Observations on the Respiratory System of *Rhodospirillum rubrum**

T. HORIO† AND M. D. KAMEN‡

WITH THE TECHNICAL ASSISTANCE OF H. DE KLERK‡

From the Department of Chemistry, University of California at San Diego, La Jolla, California; Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania; and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

Received April 26, 1962

The respiration of *Rhodospirillum rubrum* has been studied with resting wild-type cells. A few experiments on "blue-green" mutant cells of *Rhodopseudomonas spheroides* have been performed. With succinate as substrate, rates of oxygen uptake in the dark, and in light at intensities sufficient to saturate photometabolism, are compared. Variables studied have been *p*H, temperature, physical manipulations (freezing and thawing, degree of anaerobiosis during harvest and storage), and concentration of various respiratory inhibitors and alcohols (narcotics). Light suppression of oxygen uptake can be considered the result of two processes, one of which is light-sensitive. Oxygen uptake in the absence of light ("dark respiration") is the sum of these two processes, "light-sensitive" plus "light-insensitive"; oxygen uptake in light ("light respiration") represents the "light-insensitive" process alone. Observations were made with several purified components of the chromatophore electron-transport chain, *i.e.*, the two heme proteins (cytochrome c_2 and *Rhodospirillum* heme proteins) and the heme protein reductase(s), from which their roles in these processes can be inferred. Data obtained in these researches, as well as those accumulated in previous researches, are consistent with the existence of a chain of electron-transport carriers, which operates in cyclic fashion, with the photoactive pigments (bacteriochlorophyll, carotenoids, etc.) and *Rhodospirillum* heme protein as competitive H acceptors.

The inhibition of respiration by light in *Athiorhodaceae*, first noted a quarter century ago by Nakamura (1937), has been documented in numerous researches, notably by Van Niel (1941, 1944); see also Gest and Kamen (1960). Quantitative observations by Clayton (1953, 1955) and by Morita (1955) have established a strictly reciprocal relation between assimilation of substrate and uptake of oxygen in the light. Furthermore, it has been demonstrated that the bacterial chromatophore is the site of this competitive interaction between light and dark metabolism (Katoh, 1961). The participation in photoactivated processes of heme proteins which occur in chromatophores has been established (Kamen, 1961; Duysens, 1954, 1957; Chance and Smith, 1955; Smith and Ramirez, 1959; Horio and Kamen, 1961; Smith and Baltscheffsky, 1959). Purified individual components, such as cytochromes, variant heme proteins (Vernon and Kamen, 1954; Kamen and Vernon, 1953; Bartsch and Kamen, 1960; Horio and Kamen, 1961), and a pyridine nucleotide–linked flavoprotein heme reductase (Horio and Kamen, 1960) have been described as participants in electron transport processes coupled to biosynthesis (Kamen, 1961).

The complexity of the interactions involved is attested by findings such as those of Clayton and Morita, who have demonstrated that quantitative relations vary with the nature of substrate, with species tested, and with the natural history of any given culture. Examination of the depression in oxygen uptake in any specific instance shows that, as light intensity is raised, oxygen uptake rate drops, but not in a linear fashion. As a first approximation, these findings are consistent with the existence of two respiratory systems, one of which is much more sensitive to illumination than the other. The former system we call "light-sensitive," the latter we term "light-insensitive." Thus, respiration in the absence of light ("dark respiration") is the sum of light-sensitive and light - insensitive respirations, whereas respiration in the light ("light respiration") represents light - insensitive respiration alone.

The components of the respiratory system which we have studied are the two heme proteins, cytochrome c_2 and RHP, and a soluble flavin-mediated enzyme system which catalyzes electron transfer from reduced pyridine nucleotide to

* No. 170 in the series "Publications of the Graduate Department of Biochemistry, Brandeis University," Waltham, Mass.

† Present address: Institute for Protein Research, Osaka University, Nakanoshima, Osaka, Japan.

