans and his colleagues could not detect rotation when they examined the α -compound (allophanate). In the Liebermann-Burchard reaction it gives a straw colour which tends to darken on standing and which shows a rather strong green fluorescence.

The i.v., 147–170, suggest the presence of three ethylene linkages; theoretical value 177. Biological tests are in progress.

The apparent presence of two oxygen atoms in the molecule is a difficulty. We showed [1935, 2] that one is present as a non-phenolic and reactive hydroxyl group. Determinations of reactive hydrogen (Zerewitinoff) confirm the presence of one such grouping. (Found: H, 0.27 and 0.30%.) The other oxygen atom is certainly not ketonic. It may therefore possibly be present as an ether linkage.¹

Hydrogenation of E fractions

In one previous communication [Drummond *et al.* 1935, 2] we reported that hydrogenation in the presence of platinum oxide catalyst reduced the i.v. of vitamin fractions with values of $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 55, from about 170 to 55. The reduction proceeds with difficulty and is slow at room temperature. The reduced

¹ Since this paper was prepared for publication we have seen the communication of Fernholz [1937] in which evidence for the ester nature of α -tocopherol is advanced. It is probable that the duroquinone isolated by this investigator from the products of thermal decomposition of tocopherol arises by rearrangement of molecular fragments. If tocopherol is an ester of duroquinone the attached grouping $\text{C}_{19}\text{H}_{37}$ must be of such a character as to give a surface area of 65 sq. Å.; about twice that of the quinone itself.

material rather surprisingly showed the same spectroscopic properties as the original substance and was equally active in biological tests. In the present study we reduced a distilled β -tocopherol concentrate, $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 55, persistence 4.7. At three atmospheres pressure and with frequent changes of catalyst it required 3 days at room temperature to carry the reduction to the stage when the i.v. was 52; this corresponding to one ethylenic linkage. The reduced material was a soft waxy solid. From methyl alcohol solution on cooling at 0° five fractions of crystalline material were separated. All softened at room temperature to a waxy consistency. The spectroscopic examination gave the following results.

Fraction	Wt. mg.	$E_{1\text{ cm.}}^{1\%}$, 2960 Å.	Persistence
1	28	48	3
2	66	44	3.7
3	113	52	6.5
4	66	55	7
5	175	59	4.8

Fractions 3, 4 and 5 were united, 350 mg. in all, and 150 mg. were converted into allophanates. Two main crystalline fractions were separated from methyl alcohol solution. The less soluble 40 mg. melted at 230° and appeared to be identical with that described on p. 1856. The next weighed 55 mg. and melted sharply at 138°. On hydrolysis the latter gave 48 mg. of β -tocopherol showing $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 79, and a very high persistence (Fig. 1). The i.v. was 149. This indicates that the tocopherol itself had not been reduced and that the uptake of hydrogen had been mainly by other substances present. This explains the survival of the selective absorption and biological activity after hydrogenation in earlier experiments.

The mother-liquors yielded a curious waxy product showing no selective absorption in the ultraviolet. It may be the allophanate of partly reduced tocopherol but it has not been fully examined yet. The product yielded on hydrolysis is very slightly soluble in the common organic solvents.

Our failure to isolate from distilled material the α -tocopherol allophanate is curious. We followed very closely the details of the experiments of Evans *et al.*¹ but without success. On the other hand, we have several times prepared what appears to be the pure allophanate of the β -compound, m.p. 138°. There is one possible explanation. In our earlier work we found reduced biological activity after distillation in high vacuum and this has also been the experience of other workers. Evans *et al.* believe β -tocopherol to be less active than the α -compound. It is possible therefore that during distillation a conversion of α - into β -tocopherol occurs. This would explain the lower biological activity of distilled material and also our failure to isolate the allophanate of the α -compound.

