of one hundred hours would be slightly more than 1%.

Summary

The vapor pressure of phenothiazine within the temperature range of 63–121° and the rate of evaporation of the finely powdered compound at 45° have been determined.

The results obtained show that the loss in toxicity of phenothiazine when used for the control of insect pests is not due to evaporation.

Tracer Studies with Radioactive Hydrogen. Some Experiments on Photosynthesis and Chlorophyll

BY T. H. NORRIS, S. RUBEN AND M. B. ALLEN

Since nothing is known regarding the role of chlorophyll in green plant photosynthesis, there has been, and still is, considerable speculation on the subject. The many theories that have been proposed may be divided into two classifications: (1) chlorophyll participates as a reducing agent (hydrogen donor), (2) chlorophyll merely acts as a sensitizer (as certain dyes function on photographic plates, for example). The first category is by far the larger and we may cite a few of the more interesting proposals found in this group.

Dixon and Ball[1] have suggested that chlorophylls a and b are involved in a reversible oxidation–reduction cycle in which chlorophyll a (GH_2) is oxidized to b (GO).

$$GH_2 + CO_2 + \text{light} \longrightarrow GO + 1/n(H_2CO)n \quad (1)$$
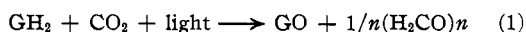
and b in turn is reduced to a as follows[2]:

$$GO + H_2O + \text{light} \longrightarrow GH_2 + O_2 \quad (2)$$

The discovery that the chlorophylls contain a readily oxidizable group led Conant[3] and co-workers to propose that the pigment might act as a two electron reducing agent.

$$12GH_2 + 6CO_2 \xrightarrow{\text{enzyme}} 12G + C_6H_{12}O_6 + 6H_2O \quad (3)$$
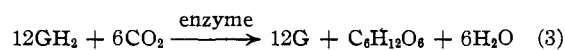
in a dark reaction,[3] chlorophyll being regenerated by a photochemical process.[4]

$$12G + 12H_2O + \text{light} \longrightarrow 12GH_2 + 6O_2 \quad (4)$$
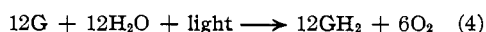
Stoll[4] and Willstätter[5] independently consider chlorophyll to function as a hydrogen donor in a photochemical reaction producing monodehydrochlorophyll (GH). This free radical is supposed to revert to the original dye via another photochemical process.

$$GH + H_2O + h\nu \longrightarrow GH_2 + OH \quad (5)$$
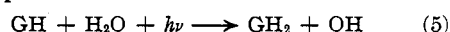
Franck[6,7] has a somewhat similar point of view, but has formulated the chlorophyll regeneration as

$$GH + R'OH + h\nu \longrightarrow GH_2 + R'O \quad (6)$$
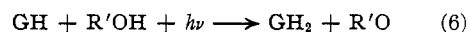
where $R'OH$ is different from water.

It occurred to us that using radioactive hydrogen[8] H^3, as a tracer it might be possible to learn whether or not chlorophyll is participating in photosynthesis as a donor of hydrogen. If photosynthesis is allowed to proceed for a sufficiently long time in water containing HTO[9] chlorophyll containing T should be formed[10] if the idea underlying equations 2, 4, 5, 6 is correct.

Experimental

Eleven cc. of the unicellular green alga *Chlorella pyrenoidosa* was suspended in 220 cc. of 0.05 M potassium bicarbonate solution containing HTO and strongly illuminated for three hours. During this period 2.5×10^{-3} mole of oxygen was evolved and a simple calculation shows that if the donor scheme is correct each chlorophyll should have been oxidized and reduced at least 100 times.[10a] The algae were centrifuged and the chlorophyll removed by exhaustive extraction with 95% acetone. All operations from the end of the illumination to the final burning of the chlorophyll were performed in strict darkness. In order to facilitate operations in the dark, the volumes of all solutions and vessels were carefully predetermined. To remove a lower layer from a separatory funnel, an evacuated

