## Summary

Directions are given for the preparation in about 28% yield of 2,3-dihydroxybutyric lactone from glycerol monochlorohydrin. The lactone has been obtained in crystalline form of melting range 22–26°. The phenylhydrazide melts at 109°.

A valuable by-product, 3-hydroxyisocrotonic lactone, was isolated in 23% yield. The compound which this lactone forms with phenylhydrazine (not the simple phenylhydrazide) melts at 183°.

2,2'-Dihydroxyisobutyric acid cannot be prepared by treatment of glycerol-$\beta$-chlorohydrin with sodium cyanide followed by hydrolysis. The product is 2,3-dihydroxybutyric lactone whether the $\alpha$- or the $\beta$-chlorohydrin of glycerol is used in the synthesis.

CHICAGO, ILLINOIS

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## STUDIES IN THE CHLOROPHYLL SERIES. VI. THE MECHANISM OF THE PHASE TEST

BY CATHERINE C. STEELE[1]

The essential change in allomerization and phase test saponification in the derivatives of chlorophyll $a$ is the dehydrogenation of the grouping —CHOHCO— to —COCO—, and it has now been shown that the two hydrogen atoms are removed by oxygen from the air.[2] If the phase test saponification of methyl phaeophorbide $a$ or chlorin $e$ trimethyl ester is carried out in a modified Warburg apparatus[3] the amount of oxygen absorbed can be measured. Since the products are the "unstable chlorins," the oxidation is also shown to take place in the alkaline medium, and not in the subsequent acidification nor in the conversion of the chlorins to the phaeopurpurins.

The inherent difference between Willstätter's methods of "phase test" and "hot quick" saponification, whereby he obtained either phytochlorin $g$ or chlorin $e$ from phaeophorbide $a$, is now apparent.[4] Phase test saponification is an atmospheric oxidation process: with phaeophorbide $a$ it also opens a ring but effects only partial saponification of the methyl group,

[1] Commonwealth Fund Fellow at Radcliffe College.

[2] A preliminary statement of these results appeared in a Communication to the Editor, THIS JOURNAL, 53, 1615 (1931), and the allomerization experiments are discussed in detail in Paper V, *ibid.*, 53, 2382 (1931). In this present paper are discussed the corresponding experiments in the phase test.

[3] Hyde and Scherp, *ibid.*, 52, 3359 (1930).

[4] Willstätter and Stoll, "Investigations on Chlorophyll," translated by Schertz and Merz, Science Printing Co., 1928, p. 263.

since the product is a mixture of unstable chlorins which change to the two phaeopurpurins, one of which (7) still retains a methoxyl group.[5] Hot quick saponification of phaeophorbide $a$ is an hydrolysis (or complete demethylation) process, and it is now obvious that the reason why the methyl alcoholic solution must be kept boiling during the entire process is that the alcoholic vapors above the solution eliminate practically all the air from the reaction, and hence hydrolysis is accomplished before the material has had a chance to oxidize. Conant and Moyer[6] found that in the phase test on phaeophorbide, the formation of chlorin $e$ can be suppressed completely by substituting ethyl or $n$-propyl alcohol for methyl alcohol in the solution of potassium hydroxide, or by keeping the reaction mixture at $-10°$. In the latter case, hydrolysis is inhibited by the low temperature, and the oxidation has time to go to completion. In the former case it is well known that concentrated ethyl and $n$-propyl alcoholic alkali solutions absorb oxygen readily with the possible formation of peroxides, and therefore accelerate the oxidation in the phase test. This is borne out by the fact that, in the measurement of the oxygen absorbed in the phase test, it was found impossible to obtain an initial equilibrium in the Warburg apparatus by the use of propyl alcoholic alkali, due to its continued absorption of oxygen (see experimental portion of this paper).

Previously, several attempts to inhibit the phase test by using an atmosphere of nitrogen were made, but these were unsuccessful, owing to the minute quantity of oxygen necessary for the reaction (0.008 millimole = 0.16 cc. for 5 mg. of methyl phaeophorbide $a$). The use of oxygen absorbers was then tried; here it was found possible on a small scale to treat methyl phaeophorbide $a$ with 25% methyl alcoholic potash in an atmosphere of nitrogen and in the presence of sodium stannite, and obtain alkali-soluble products which on methylation with diazomethane gave chlorin $e$ trimethyl ester and methyl phaeophorbide $a$ and no phaeopurpurin 7. Similarly chlorin $e$ trimethyl ester gave an alkali-soluble product which on methylation gave back chlorin $e$ trimethyl ester. The hydrolyzed product was apparently a monomethyl chlorin $e$, as it contained one methoxyl group.

