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ON THE ENZYMATIC CONVERSION OF ^{14}C-LABELED CHLOROPHYLL *a* TO ^{14}C-LABELED CHLOROPHYLL *b*

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SUMMARY

Through the addition of NADP$^+$ to homogenates of soybean leaves incubated in the dark at room temperature with uniformly ^{14}C-labeled chlorophyll *a*, significant yields of ^{14}C-labeled chlorophyll *b* (purified as pheophytin *b*) were obtained. The activity of the enzyme proposed as responsible for this conversion appears to be relatively unaffected by acetone, but is at least partially denatured by heating.

INTRODUCTION

Recently, SHLYK AND PRUDNIKOVA[1] have reported evidence for the dark conversion of ^{14}C-labeled chlorophyll *a* to ^{14}C-labeled chlorophyll *b* by a barley homogenate. Because of the lack of quantitative radiotracer data in the report, and of certain comments made therein, we concluded that the yield of ^{14}C-labeled chlorophyll *b* obtained by these authors was so low that the question of whether or not chlorophyll *a* was converted enzymatically to chlorophyll *b* remained equivocal.

This paper presents results of experiments performed similarly to those of SHLYK AND PRUDNIKOVA[1], but using soybean homogenates with added NADP$^+$ and, in addition, refined analytical procedures. The results of these experiments indicate relatively high yields of ^{14}C-labeled chlorophyll *b* from ^{14}C-labeled chlorophyll *a* and thereby strongly support the hypothesis of SHLYK AND PRUDNIKOVA[1] that chlorophyll *b* can be synthesized enzymatically from chlorophyll *a* in the dark.

METHODS AND MATERIALS

Preparation of uniformly ^{14}C-labeled chlorophyll a

Some of the uniformly ^{14}C-labeled chlorophyll *a* used in these experiments was purified from a commercial uniformly ^{14}C-labeled algal lipid extract (obtained through the generosity of the New England Nuclear Corp., Boston, Mass.). The uniformity of label in this extract had previously been verified in this laboratory[2]. Other uniformly ^{14}C-labeled chlorophyll *a* was obtained through purification of leaf pigments extracted from intact soybean leaves fed $^{14}CO_2$ for 8 h and then grown an additional 2 weeks to insure equilibration of the ^{14}C in the pigments[3]. ^{14}C-labeled chlorophyll *a* was repeatedly purified by sucrose column[4] or thin-layer chromatography[5] until constant specific activities and the proper "blue to red" ratios were obtained[6]. The radiochemical purity of the ^{14}C-labeled chlorophyll *a* was further verified by conversion of a portion of it to pheophytin *a* and the specific activities of the ^{14}C-labeled chlorophyll *a* and its pheophytin *a* compared[4]. All solvents used were reagent grade. The diethyl ether used was anhydrous and peroxide-free. Sucrose used for chromatography (column and thin layer) contained approx. 3 % (by weight) of corn starch (Domino confectioners' sugar).

Assay of enzymatic activity

Preparation of the soybean homogenate

Expt. 1. Fifteen green, expanding trifoliate soybean (*Glycine max* L.) leaves were thoroughly ground with sand in a mortar in 120 ml of a solution of 60 % aqueous sucrose, and then the suspension was centrifuged at 3500 × *g* for 25 min at room temperature. The resulting supernate was the homogenate used.

The homogenate (above) was divided in half (Fractions A_1 and B_1). To fraction A_1 approx. 5 mg of uniformly ^{14}C-labeled chlorophyll *a* (specific activily, $1.36 \cdot 10^5$ disint./min per mg) dissolved in 3 ml acetone, 5 ml of 0.05 % (v/v) aqueous Tween-80 solution, and 10 mg of $NADP^+$ were added. (No buffer was added. The pH of the incubation mixture was 7.6 before and after the allowed reaction period.) The flask was tightly stoppered and covered with alumininum foil to exclude light, and then shaken at room temperature for 20 h on a wrist action shaker.