(1) Dixon and Ball, *Sci. Proc. Roy. Dublin Soc.*, **16**, 435 (1922).
(2) For an attempt at an experimental check on this proposal *cf.* Ruben, Frenkel and Kamen, *J. Phys. Chem.*, **46**, 710 (1942).
(3) Conant, Dietz and Kamerling, *Science*, **73**, 268 (1931).
(4) Stoll, *Naturw.*, **20**, 955 (1932); **24**, 53 (1936).
(5) Willstätter, *ibid.* **21** 252 (1933)

(6) Franck and Herzfeld, *J. Phys. Chem.*, **45**, 978 (1941).
(7) Franck and Gaffron, "Advances in Enzymology," I, Inter-Science Publishers, Inc., New York, N. Y., 1941, p. 215.
(8) Alvarez and Cornog, *Phys. Rev.*, **56**, 613 (1939).
(9) We use the symbol T for H³ (*cf.* Libby and Barter, *J. Chem. Phys.*, **10**, 184 (1942)).
(10) The possibility of an isotope separation will be discussed below.
(10a) Since the quantum yield is 0.1–0.08 (*cf.* Manning, Stauffer, Duggar and Daniels, THIS JOURNAL, **60**, 266 (1938), and Emerson and Lewis, *Am. J. Bot.*, **28**, 789 (1941)).

vessel of known volume was attached to the bottom. The chlorophyll was transferred by extraction to pentane and this solution washed twice with 80% acetone. The pentane layer was thoroughly washed with water. Under such conditions chlorophyll will usually precipitate, but the solution was too dilute in the present case for this to occur to an appreciable extent. The solvent was distilled off *in vacuo* at room temperature and the chlorophyll[11] carefully dried under high vacuum for several days. It was then burned in a stream of dry oxygen to carbon dioxide and water, the gases being passed over heated cupric oxide and the water collected in a cold trap. This water was converted quantitatively to magnesium oxide and hydrogen with excess fresh magnesium turnings at ~625°.[12] The conversion was carried out in a large test-tube filled with turnings, connected to the vacuum system through a rubber stopper sealed with de Khotinsky cement. The water was frozen in the bottom of the tube by immersion in liquid air. After evacuation the upper section was heated to ~625° electrically and the water then distilled up through the turnings, one distillation usually being sufficient for complete reaction. The evolved hydrogen was dried by passage through a liquid air trap and introduced directly into a Geiger counter containing 1.5 cm. of ethanol vapor and its radioactivity measured.[13] By using a new test-tube and stopper for each conversion the difficulty of decontamination was greatly reduced. The counter and the vacuum line were rid of adsorbed radiohydrogen by repeated flushing with ethanol vapor. The results of three separate experiments are summarized in Table I.

TABLE I

FORMATION OF RADIOACTIVE CHLOROPHYLL DURING CHLORELLA PHOTOSYNTHESIS IN HTO

				Radioactivity (counts/min.)	
Expt.	Algae,[b] cc.	Conditions	O_2 evolved (milli-moles)	Found experi-mentally	Theoretical[a] for complete exchange
1	11.0[c]	175 min. in light	2.0	< 80	2200
2	3.1[d]	185 min. in light	0.5	< 65	5260
3	5.5[e]	180 min. in dark	...	<100	9350

[a] Calculated on the assumption that each chlorophyll molecule in the algae has one hydrogen atom capable of undergoing photodissociation and moreover that each cycle involves the same C—H bond. [b] 1 cc. of algae contains 2×10^{-5} mole of chlorophyll. [c] Suspended in 220 cc. of 0.05 M $KHCO_3$ containing 2.0×10^7 c./min./mole H_2O. [d] Suspended in 55 cc. of 0.05 M $KHCO_3$ containing 1.7×10^8 c./min./mole H_2O. [e] Suspended in 50 cc. of 0.05 M $KHCO_3$ containing 1.7×10^8 c./min./mole H_2O.

It is apparent from Table I that no evidence for a photochemical exchange between chlorophyll and H^+ (e. g., H_2O, —OH, —NH_2, —COOH) was obtained. Before concluding that chlorophyll does not act as a hydrogen donor in photosynthesis we must consider two possible objections:

(1) loss of H^3 from chlorophyll by thermal exchange during the extraction process; (2) an isotope effect—*i. e.*, H^3 is not an ideal tracer for hydrogen of mass 1.