In connection with this work, the difference in the time and optimum temperatures for the phase test on chlorin $e$ ester and phaeophorbide $a$ was noted, and led to a comparison of the rate of hydrolysis of several esters under phase test conditions (including both those which do and those which do not undergo the phase test). While these results cannot be entirely explained, we append in the following table a summary of the results obtained; the figures represent averages of several runs and are probably significant within 20%.

The usual procedure was to dissolve 5 mg. of ester in about 0.5 cc. of

[5] Paper V, THIS JOURNAL, **53**, 2382 (1931).
[6] Paper III, *ibid.*, **52**, 3014 (1930).

pyridine and transfer it to 15 cc. of ether in a 50-cc. Erlenmeyer flask. When the solution had attained the required temperature, 1.5 cc. of 25% methyl alcoholic potash was added and the mixture stirred mechanically. It was then shaken up with water and ether, and the material in the alkaline layer transferred to fresh ether (and methylated, where the products were the unstable chlorins). The amounts of hydrolyzed and unhydrolyzed material were determined in a Zeiss comparison spectroscope. Where the two solutions were of the same material (e. g., porphyrins) the ether solutions were compared directly. It was found to be immaterial whether the hydrolyzed solution was remethylated or not. Where the solutions were of different compounds, the amount of substance in mg. in each was obtained by comparison with a standard solution of the material in question.

TABLE I

COMPARATIVE RATES OF HYDROLYSIS UNDER PHASE TEST CONDITIONS

| | Percentage hydrolyzed in | | | |
| --- | --- | --- | --- | --- |
| | 5 min. at $-8°$ | 10 min. at $-8°$ | 15 min. at 20° | Products |
| Pyrroporphyrin monomethyl ester | | 0 | 50 56 | Pyrroporphyrin |
| Phylloporphyrin monomethyl ester | | 0 | 52 43 | Phylloporphyrin |
| Rhodoporphyrin dimethyl ester | | 0 | 57 52 | Rhodoporphyrin (spectrum) |
| Chlorin $a$ trimethyl ester | | 20 | 100 | Chlorin $a$ (spectrum) |
| Chlorin $e$ trimethyl ester | | 28 | 100 | Dimethyl phaeopurpurin 7 after methylation |
| Dehydrophaeophorbide $a$ methyl ester | 87 | 100 | 100 | Dimethyl phaeopurpurin 7 after methylation |
| Phaeophorbide $a$ | 51 | 100 | 100 | Dimethyl phaeopurpurin 7 after methylation |
| Phaeophorbide $a$ monomethyl ester | 28 | 100 | 100 | Dimethyl phaeopurpurin 7 after methylation |
| Phaeopurpurin 7 dimethyl ester | 100 | 100 | 100 | Phaeopurpurin 7 (spectrum) |
| Phaeopurpurin 18 monomethyl ester | 100 | 100 | 100 | Chlorin $a$ |

Pyrroporphyrin and phylloporphyrin esters contain only the propionic ester group; this apparently cannot be hydrolyzed under the phase test conditions at $-8°$ for thirty minutes. Rhodoporphyrin ester, which has in addition a methyl group on the $\beta$-carboxyl, hydrolyzes at about the same rate. Hydrolysis under phase test conditions for five minutes at $-8°$ of methyl phaeophorbide $a$, phaeophorbide $a$, and methyl dehydrophaeophorbide $a$ gave the following percentages of dimethyl phaeopurpurin 7 after methylation (this represents the total alkali-soluble material): 28, 51 and 87%. Demethylation and oxidation, oxidation without demethylation, and demethylation alone are the corresponding reactions involved. These results show that the group which gives alkali solubility is a grouping which is more easily hydrolyzed than the ester groups of the porphyrins. If one considered only these results, the conclusion would be that hydroly-

sis of the propionic ester group is not involved in the phase test; but we know that the propionic ester group is hydrolyzed in the case of methyl phaeophorbide because the product, phaeopurpurin 7, is a monomethyl compound with the methyl group on the carboxyl directly attached to a pyrrole ring. (The proof of this follows from its pyrolysis to monomethyl chlorin $f$ and is supported by the fact that phaeophorbide $a$ also yields phaeopurpurin 7.) These results indicate that the hydrolysis of the propionic ester is not the first result of the action of alkali: it is more likely that the anhydride ring (or a lactam ring) in phaeophorbide $a$ first opens.[5] If this is the case, no comparison can be drawn between the hydrolysis rates of the propionic ester in a neutral porphyrin and that in a compound in which a potentially acidic group has already been converted into a salt.