It is interesting to compare the properties of our preparations of β -tocopherol with those of α -tocopherol regenerated from a specimen of 60 mg. of the allophanate which Dr O. H. Emerson very kindly sent us. As his preparation melted rather indefinitely between 156 and 159° it was recrystallized from methyl alcohol. Two fractions were obtained. The less soluble melted at 157–159°, the second at 152–154°. Both seemed to contain a small trace of oily material. On hydrolysis pale yellow oils were obtained which gave on spectroscopic examination $E_{1\text{ cm.}}^{1\%}$, 2960 Å. = 75, persistence 7.5 (solvent ethyl alcohol). Evans *et al.* [1936] state that the value of $E_{1\text{ cm.}}^{1\%}$, 2960 Å. for their preparations of the α -com-

¹ Dr O. H. Emerson most kindly sent us additional and very detailed information regarding their method of preparing and fractionating the allophanate. For this assistance we are very grateful.

pound is 90 ± 10 . In a private communication from Dr O. H. Emerson measurements on α -tocopherol made by Dr T. R. Hogners of the University of Chicago were given. These show $E_{1\text{cm}}^{1\%}$ 2960 Å. = 73 (solvent *isooctane*). The accompanying letter, 15. ii. 37, states "His apparatus is better than ours, and his results are probably more trustworthy."

The alcohol we have isolated from α -tocopherol allophanate appears similar to that from the β -allophanate. This confirms the findings of Evans *et al.* Once again we found the figures for C to be low for the provisional formula $\text{C}_{29}\text{H}_{50}\text{O}_2$. One oxygen is present as a reactive hydroxyl group. It forms mono-derivatives and takes up 2 mol. of cyanic acid to form an allophanate. This is confirmed by estimations of active hydrogen by Zerewitinoff's method. Methane corresponding to approximately 0.3% of hydrogen is liberated. The i.v. 170, corresponds to three ethylenic linkages. Measurement of hydrogen absorbed with platinum oxide as catalyst indicates the presence of three, or possibly four, double bonds:

- (a) 4.68 mg. absorbed 1.025 ml. H_2 , 759.25 mm. and 19.8° .¹
- (b) 2.48 mg. absorbed 0.565 ml. H_2 , 757 mm. and 19.8° .

The material reduced in these quantitative experiments showed no selective absorption when spectroscopically examined. The disappearance of the band, which is probably due to saturation, tends to confirm our belief that the survival of selective absorption in other reduced preparations was due to preferential reduction of substances accompanying the tocopherol. This point requires further study.

Dr Danielli has very kindly examined the surface spreading properties of our purest material for us. His measurements agreed with those made by Dr Askew on our earlier and less pure preparations [1935]. He has also spread it upon *N/100* neutral potassium permanganate and finds that the film is rapidly oxidized to a stage beyond which further oxidation is very slow. This second film which establishes itself after a few seconds is very stable and tends to a limiting area of 80 sq. Å., as compared with about 65 sq. Å. for the original tocopherol. The surface potential is lower for the new film. He comments on these observations as follows: "There are two possible reactions involved, either oxidation of CHOH to CO , or else oxidation of a double bond. The latter is much more probable owing to the low surface potential. The double bond will be at least 3 Å. from the original hydrophilic area. One cross-section of the vitamin must therefore be at least 80 sq. Å., and the other less than 60 sq. Å. This is compatible with a sterol or similar structure." If there is a sterol-like nucleus then there is almost certainly a fairly long side chain because of the behaviour with permanganate. We have already suggested [1935, 2] that the molecule probably has a long, unsaturated side chain.

The preparation of tocopherol by the formation of the allophanates is not, in our experience, a very practicable method because of the high losses. This is also apparent from a close study of the paper by Evans *et al.* whose yields were very poor. If one closely follows their procedure it will be found that a very large proportion of the substance showing selective absorption at 2960 Å. remains in the original methyl alcohol mother-liquors. The solubility in organic solvents is unexpectedly high and on the small scale on which we were obliged to work it is difficult to obtain enough crystalline material for adequate purification.

¹ These estimations were carried out for us by Prof. I. M. Heilbron, to whom we are very grateful.

Part of the expenses of this work were defrayed from a grant from the Medical Research Council which we wish gratefully to acknowledge. Messrs Glaxo Laboratories very generously gave us a large amount of crude wheat germ oil concentrate. For this assistance and for willing help in testing certain fractions we are greatly indebted to them and to their chief chemist, Mr A. L. Bacharach. We also wish to thank Dr J. R. Edisbury of Liverpool University for very kindly checking some of our spectroscopic measurements.

Elementary analyses were made by Dr G. Weiler and Dr F. B. Strauss, and by Dr Ing. A. Schoeller.

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