‡ Present address: Department of Chemistry, University of California at San Diego, La Jolla, Calif.

[1] Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; DCPI, 2,6-dichlorophenolindophenol; RHP, *Rhodospirillum rubrum* heme protein; cytochrome c_2, *c*-type cytochrome of *R. rubrum;* FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Tris, Tris(hydroxymethyl)aminomethane-HCl buffer.

oxygen, 2,6-dichlorophenolindophenol, RHP, and cytochrome c_2.[1] In a previous paper (Horio and Kamen, 1960) we have described the isolation and partial purification of this heme protein reductase(s), and conditions for separation of some of the oxidation-reduction reactions catalyzed. Also, we have advanced a tentative proposal for the mechanism of electron transport based on kinetic studies of reduction of the two endogenous heme proteins.

In our present studies, we have carried purification of the reductase further by means of ion-exchange column chromatography so that, although it appears that ultimate purification still has not been attained, we have been able to establish the flavin nature of the co-factor for heme protein reduction and the relation between reductase and oxidase activities in photometabolism.

Materials and Methods

Wild-type cells of *R. rubrum* were grown anaerobically in 500-ml reagent bottles with continuous illumination at approximately 30° in the medium described by Gest *et al.* (1950). For production of light-grown cells, the culture medium was incubated for 3–5 days, during which the *p*H of the medium remained almost constant at *p*H 7. In some experiments, wild-type cells were grown aerobically in 2-liter Erlenmeyer flasks containing 500 ml of the medium supplemented with 0.15 M sodium phosphate buffer, adjusted to *p*H 6.0 initially. The culture flasks were covered with aluminum foil and shaken vigorously. Incubation was stopped when the *p*H of the medium rose to approximately 8.0. This rise in *p*H occurred so rapidly that dark-grown cells required harvest in about 1 day. After incubation, the growth medium was centrifuged at 6,000 × *g* for 10 minutes to separate the suspension into cells and supernatant fluid, labeled "cell-free medium"; this term was applied only to the supernatant fluid from the light-grown cultures. The collected cells were suspended in a small amount of the cell-free medium after supplementation with substrate, 0.05 M sodium succinate (see below). During this procedure, precautions were taken to minimize oxidation of the cell-free medium by air. The thick cell suspensions, if stored anaerobically in darkness in a refrigerator, could be used for a week without noticeable alteration in properties examined in these studies. Such cells we called "young cells." On the other hand, cell suspensions rapidly changed character if stored under air. In particular, the light-induced inhibition of respiration of light-grown cells became less and less marked. These cells we called "aged." In some experiments, young cell suspensions were aerated at room temperature in darkness just before use. Such cells we termed "aerated."

Chromatophores were prepared according to the method of Smith and Baltscheffsky (1959) with slight modifications. The harvested light-grown cells of wild type were washed twice with Tris HCl buffer (0.1 M, *p*H 8.0). The washed cells were ground in the cold in a mortar with three times their wet weight of alumina (Alcoa A-301). The mixture was extracted with 4 ml 0.1 M buffer for each gram wet weight of bacteria. After centrifugation at 10,000 × *g* for 10 minutes, the deep purple supernatant fluid was collected by decantation. This extract was used as chromatophore suspension without further purification. Its respiratory activity remained fairly constant on storage if it was refrigerated in a test tube filled to the top with extract so that no gas phase was present.

The "blue-green" mutant of *Rhodopseudomonas spheroides* was grown as described by Griffiths *et al.* (1955).

