The Origin of Naturally-occurring Acetylenes

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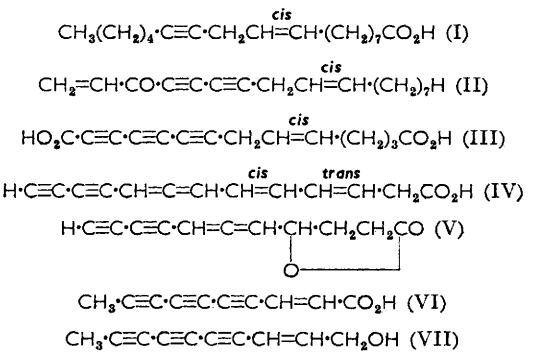
The hypothesis that the triple bond of natural acetylenes is formed by a dehydrogenation and that these compounds are derived from common fatty acids by various oxidation processes is confirmed by the demonstration that [10-^{14}C]oleic acid is converted by *Tricholoma grammopodium* into [10-^{14}C]octadec-9-en-12-ynoic acid and into [2-^{14}C]dec-2-en-4,6,8-triyn-1-ol, and by the presence in polyacetylene-producing fungi of two of the postulated intermediates, *cis*-octadec-9-en-12-ynoic (crepenynic) and *cis,cis*-octadec-9,14-dien-12-ynoic (dehydrocrepenynic) acids.

AMONGST the many naturally-occurring acetylenes now known, the discernment of structural regularities, and the results of precursor-incorporation and substrate-conversion experiments, clearly define the initial and final stages of biosynthesis but leave the intermediate stages obscure.[1] In the final stages we know that the full variety of the natural products arises from a relatively short series of intermediates, *viz.*, polyunsaturated straight-chain acids of even carbon number. As for the initial stages, it is known that these acids are assembled from acetyl- and malonyl-coenzyme A. The intervening problem is whether this assembly process is a special variant of fatty-acid synthesis, with the formation of triple bonds replacing the usual reduction steps, or whether the parent acetylenic acids are formed by special transformations of normal fatty acids. The distinction involves not only the mode of formation of the triple bonds; it concerns also the origin of the chain-lengths of natural acetylenes. Whereas a special variant of fatty-acid synthesis might well lead directly to acids of the correct chain-length and unsaturation, synthesis by way of standard fatty acids must involve chain-shortening steps, since these are mainly C_{18} and C_{16} compounds whereas the most common acetylenes are C_{14} and C_{10} derivatives.

Some natural acetylenes have obvious structural (and functional) relationships to common fatty acids, for example octadec-6-ynoic (tariric) acid, cf. octadec-6-enoic (petroselenic) acid. In recent years the series of acetylenic acids of this type has been considerably increased, and for these it seemed very plausible that the more highly unsaturated members could derive from less unsaturated acids by dehydrogenation steps. The conversion of a double bond into a triple bond could be seen as analogous to a mechanism which is now established [2] as the main route to double bonds in fatty acids, *viz.*, the dehydrogenation of saturated acids by structurally and stereochemically specific enzymes. The exploration of this biogenetic hypothesis has been given elsewhere,[3] but it is necessary to introduce some examples to show the context of the experimental verification we now describe.