Hydrolyses of phylloporphyrin ester and methyl phaeophorbide $a$ were carried out in homogeneous methyl alcoholic solution, using the same proportion of methyl alcoholic potassium hydroxide as in the phase test, and the phylloporphyrin ester was completely unhydrolyzed after thirty minutes at $-8°$, while the methyl phaeophorbide gave 25.5% (compare 28% in ether) of hydrolyzed material in five minutes. The presence of a two-phase system consisting of the ether and the alcoholic layer of the potassium salt of phaeophorbide is therefore not responsible for the differences shown in Table I between the porphyrins and the phaeophorbides. The following formulas are suggested as possibilities for the trimethyl ester of chlorin $e$
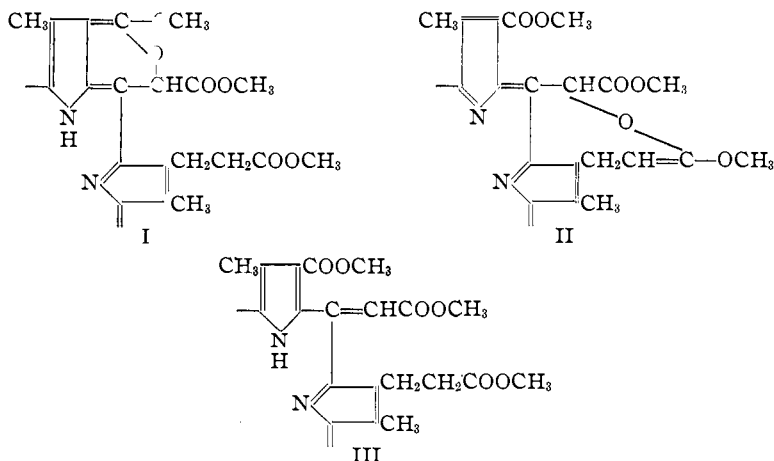


Formula II contains a more labile form for the propionic ester grouping than I or III, which require the peculiarly rapid hydrolysis of the propionic ester group as discussed above in the case of the phaeophorbide. The extraordinary rapidity with which dimethyl phaeopurpurin 7, methyl phaeopurpurin 7 and methyl phaeopurpurin 18 hydrolyze under phase test

conditions (five minutes at $-8°$, 100% alkali soluble) is surprising. **The** formula for phaeopurpurin 7 must contain the grouping (IV), or the corresponding enol modification (IVa) and hydrolysis of the trimethyl



ester (dimethyl phaeopurpurin 7) must again involve hydrolysis of a propionic ester group. That there is some difference in the ease of hydrolysis of the two ester groups was shown by stirring dimethyl phaeopurpurin 7 with phase test alkali at $-8°$ for five minutes; the resulting product had a methoxyl content of 7% (calcd. for 1 $(OCH_3)$: 4.95; for 2 $(OCH_3)$: 9.65). It is interesting to note that although dimethyl phaeopurpurin 7 hydrolyzes under phase test conditions to phaeopurpurin 7, long standing or shaking of the dimethyl ester with phase test alkali converts it back to the unstable chlorins.

The author wishes to acknowledge her indebtedness to Professor J. B. Conant for suggesting this problem.

## Experimental

**Absorption of Oxygen in the Phase Test.**—The oxygen absorption during the phase test was demonstrated by using a modified Warburg apparatus. The use of propyl alcoholic alkali was abandoned after it was found impossible with equal volumes present to equalize the pressures in the two arms of the apparatus, due to an increasing absorption of oxygen by the alkali alone. The improved yields of phase test products by the use of propyl alcoholic alkali is therefore due to the latter acting as an oxygen absorber.[7] Equilibrium was easily obtained by using methyl alcoholic potassium hydroxide and carbon dioxide-free air.