The possibility that chlorophyll contains labile hydrogen which can undergo photochemical or thermal exchange with water, etc., was therefore investigated. Pure chlorophyll was prepared from fresh spinach leaves following the procedure of Mackinney.[14] After drying in high vacuum for several weeks, the final product was analyzed spectrophotometrically[15] and found to be 97 ± 1% pure while the a/b ratio was 1.77. A weighed portion (40 mg.) of this chlorophyll was dissolved in 8 cc. of absolute ethanol and then 2 cc. of water (HDO) containing HTO and DTO was added.[16] This solution was shaken in the light for thirty minutes and the solvent distilled off *in vacuo* at room temperature. An attempt was made to free the chlorophyll of adsorbed or trapped solvent by maintaining a high vacuum (10^{-6} mm.) over it for several days, after which the chlorophyll and its glass container (crushed) were introduced into a combustion tube and burned in a stream of dry air. In order to obtain sufficient hydrogen for several entirely independent radioactivity determinations, a weighed portion of anhydrous glucose (Merck reagent grade) was added to the chlorophyll just prior to combustion. The resulting water was converted to hydrogen and counted. The results of this and similar experiments are summarized in Table II.

TABLE II

SEARCH FOR EXCHANGEABLE HYDROGEN IN CHLOROPHYLL

Expt.	Chloro-phyll, g.	Specific activity of hydroxyl hydrogen in solvent (80% EtOH) counts/min./ g. atom H	Experimental conditions	Tritium content of chlorophyll Exptl.	Theoretical[a]
1	0.040	2.78×10^8	30 min. light	187 ±30	3820
2	.095	0.53×10^8	60 min. light[b]	51 ±20	2430
3	.034	2.78×10^8	30 min. light[c]	100 ±25	3240

[a] Calculated on the assumption that each chlorophyll molecule has one exchangeable hydrogen atom. In experiments (1) and (3) 30.8% of the total H_2 generated was introduced in the Geiger counter; in (2) 43.4% of the H_2 was counted. [b] O_2 excluded at all times during experiment. After removing active solvent (25 cc.) the chlorophyll was dissolved in 10 cc. of 95% ethanol (inactive) which after ~five minutes was distilled off *in vacuo* at room temperature. This operation was repeated twice, the intention being the removal of firmly adsorbed or trapped radio-active solvent. [c] Washed once with 10 cc. of inactive 95% ethanol.

Since the small amount of radiohydrogen found in Experiments 1–3 was approximately 10^{-6} of the T used in each experiment, it was of interest to determine if exchange with the glass occurred. To this end Experiment (1) was repeated except that the chlorophyll was omitted. The hydrogen obtained in this experiment gave 30 ± 10 counts/minute in the Geiger counter.

(11) This probably contained small amounts of yellow pigments, etc., *cf.* Mackinney, *J. Biol. Chem.*, **132**, 91 (1940).

(12) In order to avoid any isotope separation it is essential that this conversion be quantitative. This method was found to be more rapid than the reaction with hot zinc.

(13) For a brief discussion of counting radiohydrogen see Allen and Ruben, THIS JOURNAL, **64**, 948 (1942).

(14) Mackinney, *J. Biol. Chem.*, **132**, 91 (1940).

(15) We are indebted to Dr. S. Aronoff for this analysis.

(16) Radio-water of this composition was obtained by condensation of the exhaust gases from the acceleration chamber of the 60" Berkeley cyclotron.

Discussion

It would appear from Tables I and II that $< 5\%$ of the chlorophyll has exchanged one hydrogen. However, due to the possibility of an appreciable isotope separation, the low values shown in Tables I and II do not exclude the possible existence of a cycle (Equations (7) and (8)) in which a photoactivated chlorophyll (G^*H_2) acts as a donor of

$$hv + GH_2 + A \longrightarrow G^*H_2 + A \longrightarrow GH + HA \quad (7)$$

hydrogen to some unknown substance (A)[17] and the chlorophyll free radical (GH) formed in this process is reduced to native chlorophyll (GH_2) by a thermal reaction such as (8).

$$GH + ROH \longrightarrow GH_2 + RO \quad (8)$$
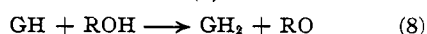
Process (8) very likely has a lower activation energy than the analogous reaction (9) involving an oxygen–tritium bond.