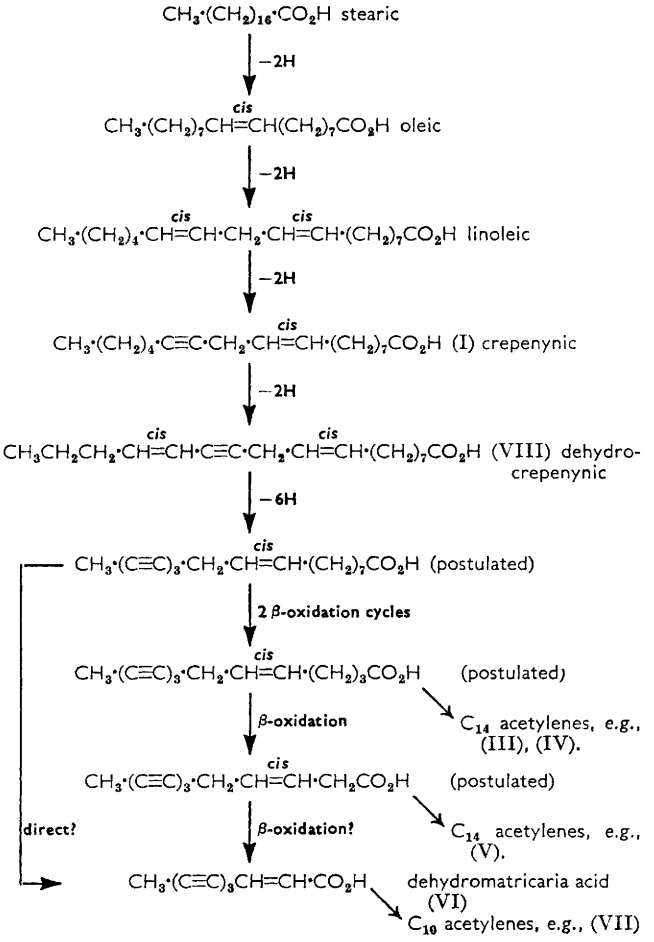
In this hypothesis the entire range of acetylenes found in Basidiomycetes and in higher plants of the Compositae, Araliaceae, and Umbelliferae can be derived from linoleic acid *via* its acetylenic 12,13-dehydro-derivative, crepenynic acid (I), first described in the seed-oils of *Crepis* spp. (Compositae, section Liguliflorae),[4] which we now know to occur more widely. The necessary transformations involve further dehydrogenations in the



" distal " part of the molecule (*i.e.*, the part further from the carboxyl group), some rearrangements of the unsaturation thus introduced, and chain-shortening in the " proximal " part of the molecule, together with the final transformations already alluded to. In some cases, structural features of crepenynic acid remain recognisable throughout this series of changes whilst in others they are eventually obscured, and the postulate that crepenynic acid is the parent of the entire series is justified only by economy. For example, in the C_{17} compound falcarinone (II) most of the molecule of (I) is recognisable; it is still apparent in the fungal C_{14} acid (III). In the fungal allene (IV) we conclude by comparisons that the allene unit represents the ·C≡C·CH_2 group of (I) and note the adjacent *cis*-double bond, which in another fungal allene (V) has disappeared. In the C_{10} acid (VI), common in Compositae and fungi alike, all superficial relationship to (I) has disappeared. However, if we write a stepwise Scheme such as shown, the biogenetic outlines of the entire series form a consistent pattern linking the clear examples with the more obscure cases. One consequence of such a Scheme is the formation of the group CH_3·$(C{\equiv}C)_3$·CH_2; if prototropic rearrangements of such a group lead to fungal allenes, we should expect these to contain, characteristically, the diyne-allene group $(C{\equiv}C)_2CH{=}C{=}CH$, as is indeed the case.[5]

[1] J. D. Bu'Lock, *Progress in Organic Chemistry*, 1963, **6**, 86.
[2] *E.g.*, G. J. Schroepfer and K. Bloch, *J. Biol. Chem.*, 1965, **240**, 56; D. K. Bloomfield and K. Bloch, *ibid.*, 1960, **235**, 337.
[3] J. D. Bu'Lock, " Comparative Phyto-chemistry," ed. T. Swain, Academic Press, London, 1966, p. 79.
[4] K. L. Mikolajczak, C. R. Smith, M. O. Bagby, and I. A. Wolff, *J. Org. Chem.*, 1964, **29**, 318.
[5] R. E. Bew, J. R. Chapman, Sir Ewart R. H. Jones, B. E. Lowe, and G. Lowe, *J. Chem. Soc.* (*C*), 1966, 129.

For an experimental test of this general hypothesis we chose first a typical C_{10} polyacetylene, the alcohol (VII) (dehydromatricarianol) which is produced by the



Possible sequence from stearic acid to the various C_9—C_{14} acetylenes, showing the role of crepenynic and dehydrocrepenynic acids. Presumably, as in the first step, special derivatives rather than the free acids are the actual intermediates.

Scheme

fungus *Tricholoma grammopodium* [6] and is one of several known derivatives of the acid (VI); [7] it offers a particularly good test since in the molecule of (VII) little or no structural relationship to crepenynic acid (I) is apparent. The aim was to detect precursors of (VII) in the fungal lipids and to establish radiochemically the type of overall relationship in the Scheme. The initial observations were made in the radiochemical experiment; [8] further studies were made with the lipids of *T. grammopodium* and with lipids from the Basidiomycete B.841 [which produces allenes such as (V)]. These studies are incomplete but some results have been used here to clarify the initial observations.

