ACTA CHEMICA SCANDINAVICA 2 (1948) 209—219

Corticrocin, a Pigment from the Mycelium of a Mycorrhiza Fungus. II *

HOLGER ERDTMAN

Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden

Mycorrhiza, »fungus root», is a product of symbiosis between the roots of most higher plants and mycelia of certain fungi. Although, on a suitable substrate, each participant usually grows quite normally it now appears to be assumed by most botanists that under certain conditions frequently occurring in Nature mycorrhiza is essential to the plants involved in this symbiosis, a mutual exchange of metabolic products probably taking place.

Although the mycorrhiza symbiosis possesses great economical importance in agriculture and forestry, and therefore has been extensively studied by physiologists [1], very little is known about its chemistry.

Some of the mycorrhiza-forming fungi have been subjected to chemical investigation *e. g. Amanita muscaria*, the fly agaric, but only the fruit bodies, not the mycelium.

On the roots of *e. g.* Norway spruce, Scots pine and whorthleberry (cowberry, *Vaccinium vitis idaea*) growing on poor, acid soil very frequently a mycorrhiza is found which is characterised by its bright yellow colour. Melin assumes that the mycelium belongs to the fungus *Corticium croceum* Bres. (= *Corticium sulphureum* (Fr.) Fr.). This question, however, is not yet settled (Melin, private communication).

Since this yellow mycorrhiza is very important in Swedish forests attempts were made to isolate the pigment responsible for its colour. Colouring matters are frequently physiologically active and this pigment may perhaps play an essential *rôle* in the metabolism of the fungus, therefore being of importance

* Part I, preliminary note, *Nature* **160** (1947) 331. Through an editorial change of the nomenclature from the »diacid» to the »dicarboxylic acid» basis in this report, $(CH_2)_{12}(COOH)_2$ and corticrocin were given erroneous names (»tetradeca» instead of »dodeca»). The paper was unfortunately published before the author had an opportunity to read the proofs.

for this particular mycorrhiza. According to Melin [1] also the isolated mycelium is characterised by a yellow colour. It is not known with certainty, but appears to be very probable, however, that the pigment of the isolated mycelium and that of the mycorrhiza are identical. Several species of the genus *Corticium* (natural order *Thelephoraceae*) produce pigments *e. g. Corticium salicinum*, and an investigation of the pigment of the fruit body of the true *Corticium croceum* and other *Corticium* species may contribute to the elucidation of the systematic position of the fungus of the yellow mycorrhiza. Such investigations are at present in progress.

Most mycorrhiza fungi grow very slowly on a synthetic medium and the mycelium of the yellow mycorrhiza is not exceptional [1]. Hence it was necessary to gather the starting material directly from the forest soil. I take this opportunity of expressing my gratitude to all those collaborators who assisted me in this very tedious work. This resulted in the recovery of about thirty rucksacks (450 liters) of soil containing roots with mycorrhiza heavily contaminated by the normal constituents of the raw humus *e. g.* decomposing branches, needles, bark, cone fragments, sand and mud.

It was of course impossible to isolate the mycorrhiza from this material manually, except for micro tests performed in order to gain experience regarding the best method for the isolation of the pigment. This could be extracted from the mycorrhiza with hot pyridine or long extraction with acetone. The pigment was found to be extremely sparingly soluble in most organic solvents. It readily formed crystals and could be purified by carefully conducted sublimations in a high vacuum. When the mycorrhiza was ground with water a turbid yellow solution was obtained, perhaps containing the pigment in combination with proteins. Sublimed crystals were completely soluble in very dilute alkali but not in more concentrated, when insoluble salts were formed. Consequently, its acidic nature was at first overlooked.

The isolated mycorrhiza appears to contain about 4 % of this pigment.

After previous concentration of the mycorrhiza, mainly involving washing with large amounts of water, procedures which are described in the experimental part, the dried soil was exhaustively extracted with acetone. The isolation of the pigment from the accompanying large amount of bituminous substances is facilitated by its extremely low solubility. From hot pyridine long yellow needles containing pyridine easily removable in a vacuum over concentrated sulphuric acid were obtained.

Elementary analysis of different preparations gave very inconsistent results. The percentage of carbon varied from 64 to 68 and that of hydrogen from 5 to 6. The preparations were free from sulphur and nitrogen. They were almost ashfree but sometimes contained a little »methoxyl» (0—1.3 %).