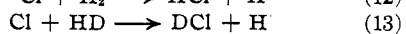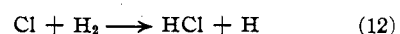$$GH + ROT \longrightarrow GHT + RO \quad (9)$$

The difference in energies of activation could be of the order of 2 kcal. and consequently the specific reaction rate for (8) would be ~ 20 times greater than that of (9).

It might be of interest to mention some simple reactions involving hydrogen and deuterium in which the differences in activation energies have been obtained by kinetic studies. Rollefson[18] found a difference of 1630 calories in the energies of activation for the following

$$Cl + H_2 \longrightarrow HCl + H \quad (10)$$
$$Cl + D_2 \longrightarrow DCl + D \quad (11)$$
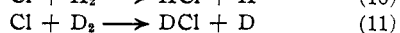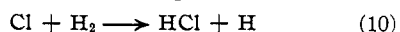
Farkas and Farkas[19] studied

$$Cl + H_2 \longrightarrow HCl + H \quad (12)$$
$$Cl + HD \longrightarrow DCl + H \quad (13)$$

and found the difference to be 600 calories.

In the photochemical reaction (7) the absorption of a light quantum would raise the photoactivated chlorophyll to an energy level well over the top of the energy barrier and therefore the specific reaction velocities for (7) and (14) would be the same and no isotope separation would occur.

$$hv + GHT + A \longrightarrow GH^*T + A \longrightarrow GH + TA \quad (14)$$
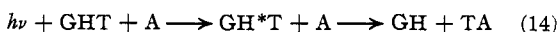
This seems to be a reasonable assumption since the quantum yield of photosynthesis is essentially the same[20] for wave lengths of light from 4500 to 6900 Å.
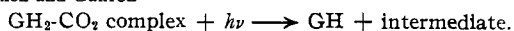
On the other hand, if (7) and (14) were thermal or if the photoactivated compound dropped to a lower energy level (by fluorescence or by a tautomeric change such as suggested by Franck and Livingston[21] such that the energy content was within say 1 kcal. of the activation energy of (7), the isotope separation would result in which GHT would tend to accumulate over GH_2. Moreover, if (8) and (9) were photochemical no isotope separation would occur.

It should be noted that Trelease and co-workers[22,23,24] have found the rate of photosynthesis in pure deuterium oxide (heavy water) to be 0.41 the rate in water. They have also shown that the thermal reactions and not the photochemical processes are slowed by the deuterium. If a reaction such as (8) is the rate determining step the difference in activation energies of (15) and (8)

$$GH + ROD \longrightarrow GHD + RO \quad (15)$$

is ~ 600 calories. Assuming the difference in energies of activation is due primarily[25] to the difference in zero point energies, one calculates that the difference in activation energies in rupturing a carbon–tritium and a carbon–hydrogen bond is $\sim 25\%$ greater than the corresponding difference between a carbon–deuterium and a carbon–hydrogen bond. However, it is possible that photosynthesis involves several thermal reactions in which isotope separations may occur. Therefore, in the tritium experiments where there is competition between ROH and ROT the isotope separation may be greater or less than one would expect from a simple consideration of the rate in pure deuterium oxide.

If the experiments described in this communication were repeated using 100% D_2O and the chlorophyll analyzed for deuterium content, all uncertainties due to isotopic effects would be completely eliminated and an unequivocal conclusion regarding the role of chlorophyll in photosynthesis could be reached. We hope to do this experiment in the near future.

It is evident that the results obtained in this research cannot be considered as definite evidence for or against the hydrogen donor hypothesis, but we may reasonably conclude that *either no isotope separation accompanies (7) and does take place in*

(17) Equation (7) is essentially similar to the formulation by Franck and Gaffron

$GH_2\text{-}CO_2$ complex $+ hv \longrightarrow GH +$ intermediate.

(18) Rollefson, *J. Chem. Phys.*, **2**, 144 (1934).
(19) Farkas and Farkas, *Naturw.*, **22**, 218 (1934).
(20) Emerson and Lewis, *J. Gen. Phys.*, **25**, 579 (1942).