To Fraction B_1 were added 5 mg of uniformly ^{14}C-labeled chlorophyll *a* in 3 ml acetone, 5 ml of an 0.05 % (v/v) aqueous Tween-80 solution, and 10 mg of $NADP^+$. Immediately after the initial mixing, 3 vol. of acetone (approx. 210 ml) were added in an attempt to denature the enzyme being studied. This reaction mixture was covered and shaken as was Fraction A_1 above.

Expt. 2. Fifteen green, expanding trifoliate soybean (*Glycine max* L.) leaves were thoroughly ground with sand in a mortar in 120 ml of a solution of 60 % (w/v) aqueous sucrose, and then centrifuged for 10 min at 2000 × *g* at 0–5°. The supernate was then centrifuged again for 15 min at 5000 × *g*, once again, at 0–5°. The resulting supernate was the homogenate used.

A homogenate prepared as above was divided into three fractions (A_2, B_2, and C_2). To Fraction A_2 were added approx. 1.13 mg of uniformly ^{14}C-labeled chlorophyll *a* (specific activity $1.58 \cdot 10^6$ disint./min per mg) in 3 ml acetone, 5 ml of 0.05 % (v/v) aqueous Tween-80 solution, 2 mg $NADP^+$, and 0.33 mg unlabeled chlorophyll *b*. The latter was added as carrier to "trap" any labeled chlorophyll *b* formed in the homogenate.

Fraction B_2 was prepared identically with Fraction A_2, but an additional 40 ml of acetone were added, once again in an attempt to denature the enzyme, if present, responsible for the conversion of chlorophyll *a* to chlorophyll *b*.

Fraction C_2 was heated 5 min on a boiling water bath and then, after cooling, was prepared as was Fraction A_2 above.

All three reaction mixtures (*i.e.*, A_2, B_2, and C_2) were covered with aluminum foil to exclude light and then shaken 20 h at room temperature on a wrist action shaker.

Preparation of blanks

To check the validity of our purification procedure, blanks were prepared and analyzed in the following manner: (A_3) All the additives as in Expt. A_1 except leaf homogenate were included in 30 ml of 60 % aqueous sucrose (*i.e.* ^{14}C-labeled chlorophyll *a* in acetone, NADP$^+$, and Tween-80 solution) with an additional 10 mg unlabeled plastid pigment extract being added. Blank B_3 was prepared identically with A_3 and then 210 ml acetone were added. These blanks were covered and shaken as were the experimental tubes.

Pigments from each incubation were extracted through the addition of 3 vol. of acetone and then mixing with 1 vol. of diethyl ether. The resulting mixture was washed with distilled water to transfer the pigments into the ether layer. The ethereal solution, following repeated washing with distilled water to remove acetone, was dried with anhydrous Na_2SO_4 prior to chromatography (as described in RESULTS below).

Solutions of the chromatographic bands in ether were adjusted to absorbances between 0.2 and 0.8, aliquots were taken, and then were counted to insure less than 1.5 % counting error at the 95 % confidence level in either a Beckman Lowbeta II low-background proportional planchet counter or an Ansitron Model 1300 scintillation counter using Liquifluor (New England Nuclear Corp., Boston, Mass.) in toluene as a "cocktail".

Chemical Methods

Preparation of pheophytins. Pheophytins *a* and *b* were prepared by hydrolysis of Mg with aqueous HCl as described by FISCHER AND STERN[7].

Preparation of rhodin g_7. Rhodin g_7 was prepared through the saponification of pheophytin *b* in alcoholic KOH (ref. 7). Purification was achieved by partitioning the crude rhodin g_7 between ether and aqueous HCl using, successively, 8 % (w/v) HCl solution and 9 % (w/v) HCl solution to separate the rhodin g_7 from other products of the reaction having, respectively, HCl numbers lower than, or higher than, that of rhodin g_7 itself.