In the lipids of these fungi linoleic acid predominates, with oleic and palmitic acids in substantial proportions.

[6] J. D. Bu'Lock and H. M. Smalley, *J. Chem. Soc.*, 1962, 4662.
[7] P. Hodge, Sir Ewart R. H. Jones, and G. Lowe, *J. Chem. Soc.* (*C*), 1966, 1216.

In *T. grammopodium* grown as described, the lipids also contain two acids which we have identified as crepenynic acid (I) and the new acid (VIII) (cf. Scheme) which for convenience we term dehydrocrepenynic acid. Together these make up about 1% of the total fatty acids; in B.841 they make up about 3—5% of the total, mainly as (VIII). The corresponding methyl esters can be separated from methyl linoleate and from each other by repeated development of thin-layer chromatograms or, more easily, by partition chromatography. On ordinary silica gel plates the order of decreasing R_f is Me linoleate > Me crepenynate > Me dehydrocrepenynate, and on silica gel impregnated with silver nitrate the order is Me crepenynate > Me dehydrocrepenynate > Me linoleate. Gas–liquid chromatographic results for the methyl esters of (I) and (VIII), with some comparative data, are summarised in Table 1. Here the behaviour on

TABLE 1

Equivalent chain-lengths of fatty acid methyl esters in gas–liquid chromatography

Column *	1	2	3	4	5
Methyl stearate	18·0	18·0	18·0	18·0	18·0
,, oleate	17·7	17·95	18·3	18·4	18·2
,, linoleate	17·7	18·0	18·9	19·05	18·65
,, linolenate	17·7	18·1	19·7	19·8	19·3
,, stearolate	17·95	18·5	19·7	19·85	19·55
,, crepenynate	18·05	18·95	20·5	20·85	20·3
,, dehydrocrepenynate	18·07	19·0	—	21·35	20·8
,, ximenynate †	—	19·75	21·9	22·1	21·9

* Column 1 1·5% "SE301," 6 ft. 200°
2 15% polypropyleneglycol, 6 ft., 200°
3 20% polyethyleneglycol succinate, 6 ft., 180°
4 9% polyethyleneglycol adipate, 4 ft. 200°
5 8% "Carbowax 1540," 6 ft., 150°

† Methyl *trans*-octadec-9-yn-11-enoate.

columns 1 and 2 establishes the chain-length and the presence of a triple bond, respectively, whilst columns 3—5 indicate the total unsaturation.

The gas–liquid chromatographic results for (I) correspond well with those reported [4] for methyl crepenynate from *Crepis* sp. which is known to be the *cis*-isomer. The structure is further confirmed by the results of the oxidative degradation described in the Experimental section. The structure of dehydrocrepenynic acid (VIII), follows first from the chromatographic behaviour, secondly from the results of the oxidative degradation, and thirdly from spectroscopy. The ultraviolet spectrum is that of a conjugated enyne; the infrared spectrum of the methyl ester is very like that of methyl *cis-cis*-linoleate save for the appearance of an extra band at 1290 $cm.^{-1}$ and an intensification of the band at 740 $cm.^{-1}$. Since there is no strong absorption in the region between 800 and 1100 $cm.^{-1}$ we conclude that (VIII) has no *trans*-double bonds [9] and is therefore *cis,cis*-octadec-9,14-dien-12-ynoic acid. A small sample of the methyl ester of (VIII) was converted into the corresponding keto-ester, methyl 12-oxo-octadec-9,14-dienoate, by mercuric-catalysed hydration of the triple bond.

[8] J. D. Bu'Lock and G. N. Smith, *Biochem. J.*, 1966, **98**, 6P.
[9] J. L. H. Allan, G. D. Meakins, and M. C. Whiting, *J. Chem. Soc.*, 1955, 1874.

The mass spectrum of this keto ester showed the molecular ion 308, and major fragments at mass numbers 97, 112, and 151, which we ascribe to the ions $(C_3H_7CH{=}CH{\cdot}CO)^+$, $[C_3H_7CH{=}CH{\cdot}C(OH){=}CH_2]^+$, and $[(CH_2)_7CO_2Me]^+$, respectively, in accordance with structure (VIII) for the original acetylene.