The melting points were high and of very little use as indications of degrees of purity.

Repeated recrystallisations, involving great losses of pigment, appeared to yield purer products, the percentage of carbon ultimately rising to about 68.

It appeared to be possible that this inconstancy in the composition of the preparations, which all »looked very pure», might be due to an admixture of compounds deriving from the soil and possessing the same low solubility as the pigment. Therefore raw humus from the same source but without yellow mycorrhizas was pretreated and extracted in exactly the same manner as soil containing the fungus. The yellow pigment was now replaced by an almost colourless material, which according to its elementary composition and chemical behaviour, *e. g.* hydrolysis with strong alkali was obviously a »wax». This material was soluble in hot ethyl benzoate in which, fortunately, the pigment is almost insoluble. After extractions with boiling ethyl benzoate and subsequent recrystallisations from pyridine different preparations, previously yielding varying analytical results, furnished materials which all had a composition agreeing with the formula $C_{14}H_{14}O_4$. Six consecutive recrystallisations from pyridine followed by sublimation in a high vacuum and a final recrystallisation from pyridine yielded a material of unchanged composition.

The yield of pure pigment from one rucksack of raw humus generally amounted to 0.2—0.3 grams.

The pigment, which has been termed *corticrocin*, dissolves in concentrated sulphuric acid to a red solution, which soon turns yellow, green and finally colourless. Corticrocin reacts with diazomethane with brisk evolution of nitrogen and formation of a yellow, sparingly soluble methyl derivative, which may be recrystallised from chloroform yielding orange-red needles or from acetic acid when golden yellow, glistening plates are obtained. Both materials melt at 230—232° (uncorr.). The molecular weight of this substance, corticrocin dimethyl ester $C_{12}H_{12}(COOCH_3)_2$ could be estimated by the Rast method, but it was necessary to work very quickly since the ester is liable to decompose in hot camphor giving too high values.

Corticrocin dimethyl ester is sufficiently soluble in alcohol to permit the investigation of the U. V. absorption, the complete curve being given in Fig. 1.

The curve shows three characteristic maxima of high absorption ($\lambda = 374$ mμ, $\log \varepsilon = 4.79$; $\lambda = 393$ mμ, $\log \varepsilon = 4.95$; $\lambda = 416$ mμ, $\log \varepsilon = 4.92$) and is very similar to those of several carotenoids. The absorption is especially reminiscent of dihydrobixin methyl ester[2], which contains eight conjugated double bonds. On the assumption, however, that corticrocin is a dicarboxylic acid of true carotenoid type (derived from isoprene) it is impossible to accommodate eight conjugated double bonds in the molecule.

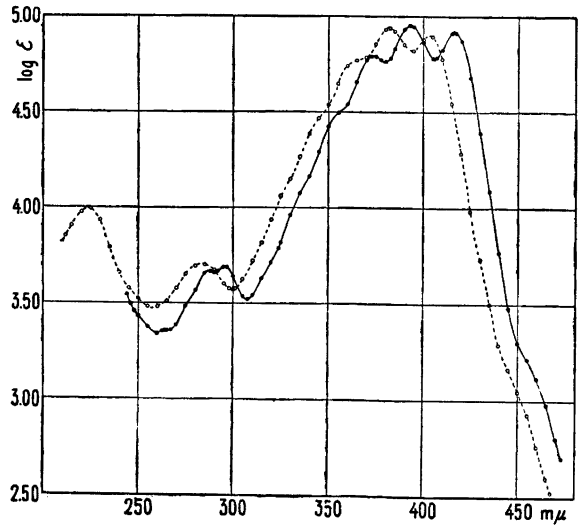


Fig. 1. Ultraviolet absorption of corticrocin dimethyl ester in chloroform (●——●) and of corticrocin in dilute alkali (○- - -○).

Moreover, chromic acid oxidation indicated that corticrocin contains no C—CH_3 groups. This led to the conclusion that corticrocin is not a normal carotenoid dicarboxylic acid but a polyene carboxylic acid of the structure

$$HOCO \cdot (CH = CH)_6 \cdot COOH$$

This was confirmed by the result of the catalytic hydrogenation of corticrocin dimethyl ester. Six molecules of hydrogen were absorbed and the dimethyl ester of *n*-dodecane dicarboxylic acid-1,12 was formed and identified by mixed melting point determination.