Oxygen respiration was measured in two ways. One, the oxygen electrode method of Davies and Brink (1942), was convenient for rapid comparisons of oxygen uptake under a variety of conditions. Reaction systems were constituted in the following manner: immediately prior to a series of experiments, 0.1 ml of cell suspension was diluted to an appropriate cell concentration by addition of 0.9 ml cell-free medium supplemented with 0.05 M sodium succinate for the light-grown cells, or by addition of 0.9 ml fresh medium supplemented with 0.05 M succinate for the dark-grown cells. One ml of 0.1 M sodium phosphate buffer was then added. The *p*H of this buffer had been adjusted previously so that it would give an appropriate *p*H after mixture of all reaction components. All mixing operations were performed at room temperature. Reaction systems were usually maintained in a water bath cooled with tap water (20–23°), with or without illumination by white light from tungsten lamps, intensity approximately 450 foot-candles. Little effect on residual respiration was noted with light intensities greater than 450 foot-candles. This level of light intensity was also close to that required for saturation of photometabolism.

The second method employed the conventional Warburg respirometer (manometric) method. It was used for measurement of Q_{O_2} of bulk cell suspensions. Reactions were carried out at 30° in darkness. The reaction system included an appropriate volume of cell suspension in fresh medium supplemented with 0.05 M sodium succinate (*p*H 7.0); total volume of the reaction mixture was 2.0 ml.

The low temperature absorption spectrum of RHP was measured at liquid nitrogen temperature by a wave length–scanning spectrophotometer devised by Chance (1951) and Yang and Legallais (1954), and modified by Estabrook (1956), to permit the recording of spectra of samples cooled to low temperature. A three-times-recrystallized sample of RHP (see below) was dialyzed against 10^{-4} M sodium chloride. The dialyzed solution was brought to *p*H 8 and to a

final buffer concentration of 0.1 M by addition of Tris buffer. The solution, containing an appropriate concentration of RHP, was placed in a 1-mm optical path cuvet for measurement of absorption spectra. For measurements of the spectrum of RHP-CO complex, CO was bubbled through the sample solution in the presence of a small excess of sodium dithionite, added as the solid; the solution was then immediately frozen in liquid nitrogen. These procedures were repeated with 0.1 M sodium phosphate (*p*H 7.0) as buffer to compare spectra at two *p*H values.

In experiments on isolation of the heme protein reductase, *R. rubrum* was grown anaerobically in 20-liter bottles under continuous illumination at approximately 30° in the synthetic medium described by Cohen-Bazire *et al.* (1951). After 4–6 days, the cells were harvested with a Sharples centrifuge, washed once with water, lyophilized, and stored in the cold (less than 0°).

Crystalline cytochrome c_2 and RHP were obtained from lyophilized cells according to the method of Horio and Kamen (1961). Crystalline cytochrome *c* was prepared from horse heart muscle by the method of Nozaki *et al.* (1957).

To prepare oxidized *c*-type cytochromes, a concentrated solution of potassium ferricyanide (*p*H 5) was added drop-wise to the cytochrome solutions (*p*H 5) until the reduced α-absorption band at 550 mμ disappeared, as viewed with a microspectroscope. Then, the oxidized cytochrome solutions were dialyzed against 10^{-3} M sodium chloride aqueous solution in the cold (approximately 3°). RHP, because of its great auto-oxidizability, was obtained in its oxidized form directly without treatment by oxidizing agents; it was dialyzed in the same manner as the cytochrome solutions.

DPNH and TPNH were commercial preparations purchased from Pabst Laboratories, Milwaukee, Wis. FAD and FMN were obtained from California Corporation for Biochemical Research, Los Angeles, and Sigma Chemical Company, St. Louis, Mo., respectively.

Ammonium sulfate was C.P. reagent grade (Baker Chemical Company) rendered metal-free by the following treatment. The salt was dissolved in a minimal volume of warmed 10^{-3} M Versene; after cooling, salt precipitated out. The supernatant fluid was decanted and the residue was recrystallized three times more from hot, then cooled, distilled water to remove all but traces of Versene. This was necessary because enzyme activity was inhibited by the presence of Versene in appreciable concentrations (approximately 10^{-3}–10^{-4} M).

The acidic form of Amberlite resin CG-50, type II, after serial alkaline and acid treatment to clean and reactivate (Yagi *et al.*, 1956), was washed thoroughly with distilled water, mixed with sufficient 10 N NaOH to bring its *p*H near that of the buffer, and then suspended in buffer for final equilibration.