From *T. grammopodium* to which [10-^{14}C]oleic acid had been administered, labelled linoleic and crepenynic acids were isolated, and degradation of these (Table 2) satisfactorily demonstrated that the C_{18} chain of both acids was derived, intact, from oleic. There was some redistribution of the labelling into the remainder of the chain but this was not significant since both samples included material formed up to 7 days after administration of the [10-^{14}C]oleic acid, a period allowing for considerable breakdown and resynthesis of the fatty acids. As expected, there was considerable dilution of the original labelling in the oleic acid isolated (about 1200-fold), but the further dilution in going to linoleic and crepenynic acids was quite small (about 60-fold), and the specific activities of these two acids were virtually the same. It is thus established that, as in the Scheme, oleic acid undergoes dehydrogenation to the diene, linoleic acid, and to the enyne, crepenynic acid, and the discovery of the next acid (VIII) extends the sequence.

Degradations of the C_{10} alcohol (VII) from the same incubations (Table 2) similarly establish that these ten carbon atoms derive from C(9)—C(18) of oleic acid, with the ^{14}C from [10-^{14}C]oleic now located at C(2), as required by the Scheme. Here too, the sample for which more complete results were obtained (sample DB) included material formed up to 14 days after administration of tracer, so that there is a fairly high proportion of redistributed labelling in C(1) and C(3)—C(10), but sample DA, obtained after only 3½ days incubation, shows how small this non-specific labelling can be in a shorter-term experiment. These degradations, therefore, fully confirm the overall hypothesis outlined in the Scheme.

[10] J. D. Bu'Lock, H. Gregory, and M. Hay, *J. Chem. Soc.*, 1961, 3544.

The natural acetylenes can therefore be viewed as the products of a new route of fatty acid oxidation, in which only the hydrogen of the $(CH_2)_n$ chain is oxidised. Whatever the metabolic significance of this pathway, it is quantitatively important in certain fungi,[10] whilst its taxonomic distribution is already defined.[1,3] Given that the dehydrogenation pathway to a triple bond is established, we need no longer insist upon what has till now been a guiding rule, that all the natural acetylenes were derived from fatty acids, and a limited occurrence of, for example, acetylenic terpenoids becomes conceivable: the first example is already known.[11] For the main series of natural acetylenes, it may be desirable to establish further instances to parallel the overall derivation of (VII) from oleic acid; it will also be important to explore the possible sequences in such a derivation, since the stepwise route in the Scheme is only one possibility. In particular, the stages at which chain-shortening reactions intervene, and the nature of such reactions, needs to be established. Starting from C_{18} acids, there are some C_{17} acid derivatives which may well arise by α-oxidation,[3] whilst for the common C_{14} series two steps of β-oxidation may be the most probable mechanism. For the common C_{10} series further β-oxidation is at least a possibility, but the natural occurrence of, for example, deca-2,4-dienoic acid as a conjugate with a C_8 fragment,[12] clearly originating directly from a C_{18} glyceride, suggests that a direct oxidative fission might more easily explain the peculiar preponderance of C_{10} polyacetylenes in some organisms.

TABLE 2

Radiochemical results, corrected for dilution steps

	Oleic acid	Linoleic acid	Crepenynic acid	Dehydromatricarianol (VII) Sample DA	Dehydromatricarianol (VII) Sample DB
Recovered mμc	2500	300	5	13	60
μc/g.	60	0·9	1·0	5	3
Incorporation (%)	12	1·5	0·025	0·13	0·2
Distribution of ^{14}C (mμc)					
C_{18} acids C(1)—C(9)	—	50	0·9	—	—
C_{18} acids C(10)—C(12)	—	190	3·4	—	—
(VII) C(1)—C(8)	—	—	—	13	—
(VII) C(9)—C(10)	—	—	—	<0·1	—
(VII) C(1)	—	—	—	—	3·8
(VII) C(2)	—	—	—	—	24·5
(VII) C(3)—C(10) (×⅛)	—	—	—	—	4·0

EXPERIMENTAL

Culture Conditions.—*Trichloma grammopodium* was grown for 35 days in surface culture on Czapek–Dox medium with 3% malt extract, and the medium then replaced by 4% glucose solution. [10-^{14}C]Oleic acid (Calibiochem, 21 mc/mmole) was administered as the ammonium salt in water (4 μc/ml.) in 10 μc lots to each of 4 flasks: to flasks *A*, *B*, and *C* after 24 hours' replacement culture and to flask *D*

[11] R. A. Massy-Westropp, G. D. Reynolds, and T. M. Spotswood, *Tetrahedron Letters*, 1966, **18**, 1939.