RESULTS AND DISCUSSION

The pheophytin *b* obtained from the blanks contained no detectable radioactivity after the extracted chlorophyll *b* was chromatographed twice on sucrose columns, degraded to the pheophytin and run twice on sucrose thin-layer plates (the limit of detection of label in the pheophytin *b* was 1125 disint./min per mg using a Beckman low-background proportional counter having a background of 1.1 counts/min). The chlorophyll *b* pigments extracted from the blanks were contaminated with what appeared to be degraded ^{14}C-labeled chlorophyll *a*; this material could not be removed from the chlorophyll *b* by repeated chromatography. This chlorophyll *a*-like material was removed from the chlorophyll *b* when the pigments were degraded to pheophytins and then chromatographed. These observations were identical with those made during the purifications of ^{14}C-labeled chlorophyll *b* extracted from the homogenate reaction mixtures.

The specific activities and "blue to red" ratios of pheophytin *b*'s (purified identically with the blanks) obtained from Expts. A_1 and B_1 are presented in Table I. The specific activity and "blue to red" ratio of these pheophytin *b*'s remained constant after two chromatographic purifications on sucrose thin layers.

The specific activities and "blue to red" ratios of the pheophytin *b* obtained from the chlorophyll *b* separated from pigments extracted from Expts. A_2, B_2 and C_2 are presented in Table II. The specific activities of the pheophytin *b*'s were obtained after

TABLE I

SPECIFIC ACTIVITIES AND "BLUE TO RED" RATIOS * OF ^{14}C-LABELED CHLOROPHYLL *a* AND ^{14}C-LABELED PHEOPHYTIN *b* FROM EXPTS. A_1 AND B_1

Compound	*Expt. A_1*		*Expt. B_1*	
	*Specific activity*** ($\times 10^{-3}$)	*Blue to red ratio*	*Specific activity*** ($\times 10^{-3}$)	*Blue to red ratio*
Chlorophyll *a* before addition to homogenate	136	1.29	136	1.29
Chlorophyll *a* after extraction from homogenate	90.0	1.29	—	—
Pheophytin *b*	7.52	5.20	1.80	5.20

* Ratio of the absorbance at the Soret maximum to that at the major red maximum of the subject compound.

** Specific activity (disint./min per mg), determined using a Beckman Lowbeta II low-background proportional counter.

the isolated chlorophyll *b* was run 3 times on sucrose thin layers. The specific activities of the rhodin g_7 and of phytol resulting from the saponification of pheophytin *b* of Expt. A_2 (and a repeat of A_2) are also presented in Table II.

The data in Tables I and II clearly indicate that ^{14}C-labeled chlorophyll *b* (purified as pheophytin *b*) was obtained in Expts. A_1 and A_2. The data in these tables also show that the conversion activity is only partially, if at all, affected by the addition of

TABLE II

SPECIFIC ACTIVITIES AND "BLUE TO RED" RATIOS* OF ^{14}C-LABELED CHLOROPHYLL *a* AND ^{14}C-LABELED PHEOPHYTIN *b* FROM EXPTS. A_2, B_2 AND C_2

	Expt. A_2				*Expt. B_2*		*Expt. C_2*	
	*Specific activity*** ($\times 10^{-5}$)	*Blue to red ratio*			*Specific activity*** ($\times 10^{-5}$)	*Blue to red ratio*	*Specific activity*** ($\times 10^{-5}$)	*Blue to red ratio*
Chlorophyll *a* prior to addition to homogenate	15.8	1.28			15.8	1.28	15.8	1.28
Chlorophyll *a* after extraction from reaction mixture	9.77	1.28			10.2	1.28	14.2	1.28
	Expt. A_2 repeated							
Pheophytin *b*	1.30	5.12	0.25	4.98	1.29	4.90	< 0.011	5.05
Rhodin g_7	0.954	6.81	—	—	1.01	6.80	—	—
Phytol***	—		0.24	—	—	—	—	—

* Ratio of the absorbance at the Soret maximum to that at the major red maximum of the subject compound.

** Specific activity (disint./min per mg), determined using an Ansitron Model 1300 scintillation counter.

*** From pheophytin *b*.

up to 3 vol. of acetone to the homogenate. The data in Table II clearly indicate, however, that this activity is completely denatured by heat. All of these above results were reproducible in subsequent experiments although the complete denaturation of the enzyme by boiling is not always achieved. It is presumed that the enzyme exhibits less heat lability than do many enzymes.