In a series of brillant studies of the polyene dicarboxylic acids Kuhn and his collaborators [3] prepared the acids of the general structure $HOCO \cdot (CH = CH)_n \cdot COOH$ with $n = 3$, 4, 5 and 7. The missing link in Kuhns series is corticrocin, the first polyene dicarboxylic acid of this type to be found in Nature.

The melting points of the dimethyl esters $CH_3OCO \cdot (CH = CH)_n \cdot COOCH_3$ are placed together in Table 1.

The melting point of corticrocin dimethyl ester (232°) fits well in this series.

In their papers on polyene dicarboxylic acids Kuhn and his co-workers only give wavelength data for the position of the maxima. Quantitative

Table 1. Melting points of the dimethyl esters $CH_3OCO(CH = CH)_nCOOCH_3$.

n	M. p. n uneven	M. p. n even	Difference
0		53°	49
1	102		54
2		156	16
3	172		40
4		212	11
5	223		9
6		232	4
7	236		

measurements, however, had been carried out and this enabled Kuhn to predict the maxima for corticrocin with great accuracy. »Für Corticrocindimethylester in Chloroform würde ich etwa erwarten λ^1_{max} 415 mμ, λ^2_{max} 393 mμ.« (Found: $\lambda^1_{max} = 416$ mμ, $\lambda^2_{max} = 393$ mμ).

I am very much indebted to Professor Kuhn for the following figures regarding wavelengths and molar extinction coefficients of the absorption maxima of the dimethyl esters of the acids $HOCO(CH = CH)_nCOOH$ with $n = 5$ and 7 in chloroform. (Table 2.)

Table 2. Absorption maxima of the methyl esters $CH_3OCO(CH = CH)_nCOOCH_3$ in chloroform.

n	λ^1_{max}	$\varkappa^1_{max}$	log ε^1_{max}	λ^2_{max}	$\varkappa^2_{max}$	log ε^2_{max}
5	384 mμ	$153 \cdot 10^3$	4.82	365 mμ	$174 \cdot 10^3$	4.88
6	416 »	$191 \cdot 10^3$	4.92	393 »	$205 \cdot 10^3$	4.95
7	440 »	$216 \cdot 10^3$	4.97	414 »	$220 \cdot 10^3$	4.98

Corticrocin is too insoluble in alcohol to permit a quantitative study of its U. V. spectrum. The solubility of its sodium salt in weak sodium hydroxide solution, however, is sufficient for this purpose and Fig. 1 gives the absorption of the salt.

Small specimens of corticrocin dimethyl ester can be sublimed unchanged in a high vacuum. The absorption spectrum is not altered appreciably if a trace of iodine (1 % of the weight of the ester) is added to the chloroform solution and the absorption examined after 30 minutes in diffuse light [4]. A

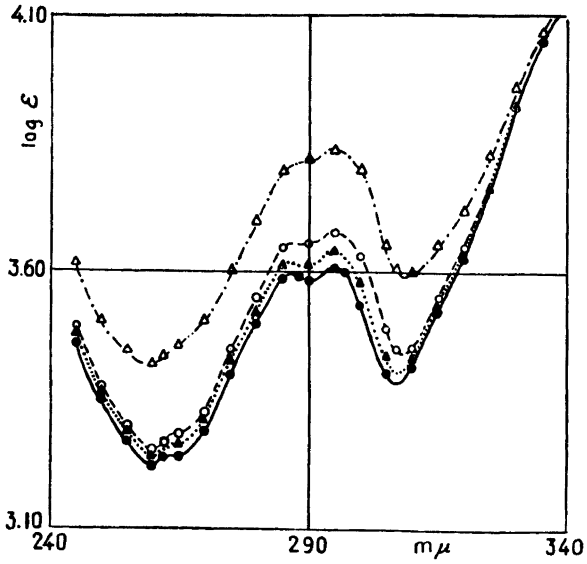
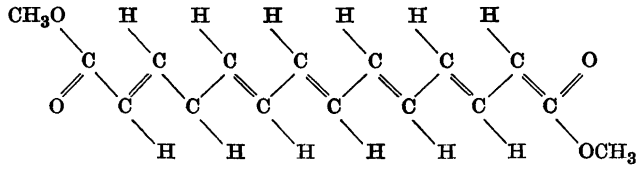


Fig. 2. Effect of traces of iodine on the absorption spectrum of corticrocin dimethyl ester in chloroform.