[12] H. W. Sprecher, R. Maier, M. Barber, and R. T. Holman, *Biochemistry*, 1965, **4**, 1856.

after 96 hours' replacement culture. Flask *A* was examined 3·5 days after addition of tracer and flask *B* 7 days after; the medium from flasks *C* and *D* was pooled, after 14 and 11 days' incubation, respectively.

Dehydromatricarianol (VII).—Two separate samples of (VII) were examined. Sample DA was derived from the medium in flask *A* alone, whilst the larger sample DB was derived from the medium in flasks *B*, *C*, and *D* and from the fractionation of mycelial extracts (see below). Each sample was diluted with approximately equal amounts of carrier (VII). The media and mycelial washings were extracted twice with ether, and the combined extracts dried, evaporated at room temperature, taken up in cyclohexane, and fractionated in ethyl acetate–hexane on a column of silica gel (Whatman SG-31). The eluates with 22·5 to 25·0% ethyl acetate were combined and further purified by partition chromatography on chloroform-loaded silanised " Embacel," eluting with 60% aqueous methanol. Final purification to constant radioactivity–optical density ratio was by thin-layer chromatography in 20% ethyl acetate–hexane on " Kieselgel H."

Mycelial Lipids.—Washed mycelium from flasks *A* and *B* was combined and extracted repeatedly with cold 3 : 1 ethanol–ether and once with hot solvent. The combined extracts were evaporated to dryness, and the lipids taken up in hexane, which was centrifuged to remove insoluble material and then evaporated. The lipid fraction was then transmethylated by shaking with 1% sodium methoxide in methanol for 2 hr., acidified with dilute acid at 0°, and extracted with 1 : 1 ether–hexane. The methylated material was then fractionated on a silica gel column with ethyl acetate–hexane. With 2·0 to 3·0% ethyl acetate the methyl esters were eluted, with some enrichment of acetylenic esters in the later fractions. A small amount of dehydromatricarianol (VII) was eluted with 25% ethyl acetate and combined with sample DB.

Resolution of the acetylenic esters from methyl linoleate (the major component of this fraction) was by repeated thin-layer chromatography in 5% ether–hexane on " Kieselgel H "; we have since found it more convenient to use a column of silanised " Embacel " loaded with decane and eluting with 90—97% methanol–water. The separation is monitored by gas–liquid chromatography and ultraviolet spectroscopy.

Methyl linoleate was finally purified by repeated thin-layer chromatography in 20% ether–hexane on " Kieselgel H " made up in 5% $AgNO_3$. Methyl crepenyate was purified by repeated development with 5% ether–hexane on normal " Kieselgel H " plates, and from the " tail " of the methyl crepenynate band a concentrate showing the ultraviolet absorption of an enyne was obtained (methyl dehydrocrepenynate concentrate).

This concentrate was treated with mercuric acetate in methanol (24 hr., dark, room temp.); the methanol was then evaporated, the residue taken up in chloroform, and the extract evaporated. The residue was treated with dilute HCl and extracted with ether. The ether extract contained the keto-ester (IX) (positive reaction with 2,4-dinitrophenylhydrazine spray) which was purified by thin-layer chromatography. Finally the keto-ester zone was scraped off and eluted for mass-spectroscopic examination.

Characterisation of Crepenynic and Dehydrocrepenynic Acids.—In the original experiments, the methyl crepenynate was characterised by its column- and thin-layer-chromatographic behaviour and contained an appreciable proportion of the enyne, methyl dehydrocrepenynate, as apparent from the results of oxidative degradation (see below). This has since been confirmed in further examinations of *T. grammopodium* and *Basidiomycete* B.841 in which the two methyl esters were completely resolved, and the results for methyl crepenynate and dehydrocrepenynate in Table 1 are derived mainly from this later work.

Degradation of [^{14}C]*Dehydromatricarianol.*—Sample DA was degraded directly by Kuhn–Roth oxidation,[13] the evolved CO_2 being collected as $BaCO_3$ and the acetic acid by steam-distillation followed by ion-exchange chromatography [14] and conversion into the potassium salt for counting.