Absorption spectra were measured with a Cary recording spectrophotometer, model 14, at room temperature (24°). A 1-ml cuvet (1-cm optical path) was used.

Enzyme activities were defined and assayed as follows:

(a) 2,6 - Dichlorophenolindophenol - reduction: Reduction of 2,6-dichlorophenolindophenol (DCPI) in the presence of enzyme and reduced pyridine nucleotide was asssayed by the change in absorbancy (optical density) at 600 mμ. Symbol: $-\Delta E_{600}^{\text{DCPI}}$/min.

(b) Heme protein reduction: Reduction of cytochrome *c*, cytochrome c_2, or RHP, in the presence of enzyme and reduced pyridine nucleotide, was assayed by the change in absorbancy at characteristic α-band maximum (550 mμ for all three heme proteins). Symbols: $\Delta E_{550}^{\text{cytc}}$/min; $\Delta E_{550}^{\text{cytc}_2}$/min; and $\Delta E_{550}^{\text{RHP}}$/min; respectively.

(c) Flavin-reduction: Reduction of flavin (FAD, FMN, or riboflavin) in the presence of enzyme and reduced pyridine nucleotide was assayed by the change in absorbancy at 450 mμ. Symbol: $-\Delta E_{450}^{\text{flav}}$/min.

(d) DPNH-oxidation: Oxidation by air in the presence of enzyme alone was assayed by the change in absorbancy at 340 mμ. Symbol: $-\Delta E_{340}^{\text{DPNH}}$/min.

Reaction rates were calculated from the initial linear portion of the activity curves obtained by observation of ΔE as a function of time. Protein concentrations were referred directly to absorbancies at 280 mμ. Specific enzyme activities were expressed as absorbancy changes for dye reduction per unit absorbancy at 280 mμ per minute. Symbol: $\Delta E_{600}^{\text{DCPI}}/E_{280}$/min. Dye reduction, as the most convenient assay, was chosen to monitor enzyme purification.

All assays were conducted in the presence of 0.25 mM DPNH and 40 μM 2,6-dichlorophenolindophenol at 24°. Other additions are noted in the figure legends. In aerobic assays, standard components were 0.50 ml 0.1 M buffer, an appropriate quantity of enzyme, 0.10 ml 3.0 mM DPNH, and distilled water to make a final volume of 1.20 ml. In anaerobic assays, it was convenient to use twice this volume in Thunberg-type cuvets with enzyme, or DPNH, in the side-arm. Continuous spectrophotometric readings commenced about 5 seconds after the final addition (usually of DPNH), unless otherwise noted.

Sedimentation velocity measurements were made with a Spinco analytical ultracentrifuge, model E (59,580 rpm, 20 ± 0.2°). Three protein concentrations were employed: approximately 0.5, 0.75, and 1.0%. Before each determination, the enzyme sample was dialyzed several times against sodium acetate buffer (*p*H 5.8, ionic strength 0.1).

Extraction and Purification of DPNH-Heme Protein Reductase

Step 1. Extraction of Enzyme.—Lyophilized cells of light-grown *R. rubrum* (100 g) were sus-

TABLE I

SUMMARY OF PURIFICATION OF DPNH-HEME PROTEIN REDUCTASE FROM *Rhodospirillum rubrum*

The reaction components were as follows: 0.50 ml 0.1 M Tris-HCl buffer (*p*H 8.0), 0.10 ml 3 mM DPNH, 0.10 ml 0.48 mM DCPI, 0.10 ml 1 mM FMN, an appropriate amount of sample solution, and water to make a total volume of 1.20 ml. Reactions were started by the final addition of DPNH and carried out at 24°. The specific activity of the purest fraction (step 5), based on content of protein in mg ($-\Delta E_{600}^{DCPI}$/min./mg) was 18.0 units, or approximately 5-fold greater than that of the purest sample reported in our previous paper (Horio and Kamen, 1960).