●——● *Without addition of iodine. No illumination.*
▲·····▲ *Without addition of iodine. Illumination 45 minutes.*
○— — —○ *1 % of iodine added. 30 minutes in diffuse day light. (No change after 43 hours in the dark.)*
△— · —△ *1 % of iodine added. 30 minutes in diffuse light. Then illumination 45 minutes.*

definite change of the absorption in the region of short waves takes place if the solution is illuminated. (45 min, 100 Watt lamp, distance 15 cm). The maxima of high absorption were practically unchanged. (Fig. 2.)
This appears to show that the corticrocin dimethyl ester preparation employed had the stable *trans* configuration throughout the chain:



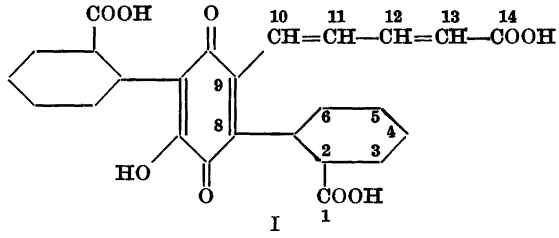
Illumination in the presence of iodine probably causes an isomerisation.
It is not yet known whether corticrocin possesses any physiological activity or plays any *rôle* in the metabolism of the fungus. If this ultimately proves to

be the case it is probable that it functions as a hydrogen carrier. Like the polyene dicarboxylic acids studied by Kuhn corticrocin and its dimethyl ester are readily hydrogenated by zinc and pyridine to the corresponding dihydro-compounds, but a description of these experiments will be the subject of a forthcoming publication.

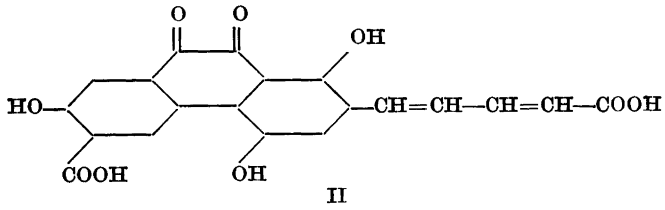
In view of the remarkable oxidation of benzene to muconic acid and especially the ω-oxidation of polyene monocarboxylic acids to the corresponding dicarboxylic acids discovered by Kuhn [5], reactions which take place in the animal body, it appears probable that corticrocin is formed in nature by ω-oxidation of the acid $CH_3(CH = CH)_6COOH$. Serious attempts were made to isolate this acid from the mother liquors but since the latter are heavily contaminated with bituminous products and waxes this task was similar to the search for a needle in a hay-stack.

Corticrocin accommodates in its molecule all the carbon atoms of phenanthrene and anthracene, derivatives of which are common products of fungus metabolism. It is interesting to note that some other components of fungi show close similarities to corticrocin.

The structure of muscarufin (I), the red colouring matter of the fly agaric *(Amanita muscaria)*, according to Kögl [6] possesses a side chain which resembles corticrocin.



I



II

Moreover, the right-hand part of the molecule indicated by the numbers 1—14 accommodates the whole molecule of corticrocin, part of which has been cyclicised to a benzene ring. If one assumes that the OH-group in the quinone nucleus of muscarufin is formed by an oxidation process during which a side chain similar to C_{10}—C_{14} is split off, the whole molecule of muscarufin appears

to be built up from two molecules of corticrocin, joined at C_8 and C_9 by two carbonyl groups.

The structure of thelephoric acid from *Thelephora* species (II), also proposed by Kögl [7], invites similar speculations but the relation to *corticrocin* is, apart from the presence of the —CH = CH—CH = CH—COOH side chain, less obvious. *Thelephora* and *Corticium* are closely related (natural order *Thelephoraceae*).

It appears not to be out of the way to assume that sometimes benzene nuclei may be formed in Nature by cyclisation of highly unsaturated fatty acids. Among the true »carotenoids» bixin and crocetin bear very close structural relationship to corticrocin. According to Kuhn and Moewus [8] crocetin possesses great biological importance *e. g.* as a sex determining factor of certain algae.

EXPERIMENTAL PART

Isolation of corticrocin

The material was collected in the neighbourhood of Stockholm. The best places to search for this subterranean fungus mycelium are on stones or rocks losely covered with mossy raw humus and inhabited by *Vaccinium vitis idaea*. It is sometimes possible to roll off a carpet of such raw humus interwoven with yellow mycorrhiza. The layer next to the rock in particular is sometimes bright yellow.

