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18 Nov 1992, rvsd 23Nov93, 20Sept94, 21Nov95, 26Nov96, 20Sept99, 18Sept00, 20Nov01, 20Sept04, 29Nov05, 27Nov07
 From DBF Notebook, C.C. III, p. 84 & CC IX, p. 107

See previous protocol for the preparation of the purified chloroplasts. Continue to keep them ice cold until the moment you add them to the prepared tubes. Work in teams of two.

Supplies:

- | | | |
|---|---|--------------|
| purified spinach chloroplasts, ice cold | 100 watt light | 1/ two teams |
| 0.1 M PO ₄ buffer, pH 6.5 (pH 7.0 OK?) | 37°C incubator (can exclude light) | |
| 2.5 x 10 ⁻⁴ M 2,6 dichloroindophenol | Set up hot block next to spectrophotometer: | |
| (36.3 mg DCIP/500 mL, A ₆₀₀ ≈ 3.0) | hot block, 37°C | 1/ 2 teams |
| displacement pipets, 10 uL and 50 uL | spectrophotometer | 1/team |
| seven unblemished cuvettes in plastic finger rack | vortex | |

cut here.....

- Set up apparatus:
 - 37°C hot block (for 13x100 mm tubes) nearby and warmed up.
 - 37°C incubator warmed up.
 - spectrophotometer on the same desk as the light exposure apparatus.
 - 100 watt bulb with reflector 25 cm from open test tube rack.
- Construct a data table in your notebook to accept data (previous page in handouts).
- Select, label and polish seven very clean, unblemished test tubes** (B for blank + six tubes).
- Prepare DCIP reaction mix.** (Read A₆₀₀ before dispensing. A₆₀₀ should be around 0.600).

Per team: 12 mL 0.1 M PO₄, pH 6.5 buffer
 12 mL 0.5 M sucrose
 12 mL 2.5 x 10⁻⁴ M DCIP (2,6 dichloroindophenol)

- Dispense 4 mL of the reaction mix** to each of your tubes. (Use 50 mL repeat pipet set on 4 mL.)

tube:	Reaction Mix mL	chloroplast suspension μL	incubation conditions:	A ₆₀₀
1	4.00	0	dark	1
2	"	0	light	2
3	"	5	dark	3
4	"	5	light	4
5	"	10	dark	5
6	"	10	light	6

- Prewarm all prepared tubes** (lacking chloroplasts) in 37°C hot block for 2 min.
- DARK TUBES (control tubes): Add chloroplasts to tubes 3 and 5.** Mix and read the A₆₀₀ of 1, 3, and 5 against a water blank. *Immediately* place in 37°C incubator, *keep light excluded*.
- LIGHT TUBES:**
 - tube 2:** Place 25 cm from a 100 watt bulb.
 - tube 4:** Add 10 uL chloroplasts, mix, read A₆₀₀ and place in light as for tube 2. Start stopwatch.
 - tube 6:** Add 20 uL chloroplasts, mix, read at 30 seconds and place in front of light. Note time. Keep this 30 second time separation between reading of 2 and 4.
- Read A₆₀₀ of 4 and 6 every minute for 10 minutes,** read tube 2 only at 0, 5, and 10 min. (Keep tubes polished, read in consistent configuration.)
- Read A₆₀₀ of tubes which were kept in the dark** again at 15 minutes.
- Plot the absorbency of each tube versus time.**
- Discuss the significance of the differences between the various six curves.

Problems 2000:

1. Emphasize the different decanting techniques for S1 (saving supernatant, do not want any pellet) and SO (saving pellet, do not want any supernatant, so pour off all supernatant in one motion, let pellet drain).
2. The chloroplasts seemed weak. (Degraded in RT conditions? Too few?) Have changed protocol to increase the concentration of chloroplasts (used to be 1:5, now have doubled SO, have no dilution, so should be 2.5x as much chloroplasts).
3. T-0 only read about 0.335. So have increased DCIP in Rxn Mx by 33%.
4. Be sure to illustrate the reduction of DCIP by NADPH.
5. Only had 3 teams, so had 50 mL Rxn Mx left over.

Problems, suggestions from 1996:

Took too long with chloroplast isolation, rushed through reduction phase

Students did not understand goal of reduction phase.

Need to set up station **for each pair of students:**

spectrophotometer

light

meter stick

vortex

hot block

Problems, suggestions from 1995:

Ran out of 17% sucrose, needed about 500 mL for 10 students

Reaction Mix had an undiluted A_{600} of about 1.9... Should either make less concentrated DCIP solution, or use less in rxn mix.

1:20 chloroplasts were not nearly strong enough. I tested 10 lambdas undiluted, got good reduction (maybe too fast)

Reaction mix made up for the entire class of 10 students:

60 mL 0.1 M PO_4 , pH 7.0

60 mL 0.5 M sucrose

14 mL DCIP 36.3 mg/500 mL

gave OD at 600 nm of around 400.