Step	Volume (ml)	Total Activity ($-\Delta E_{600}^{DCPI}$/min.) Without FMN	With FMN	Specific Activity	Yield (%)
1. Extract	857	19,200	19,900	1.29	(100)
2. *p*H 5.3 resin eluate	2200	930	10,700	4.30	54
3. Ammonium sulfate 40–80% saturated fraction	19	—	7,600	7.74	38
4. Best fractions after chromatography on *p*H 5.65 Amberlite CG-50 resin	25	—	4,600	32.9	23
5. Ammonium sulfate 50–60% saturated fraction	13	100	2,100	61.7	11

pended in 1 liter of 0.1 M sodium acetate buffer (*p*H 5.0) by homogenization with a Waring blendor at room temperature (24°); immediately thereafter the *p*H of the suspension was adjusted to 5.3. All consequent procedures were carried out in a cold room (approximately 3°). The suspension was extracted overnight with continuous stirring and then centrifuged at higher than 10,000 × *g* for 1 hour. The *p*H of the resulting supernatant fluid was adjusted to *p*H 5.3, if necessary; then it was dialyzed against 0.1 M sodium acetate buffer (*p*H 5.3) for 1 day, with three more changes of the external buffer solution. The dialyzed extract was centrifuged to remove the precipitate which formed during dialysis. This procedure yielded 857 ml of "extract" (Table I).

Step 2. Adsorption and Elution of Enzyme on pH 5.3-Amberlite Resin.—The extract was passed through a column packed with Amberlite CG-50 (type II) resin, 20 × 5 cm diameter, which had been equilibrated with 0.1 N sodium acetate buffer (*p*H 5.3). The cytochrome c_2, RHP, and enzyme present in the extract were adsorbed on the column. The emergent solution did not show absorption spectra characteristic of heme proteins and contained less than 1/20 of the total enzyme activity of the extract. The charged resin was recovered from the column and washed with the acetate buffer until the washings were colorless. The washed resin was then suspended in 3 volumes of 0.2 M Tris-HCl buffer (*p*H 8.0). The resin suspension, with continuous stirring, was frequently adjusted to *p*H 8 by addition of 5 N NaOH at ice-cold temperature. This process lasted somewhat more than 3 hours. Then, the resin suspension was filtered through a glass filter. This elution procedure was repeated at least once with the same volume of 0.2 M Tris-HCl buffer (*p*H 8.0). The cytochrome c_2 and RHP adsorbed on the *p*H 5.3 resin appeared to be more easily eluted from the resin in this manner than was the enzyme. The two eluates were mixed and adjusted to *p*H 6.0. The brownish-red solution (2.2 liters) exhibited from 1/2 to 2/3 of the total enzyme activity of the extract.

Step 3. Fractionation of Enzyme with Ammonium Sulfate.—To the *p*H 5.3 resin eluate (2.2 liters), 540 g of metal-free, Versene-free ammonium sulfate was added to approximately 40% saturation, followed by adjustment of *p*H to approximately 6. After standing for more than one hour, the turbid solution was centrifuged twice (10,000–15,000 × *g*) for 30 minutes. The resultant clear supernatant was brought to approximately 80% saturation by addition of 710 g of solid ammonium sulfate. After standing at least 1 hour, the solution was centrifuged for 30 minutes. Almost all of the enzyme activity, in addition to some RHP and cytochrome c_2, was found in the precipitate. The supernatant fraction, containing the rest of the RHP and cytochrome c_2, was stored for further purification of these heme proteins. The precipitate was suspended in a minimal volume of 0.1 M sodium acetate buffer (*p*H 5.65) and dialyzed against the buffer solution. The dialyzed suspension was then centrifuged at 30,000 × *g* for 30 minutes. The resulting 19-ml supernatant fraction, called "40–80% saturated fraction," was used for further purification of the enzyme.