(a) **Methyl Phaeophorbide** *a*.—Twenty-three cc. of 25% methyl alcoholic potash was placed in one of the bottles of the absorption apparatus, and a glass bulb having a long capillary end and containing methyl phaeophorbide *a* (5.09 mg. = 0.008 millimole) was carefully inserted in the alkali. The other bottle contained a corresponding amount of alkali to equalize the volumes. The apparatus was placed in a water-bath at 20° and shaken for ten or fifteen minutes until a constant difference in the pressure readings was obtained. The apparatus was then removed from the bath, the bulb broken by a quick shake, the apparatus replaced, and shaken continuously. Pressure readings were recorded during five-minute intervals for the first hour, subsequently at longer intervals. The number of moles of gas absorbed was calculated from a previously determined factor (1 cc. of manometer liquid =0.0017 millimole of gas). The course of the reaction is indicated by the following figures, which give the millimoles of gas absorbed at different times: ten minutes, 0.0027; thirty minutes, 0.0046; one hour, 0.0086; one and one-half hours, 0.0102; two hours, 0.0117; two and one-half hours, 0.0129; three hours, 0.0137.

[7] Paper III, THIS JOURNAL, **52**, 3016 (1930).

That the reaction proceeds at a progressively diminishing rate is shown by the following values in $10^{-6}$ mole per hour for the amount of gas absorbed during successive thirty-minute intervals: 9.2, 8.0, 3.2, 3.0, 2.4, 1.6. The maximum for the rapid absorption of oxygen is in eighty-seven minutes when 0.01 millimole of oxygen has been absorbed; this corresponds to the absorption of one mole of oxygen per mole of phaeophorbide with the formation of hydrogen peroxide.

(b) Chlorin *e* Trimethyl Ester.—In an experiment with 5.92 mg. of chlorin *e* ester ( = 0.01 millimole), the amount of gas in millimoles absorbed at different times was as follows: ten minutes, 0.0027; thirty minutes, 0.0047; one hour, 0.0062; one and one-half hours, 0.0068; two hours, 0.0071. The rates in $10^{-6}$ mole per hour for the amount of gas absorbed during successive thirty-minute intervals are therefore 9.2, 3.0, 1.2, 0.6.

In a second experiment with 4 mg. of chlorin *e* ester ( = 0.0067 millimole) the amount of gas in millimoles absorbed was 0.0014 in ten minutes; in thirty minutes, 0.0038; in one hour, 0.0054; in one and one-half hours, 0.0065; in two hours, 0.0073. The rates in $10^{-6}$ millimole per hour for absorption during thirty-minute intervals is therefore 7.6, 3.2, 2.2, 1.6. The limit to the amount of oxygen absorbed is clearly one mole of oxygen per mole of chlorin *e* trimethyl ester.

In both (a) and (b) the products were the unstable chlorins, which on standing gave mixtures of phaeopurpurins 7 and 18, or on methylation with diazomethane gave dimethyl phaeopurpurin 7.

In a control experiment, phaeopurpurin 7 was used in the bulb, and no absorption was observed during 1 hour's shaking with the alkali.

## Inhibition of the Phase Test

(i) **Methyl Phaeophorbide** *a*.—Twenty cc. of 25% methyl alcoholic potassium hydroxide and 1 g. of stannous chloride were placed in the long arm of a Thunberg tube, and 20 mg. of methyl phaeophorbide *a* was placed in the short arm. The tube was alternately evacuated and filled with nitrogen about eight times, then shaken for seven minutes. The solution was deep green in color. Water was added immediately the tube was opened, and the alkaline solution, on being shaken with ether, retained all the material. It was transferred by acidification to fresh ether, and washed with water. In one experiment the solution was methylated with diazomethane; no change in color was observed, and the product was a mixture of chlorin *e* ester (extracted with 8% acid) and of methyl phaeophorbide *a*, acid number 18. These were identified by spectrum in acid and ether; there was twice as much chlorin *e* as phaeophorbide *a*, and no phaeopurpurin 7 was obtained.