Some concentration of the mycorrhiza from the raw humus was necessary and several methods were tried, including washing with saponine solutions and different synthetic detergents. In this manner a large amount of soily material could be removed but the washings were frequently coloured faintly greenish yellow, indicating loss of pigment. Extraction of this material invariably yielded less pigment than the untreated raw humus.

The method finally adopted was to stir the raw humus for several hours with large volumes of water, then filter the mixture through a wire gauze (mesh 4 × 4 mm). When still on the screen a rapid stream of water was directed on to the raw humus to wash away impurities. The material was then kneaded manually with water containing a little fat alcohol sulphonate and washed again with large amounts of water. The residue was pressed mechanically and dried in a stream of hot air. The coarser material, *e. g.* bigger roots, stones, lumps of bark and cones were removed by hand. The material was then packed in an apparatus for continuous extraction with acetone. The black extract obtained after 0.5—1 hours extraction was collected separately and the extraction continued for about two days or until no more yellow mycorrhiza could be detected in the material.

From the first extract a sediment slowly deposited which was isolated by filtration, boiled with much acetone and filtered through a hot filter leaving undissolved a crude, yellow pigment. From the second extract the precipitate was recovered and the crude material boiled with acetone and filtered. The two acetone filtrates from the second extract were combined and concentrated. After a few days a precipitate had appeared which was treated in the same way as the sediment from the first extract, yielding a

second crop of pigment. The combined crude pigment fractions were boiled with ethyl acetate and finally with ethyl benzoate, filtered hot and washed with acetone. The dried corticrocin was dissolved in large amounts of boiling pyridine, filtered through a hot filter and left to crystallise. Sometimes supersaturated solutions were formed which crystallised on seeding. The corticrocin should not be left in contact with pyridine and air longer than necessary. Three to five recrystallisations generally yielded an analytically pure product. The mother liquors were poured into water and acidified with concentrated hydrochloric acid. The fine suspension was centrifuged and the precipitate triturated with boiling methanol and recrystallised from pyridine. The pyridine-containing yellow needles or flat, elongated prisms, sometimes attaining a length of several centimeters, were spread in a thin layer on a watch glass and dried in an evacuated desiccator over concentrated sulphuric acid. In this way the pyridine was quickly removed and the crystal form retained. The pyridine can also be removed by washing with ether, acetone or methanol but in this way the crystal form is spoiled and an orange red powder obtained.

$C_{14}H_{14}O_4$	Calc.	C 68.3	H 5.7
	Found	» 68.1, 68.4, 68.3	» 5.9, 5.7, 5.8

Less carefully purified samples which had been boiled with ethyl benzoate usually gave somewhat low values for carbon and hydrogen, (67.8—68.0 *viz.* 5.4—5.7 %).

When heated in evacuated capillary tubes pure corticrocin starts to sublime at about 270°, a red »gas» filling the tubes. The sublimate usually melts at about 317° leaving a network of a faintly coloured decomposition product. Similar results, sublimation at 260—280° and decomposition between 300 and 317° with formation of a product not melting below 340°, were obtained in tubes filled with nitrogen or in open capillary tubes. (All the melting points were observed on the »Mikroheiztisch»).

With bromine corticrocin suspended in chloroform yields a colourless product which, unlike the pigment, is easily soluble in ether, acetone and benzene, less readily soluble in chloroform and insoluble in petroleum ether and ligroin.

Percentage of corticrocin in the isolated mycorrhiza

In order to get an idea of the percentage of corticrocin in the mycorrhiza 0.4736 g were isolated manually from the raw humus and extracted for one week with acetone in a Soxhlet apparatus, by which time the material had become white. (It is much more difficult to remove the pigment from the isolated mycorrhiza than from the raw humus.)

The acetone extract was evaporated and the residue, 42.3 mg, washed with chloroform. Yield 15.0 mg of crude corticrocin (3.7 % calculated on ash free, dry mycorrhiza).

Corticrocin dimethyl ester

Pure corticrocin was treated with an excess of diazomethane in ether. A vigorous evolution of nitrogen took place and the crystals were transformed into a yellow powder. After some hours the ether was evaporated and the methyl ester recrystallised from acetic acid or chloroform. The ester is more soluble than the acid. From acetic acid golden yellow, glistening plates are obtained. From chloroform orange red, glistening needles separate. Both types of crystals melt at the same temperature. M.p. after one recrystal-

lisation generally 228—230°, after two or more recrystallisations 230—232° (open tubes, uncorrected). The ester is more easily sublimed in a high vacuum than the acid and may be purified in this way. Pure specimens of the ester could easily be obtained from crude corticrocin, which, however, had been boiled with ethyl benzoate. It is soluble in concentrated sulphuric acid with a red colour, which soon turns brownish.