Step 4. Chromatography on pH 5.65 Amberlite Resin.—The 40–80% saturated fraction was passed through a column packed with Amberlite CG-50 (type II), 30 × 1 cm diameter, which had been equilibrated with 0.1 M sodium acetate buffer, *p*H 5.65. The emergent solution contained approximately 5% of the total DPNH–2,6-dichlorophenolindophenol reducing activity in the 40–80% saturated fraction. Because this

fraction lost activity much faster during storage than the enzyme fraction recovered at a later stage (see below) and was not adsorbed upon rechromatography, it seemed certain that the enzyme in this emergent solution had been modified or denatured. We found that, compared with the "native" enzyme, this "modified" enzyme did not catalyze appreciable reduction of cytochrome c_2 by DPNH (see below).

Elution of "native" enzyme was accomplished by repeated washing with the same buffer. Of the main colored substances present in the dialyzed sample, cytochrome c_2 migrated most rapidly. This heme protein could be fractionated into its reduced and oxidized forms; the former eluted a little more readily than the latter. The brownish-yellow band of the enzyme migrated just behind the band of oxidized cytochrome c_2, while the brown band of RHP remained at the top of the column. The brownish-yellow band containing enzyme was carefully fractionated into several portions, and the best fractions, which assayed better than 15 units in specific activity, were combined. The resultant mixture showed a yellowish-red color. Immediately after almost all the enzyme had been eluted, the RHP was eluted with ammonium phosphate buffer (*p*H 8.0, 4 N as ammonium ion). The cytochrome c_2 and RHP fractions were stored in a frozen state for further purification.

Step 5. Refractionation with Ammonium Sulfate.—The mixture of enzyme fractions from step 4 was fractionated immediately with a saturated solution of ammonium sulfate (*p*H approximately 5.2), and the precipitate obtained at between 50 and 60% saturation was collected by centrifugation at 25,000 × *g* for 30 minutes. The precipitate was washed twice with 10-fold volumes of 65%-saturated ammonium sulfate, dissolved in distilled water, dialyzed against distilled water, and then stored frozen. In all experiments described below, this purified enzyme sample (specific activity, 57–69) was used, unless otherwise noted. The purification procedure is summarized in Table I.

Results

Observations on Dark and Light Respiration

Effect of pH on Dark and Light Respiration.—As expected, the light-grown cells of *R. rubrum* consumed oxygen faster in darkness than in light. However, the ratio of dark to light respiration varied from culture to culture; the highest value obtained in these studies was approximately 4.5. Optimal *p*H values were 7.7 for the dark (total) respiration, 8.0 for the light respiration, and 7.5 for the respiration which could be inhibited by the light. These values obtained whether young or aged light-grown, wild-type cells were used (Fig. 1).

We determined Q_{O_2}, at 30° and *p*H 7.0, to be 15–20 mm³/hr./mg dry weight for the dark

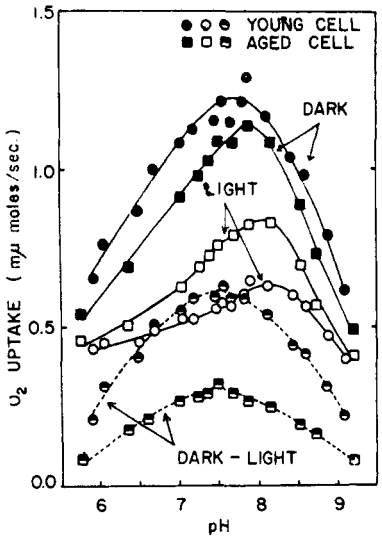

FIG. 1.—Effect of *p*H on oxygen consumption by wild-type light-grown cell suspensions of *R. rubrum*. Young and aged cells were used (approximately 5 mg dry weight cells for each assay). All other experimental conditions are described in the text.

respiration of light-grown young cells. We noted that young cells always showed more rapid oxygen uptake in darkness and less rapid oxygen uptake in the light than did the aged cells. Aeration of young cell suspensions for 15 minutes at room temperature evoked the same type of respiratory behavior in light and dark as that of aged cells. After 1 hour anaerobiosis at room temperature, from 40–70% restoration of the original respiratory rate was noted. The extent of light-induced inhibition was the greater, the more reduced were the cell suspensions.