(ii) **Chlorin** *e* **Trimethyl Ester.**—In similar experiments on chlorin *e* ester, most of the material was alkali-soluble after fifteen minutes' shaking; the alkali solution was yellow-green. On transference to ether, methylation with diazomethane gave back the trimethyl ester of chlorin *e*, identified by acid number and spectrum in acid and ether. No phaeopurpurin 7 was obtained. Fractionation of the unmethylated solution resulted in the extraction of all the material with 5 to 6% hydrochloric acid; it had the spectrum in acid and ether of chlorin *e* but contained one methoxyl group ($OCH_3$: found, 5.76; calcd., 4.95).

## Action of Phase Test Alkali on Esters

A few typical experiments are given below (see theoretical section of this paper for table of all the results).

**Phylloporphyrin Ester.**—Five mg. of ester was dissolved in pyridine, transferred to 15 cc. of ether, and 1.5 cc. of 25% methyl alcoholic potassium hydroxide added. The mixture was stirred mechanically for fifteen minutes at 20°; then, after addition of water

and ether, the alkaline layer was separated and acidified in the presence of fresh ether. The two ether solutions were compared in the spectroscope and gave the ratio hydrolyzed:unhydrolyzed material::19.6:20. The volumes of the two solutions were measured and the ratio became 19.6 × 95 cc.:20 × 87 cc. The percentage of hydrolyzed material is therefore 51.7%. The hydrolyzed fraction was methylated with diazomethane, washed and dried. On comparing it again with the unhydrolyzed ester the ratio was 20 × 46 cc.:19.2 × 50 cc., and the percentage hydrolyzed was 49.6.

**Methyl Phaeophorbide** *a*.—(i) Five mg. of ester dissolved in pyridine and transferred to ether was cooled to −8° in an ice–salt bath and 1.5 cc. of methyl alcoholic potassium hydroxide added. The mixture was stirred for five minutes, then water and ether were added. The alkaline layer was acidified under fresh ether and immediately methylated with diazomethane to convert the unstable chlorins to dimethyl phaeopurpurin 7. This was compared with a standard solution of dimethyl phaeopurpurin 7 (50 mg. per liter) and the amount determined was 1.11 mg. The original ether solution containing the unhydrolyzed material was compared with a standard solution of methyl phaeophorbide *a*. The amount obtained was 2.8 mg.; hence the percentage of hydrolyzed material was 28%.

(ii) Five mg. of ester in pyridine was added to 15 cc. of methyl alcohol in a 50-cc. flask, the solution was cooled to −8° and stirred for five minutes with 1.5 cc. of 25% methyl alcoholic potassium hydroxide. Water and ether were added and the alkaline layer was washed several times with ether, then acidified under fresh ether. This was methylated, and was found to contain 1.2 mg. of dimethyl phaeopurpurin 7. The percentage of unhydrolyzed material was 25.5.

**Phaeophorbide** *a*.—Five mg. of phaeophorbide *a* in pyridine and ether was stirred for five minutes at −8° with 1.5 cc. of 25% methyl alcoholic potassium hydroxide. Water and ether were added, the alkaline layer rapidly acidified under fresh ether, and the product methylated. A fractionation with 14% hydrochloric acid removed the dimethyl phaeopurpurin 7 and this was compared with a standard solution. The amount found was 2.4 mg. The residue from the fractionation, which consisted of unphased phaeophorbide, was washed, dried and compared with a standard solution of methyl phaeophorbide *a*. The amount was 2.3 mg.; hence the percentage of phaeophorbide phased was 51.

**Methyl Dehydrophaeophorbide** *a*.—Five mg. in pyridine–ether was stirred with alkali for five minutes at −8°. The alkaline layer was acidified under fresh ether and the solution methylated. It was compared with a standard solution of dimethyl phaeopurpurin 7, and contained 1.2 mg. The original ether, containing unhydrolyzed material, was compared with a standard solution of methyl dehydrophaeophorbide *a* (15 mg. per liter). It contained 0.6 mg.; hence the percentage hydrolysis was 87%.

## Summary

1. The phase test on chlorophyll derivatives involves oxidation by atmospheric oxygen. The amount absorbed was measured in the case of methyl phaeophorbide *a* and chlorin *e* trimethyl ester.

2. This oxidation can be prevented by the use of sodium stannite with an atmosphere of nitrogen.

3. A comparison of the rates of hydrolysis under "phase test" conditions of various esters in the "*a*" series is given.

CAMBRIDGE, MASSACHUSETTS