Sample DB (23 mg.) was hydrogenated with 5% palladium–charcoal (20 mg. in 25 ml. hexane–ethyl acetate) (7 mol. H_2 in 30 min.). Only a trace of decane was formed; carrier decanol (25 mg.) was added. The decanol (50 mg.) was dissolved in glacial acetic acid (0·6 ml.) and CrO_3 (0·1 g. in 0·3 ml. of 66% aqueous acetic acid) was added. After 12 min. at 100°, water was added and the mixture extracted with hexane. A small sample, treated with diazomethane, gave methyl decanoate substantially pure by gas–liquid chromatography; the remainder was finally purified by ion-exchange chromatography [14] and diluted with carrier.

After Schmidt degradation of this decanoic acid by the method of Phares [15] the nonylamine was separated by steam-distillation and collected as the hydrochloride. The product (90 mg.) was dissolved in 35% aqueous Bu^{t}OH (25 ml.) and brought to pH 12 with 0·5N-NaOH. After oxidation with $KMnO_4$ (8 ml. of 5% solution, room temp., 1 hr.) and treatment with potassium metabisulphite, the solution was made alkaline and the Bu^{t}OH evaporated under reduced pressure. After acidification a quantitative yield of substantially pure pelargonic acid was recovered by extraction with hexane and finally purified by ion-exchange chromatography. This was then degraded to n-octylamine and CO_2 as before.

Degradation of Methyl Linoleate.—Methyl linoleate (20 mg.) was dissolved in 70% aqueous t-butyl alcohol (40 ml.) and 0·1M-sodium borate (5 ml.) added followed by permanganate–periodate reagent ($NaIO_4$ 97·5 mmole, $KMnO_4$ 2·5 mmole in 40 ml.). After 24 hr. the solution was acidified and the liberated CO_2 swept out with CO_2-free nitrogen and collected as $BaCO_3$ (30 mg.). The solution was made alkaline and Bu^{t}OH evaporated off, then acidified and extracted repeatedly with ether. The ether-soluble material was separated into steam-volatile and non-volatile components, identified by gas–liquid chromatography as hexanoic and heptane-1,7-dicarboxylic acids, respectively, and the latter acid was recrystallised from ether–hexane for counting.

Degradation of Methyl Crepenynate.—The labelled material also contained some of the methyl dehydrocrepenynate. The sample (2 mg.) was oxidised as above with permanganate–periodate reagent, with the addition of K_2CO_3 (25 mg.) before collecting the CO_2 as $BaCO_3$ (38 mg.). Examination of the oxidation products confirmed that heptane-1,7-dicarboxylic acid was the only significant dibasic component (with $<2\%$ hexane-1,6-dicarboxylic); carrier heptane-1,7-dicarboxylic acid (40 mg.) was therefore added before

[13] H. S. Anker, *J. Biol. Chem.*, 1952, **194**, 177.
[14] T. Seki, *J. Biochem.* (*Japan*), 1958, **45**, 856.
[15] E. F. Phares, *Arch. Biochim. Biophys.*, 1951, **33**, 173.

steam-distillation. The steam-volatile acids comprised (by gas–liquid chromatography of the methyl esters) some 75% hexanoic with 25% butyric (from the dehydrocrepenynate) and a trace of valeric acid.

Radioactivity Measurements.—Definitive counts were measured at 30 mg./cm.^{2} on 0·3, 1·0, or 2·0 cm.^{2} discs to $\pm$2% counting error with an end-window counter of *ca.* 5% efficiency, calibrated against a polymer standard. Thin-layer chromatograms were counted semiquantitatively with a modified B.T.L. Radioactive Chromatogram Counter, the plates being covered with film and counted twice with 0·5 cm. displacement of the plates.

Gas–liquid Chromatography.—A Perkin-Elmer 800 instrument with 6 ft. columns of 1·5% SE-301, 8% Carbowax 1540, 15% polypropylene glycol or 20% polyethylene glycol succinate, or a Pye Argon instrument with a 4 ft. column of 9% polyethylene glycol adipate, was used. Equivalent chain-lengths are defined in standard terms.[16]

[16] F. P. Woodford and C. M. van Gent, *J. Lipid Res.*, 1960, **1**, 188.

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