(21) Franck and Livingston, *J. Chem. Phys.*, **9**, 184 (1941).
(22) Pratt, Craig and Trelease, *Science*, **85**, 271 (1937).
(23) Craig and Trelease, *Am. J. Botany*, **24**, 232 (1937).
(24) Pratt and Trelease, *ibid.*, **25**, 133 (1938).
(25) *Cf.* "Light and Heavy Hydrogen," by A. Farkas, Cambridge University Press, 1935.

(8) or else the hypothesis that chlorophyll acts as a donor of hydrogen in photosynthesis should be abandoned.

Acknowledgments.—We wish to thank Professors G. K. Rollefson and J. Franck for many helpful suggestions and discussions. We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory, particularly Dr. M. D. Kamen, for the tritium samples.

Summary

1. The formation of chlorophyll containing T could not be detected during photosynthesis of Chlorella pyrenoidosa in HTO + H_2O.

2. No (<5%) thermal exchange was observed between purified chlorophyll and 80% ethanol containing HTO.

3. The implications of these results for the theory that chlorophyll acts as a hydrogen donor in photosynthesis are discussed.

4. It is pointed out that repetition of the experiments herein described using 100% D_2O would avoid the question of isotope separation and make possible an unequivocal conclusion regarding the role of chlorophyll.

NOTES

Characteristics of β-[2,5-Dimethoxyphenyl]-β-hydroxyisopropylamine Hydrochloride

BY RICHARD BALTZLY AND JOHANNES S. BUCK

This compound was reported recently[1]; subsequent investigation makes it evident that the substance to which that formula was attributed is actually β-[2,5-dimethoxyphenyl]-β-oxoisopropylamine hydrochloride. This could have been anticipated[2] but was not at the time. The error arose partly from the difficulty of interpreting small absorptions of hydrogen when using a catalyst (palladized charcoal) that itself binds relatively large amounts of hydrogen and partly from adverse conditions over which the analyst had no control.

The substance previously obtained (m. p. 176° dec.) when dissolved in water and reduced with hydrogen and platinum-black absorbed 1 mole of hydrogen. A new hydrochloride was isolated melting at 215°.

Anal. Calcd. for $C_{11}H_{18}O_4N$:   C, 53.31;   H, 7.33. Found: C, 53.43; H, 7.53.

When the corresponding base was acetylated with acetic anhydride a diacetyl derivative was formed melting at 120° and crystallizing from ethyl acetate–hexane in parallelogrammatic plates.

Anal. Calcd. for $C_{15}H_{21}O_5N$:   C, 60.98;   H, 7.17. Calcd. for $C_{13}H_{17}O_4N$:   C, 62.12;   H, 6.83.   Found: C, 61.09; H, 7.30.

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(1) Baltzly and Buck, THIS JOURNAL, **62**, 164 (1940).
(2) Cf. Hartung, ibid., **53**, 4149 (1931).

Identification of o- and p-Sulfobenzoic Acids[1] as their S-Benzylthiuronium Salts

BY E. CAMPAIGNE[2] AND C. M. SUTER[3]

The structure of the alkylated benzenesulfonic acids may be partially clarified by oxidation to the sulfobenzoic acids. The identity of the o-, m- and p-sulfobenzoic acids may be determined by conversion to the acid chlorides and to the amides.[4] This involves separation and drying of the salts of the sulfobenzoic acids, and the somewhat tedious conversion to the chlorides with a phosphorus halide. The use of S-benzylthiuronium chloride as an analytical reagent for sulfonic acids[5] has recently been reported. Since the benzylthiuronium derivatives are prepared in water solutions, the use of this reagent should give a quick and easy method for the identification of the oxidation products of alkylbenzenesulfonic acids and related compounds.

The S-benzylthiuronium salts of o- and p-sulfobenzoic acid were obtained in good yield from water solutions of the acid sodium or ammonium salts, but the derivative of the m-sulfobenzoic acid was found to be quite soluble in water, and could not be isolated conveniently. The o-sulfo-

(1) This investigation was supported by a grant from the Abbott Fund of Northwestern University.
(2) Present Address: University of Texas, Medical Branch, Galveston, Texas.
(3) Present Address: Winthrop Chemical Company, Inc., Rensselaer, N. Y.
(4) Beilstein's "Handbuch," 4th ed., Vol. XI, p. 369.
(5) Chambers and Watt, J. Org. Chem., **6**, 376 (1941).