The fact that ^{14}C was found both in the rhodin g_7 and in the phytol moieties suggests that the phorbin *a* moiety was indeed converted to a phorbin *b* moiety. This observation also precludes the possibility that the ^{14}C content of the chlorophyll could be attributed solely to transphytylation by chlorophyllase of unlabeled chlorophyll *b* with ^{14}C-labeled chlorophyll *a* to yield the ^{14}C-labeled chlorophyll *b*. Under certain conditions we have observed the occurrence of such transphytylation but never to the complete exclusion of the occurrence of ^{14}C in the phorbin moiety.

The data in Table II show the specific activities of the rhodin g_7 samples from Expts. A_2 and B_2 to be approximately equal and, at the same time, to be somewhat lower than those of the pheophytins from which they were derived. The one phytol sample (Expt. A_2, repeat) gave a specific activity suggesting that the pheophytin *b* from which it was derived was uniformly labeled. That the rhodin g_7 data indicated otherwise is due, we surmise, to analytical difficulties experienced in determining accurately the specific activities of ^{14}C-labeled rhodin g_7 samples. Not the least of these problems is adsorption of the compound on the glassware used in its preparation and low solubility in the toluene cocktail. Furthermore, the purity of the rhodin g_7 prepared as described is open to question. Had significant amounts of transphytylation occurred during the experiments reported here, the specific activity of the phytol would be expected to be much higher than that of the pheophytin from which it was derived. This effect was not noted in this study. In some of our subsequent studies, however, we have found cases where the specific activities of phytol derived from ^{14}C-pheophytin *b* were significantly higher than those of the corresponding pheophytin, suggesting that ^{14}C-labeled phytol transfer to unlabeled chlorophyll *b* in the homogenate had indeed occurred. The seeming variations in reproducibility of transphytylation data in these experiments are most likely due to the nature of the system being studied. The chlorophyll molecules are obviously not dissolved, but rather suspended, in the reaction mixtures. These mixtures would not be expected to produce the reproducible results that one would expect to obtain from reaction mixtures of true solutions. In addition, these reaction mixtures were incubated overnight at room temperature. Because of the variation in room temperatures that occurs, one could expect other deviations which would affect the quantitative reproducibility of these experiments. In the present study, we are interested mainly in the qualitative aspects of the reaction. In one study, the ^{14}C-labeled pheophytin *b* was hydrolyzed[7] to pheophorbide *b* which was repeatedly purified by reverse phase chromatography on cellulose thin layers[9]. This pure pheophorbide *b*, which is more soluble in the solvents used than is the rhodin g_7, also dropped in specific activity, but still retained a significant amount (approx. 30 % that of its pheophytin *b*) of ^{14}C-label. It is clear, therefore, that both processes were occurring simultaneously in the homogenates: transphytylation between labeled chlorophyll *a* and unlabeled chlorophyll *b* and the biochemical conversion of the phorbin moiety of chlorophyll *a* to that of chlorophyll *b*.

These results verify the observations of SHLYK AND PRUDNIKOVA[1] of the dark conversion of ^{14}C-labeled chlorophyll *a* to chlorophyll *b* by a leaf homogenate and strongly suggest that the conversion is facilitated by adding NADP$^+$ to the homogenate. When we omitted NADP$^+$, as was the case in the experiments of SHLYK AND PRUDNIKOVA[1], the chlorophyll *a* to *b* conversion was extremely difficult to discern. Although adding the NADP$^+$ to the homogenate apparently increases the rate of formation of chlorophyll *b*, we do not yet know if this reaction is specifically dependent on NADP$^+$, or if the NADP$^+$ is at all directly involved in the reaction.

The extent to which chlorophyllide *a* (or possibly methyl chlorophyllide *a* (ref. 8)) is converted to the corresponding *b* analog by this proposed enzyme has not been established, nor has the requirement of a magnesium chelate substrate. These questions are presently being investigated in this laboratory, as is the degree to which the reaction may be reversible.

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