$C_{14}H_{12}O_2(OCH_3)_2$	Calc.	C 70.0	H 6.6	OCH_3 22.6
	Found	» 69.9	» 6.6	» 22.9

Molecular weight (Rast, camphor, depression: 13.8° 10.1°) 285, 305 (calc. 274). It was found advisable to introduce the capillary tubes into a preheated bath. If this precaution was not observed too low depressions were obtained corresponding to molecular weights varying between 300 and 400.

Catalytic hydrogenation of corticrocin dimethyl ester

Corticrocin dimethyl ester (0.5 g) was suspended in acetic acid (35 ml), platinum oxide (0.2 g) added and the mixture shaken with hydrogen. The methylester gradually passed into solution. After four hours the calculated amount of hydrogen had been consumed. The solution was now colourless and no further absorption of hydrogen took place. The acetic acid was removed in a vacuum, the high-boiling oily residue dissolved in ether and the solution shaken with sodium hydrogen carbonate. The ether was evaporated and the oil distilled in a high vacuum. The distillate crystallised on cooling and was recrystallised from methanol. M. p. 43—45°, alone or mixed with an authentic specimen of dodecane dicarboxylic acid-(1,12) dimethyl ester.

$C_{12}H_{24}(COOCH_3)_2$	Calc.	C 67.1	H 10.5	OCH_3 21.6
	Found	» 67.0	» 10.5	» 21.5

The ester was hydrolysed with alkali to the corresponding acid, which was recrystallised from water or ethanol. M. p. 125—126° alone or in admixture with an authentic specimen of dodecane dicarboxylic acid - (1, 12).

$C_{12}H_{24}(COOH)_2$	Calc.	C 65.1	H 10.1
	Found	» 65.1	» 10.2

SUMMARY

From the yellow mycorrhiza frequently found in Sweden on the roots of pine, spruce and *Vaccinium vitis idaea* (fungus probably *Corticium croceum* Bres.) an orange yellow pigment, corticrocin, has been isolated. It has been shown to be dodecahexaene-(1,3,5,7,9,11)-dicarboxylic acid-(1, 12). The U. V. absorption curve shows three characteristic maxima: $\lambda = 374$ mμ, log $\varepsilon = 4{,}79$; $\lambda = 393$ mμ, log $\varepsilon = 4.95$; $\lambda = 416$ mμ, log $\varepsilon = 4.92$.

Corticrocin is the first unbranched polyene dicarboxylic acid to be found in Nature.

The author wishes to express his sincere gratitude to *Statens Tekniska Forskningsråd* for generous financial support of this work. My cordial thanks are also due to Mrs. A. Timell for skilful assistance in the laborious preparations, to Fil.lic. G. Aulin-Erdtman for spectrographic assistance and to Mr. W. Kirsten for the microanalysis.

LITERATURE

1. Melin, E. In Abderhalden *Handbuch der biologischen Arbeitsmethoden* Abt. XI. Teil 4 : 2 (1936) 1025.
2. v. Euler, H., Karrer, P., Klussmann, E., and Morf, R. *Helv. Chim. Acta* **15** (1932) 504.
3. Kuhn, R., and Grundmann, Ch. *Ber.* **69** (1936) 1557, 1979; **70** (1937) 1318; *Angew. Chem.* **49** (1936) 928; *Chem.-Ztg* **61** (1937) 17.
4. LeRosen, A. L., and Zechmeister, L. *J. Am. Chem. Soc.* **64** (1942) 1075; Zechmeister, L., and Pinckard, J. H. *J. Am. Chem. Soc.* **69** (1937) 1930, 1934.
5. Kuhn, R., Köhler, F., and Köhler, L. *Z. Physiol. Chem.* **247** (1937) 197.
6. Kögl, F., and Erxleben, H. *Ann.* **479** (1930) 1.
7. Kögl, F., Erxleben, H., and Jänecke, L. *Ann.* **482** (1930) 170.
8. Literature: Kuhn, R. *Angew. Chem.* **53** (1940) 1.

Received February 12, 1948.