The respiration of the blue-green mutant type of light-grown young *R. spheroides* cells was much less markedly inhibited by light than was the case with wild-type cells. Thus at *p*H 8.0, where the effect was maximal, oxygen uptake in the dark fell only ∽ 10% on illumination.

Effect of Temperature on Dark and Light Respiration.—When wild-type *R. rubrum* cells were heated at *p*H 7.2 for 5 minutes in air at temperatures up to 40°, neither dark nor light respiration was altered (Table II). When heated at higher temperatures, the cells showed an acceleration in the rate of light respiration, then a decrease, while the rate of dark respiration fell continuously. In fact, the light respiration of the cells which had been heated at temperatures greater than 60° was more rapid than the dark respiration.

The cell suspension heated at 70° for 5 minutes did not consume oxygen at all unless cell-free medium was added. When cell-free medium was boiled or frozen and thawed, it lost this potentiating capacity completely. Hydroquinone showed the same capacity to support oxygen uptake as did the nontreated cell-free medium. Freezing

TABLE II
EFFECT OF TEMPERATURE TREATMENT ON RESPIRATION

	O_2 Uptake (mμmole/2 min)				Ratio
	$Light_1$	Dark	$Light_2$	$(L_1 + L_2)/2$	$2D/(L_1 + L_2)$
Untreated	78	163	78	78	2.1
Five minutes at					
35°	77	166	79	78	2.1
40°	79	161	79	79	2.0
45°	82	161	84	83	1.9
50°	97	133	102	99	1.3
55°	87	87	87	87	1.0
60°	77	77	79	78	1.0
65°	53	19	47	50	0.4
70°	18	3	15	17	0.2
Freezing-thawing					
× 1	105	115	107	106	1.1
× 2	107	107	107	107	1.0
× 5	107	107	110	109	1.0

and thawing of the cell suspension rendered respiration insensitive to light (Table II). Respiration of these suspensions was more rapid than the light respiration of the nontreated cell suspension and less rapid than the dark respiration (Table II).

Effect of Inhibitors on Light and Dark Respiration.—Carbon monoxide and cyanide, as typical respiratory inhibitors, markedly inhibited respiration of dark-grown and light-grown cells. Cyanide inhibited both dark and light respirations of light-grown, wild-type cells (Fig. 2). With either young cells or aged cells, the ratio of the dark to light respiration rose when the concentration of cyanide was increased from 10^{-4} to 10^{-3} M. Thus at 10^{-5}, 5×10^{-3}, and 10^{-3} M cyanide, the ratios for young cells were 2.7, 3.6, and 1.8, respectively, whereas for old cells they were 1.4, 1.8, and 1.0. Thus, cyanide inhibited the light-insensitive respiration more than it did the light-sensitive respiration, and the increase in ratio of dark to light respiration was less for aged cells.

Aerated young cells behaved like the aged cells in the presence of cyanide. When stored under anaerobic conditions, the aerated cells regained the characteristics of young cells with respect to response of dark and light respiration to cyanide.

Phenylmercuric acetate, unlike cyanide, inhibited both dark and light respiration (Fig. 3) to the same extent.

n-Butanol, tested as a possible narcotic, inhibited the light-sensitive respiration, but inhibited the light-insensitive respiration hardly at all (Fig. 4). Methyl alcohol, ethyl alcohol, and iso-butanol showed the same effect as found for ethyl alcohol and *n*-butanol. The concentration required to inhibit almost all of the light-sensitive respiration was approximately 3 M for methyl alcohol, 1.0 M for ethyl alcohol, and 0.05 M for iso-butanol. Allyl alcohol inhibited both dark and light respiration. Cells pretreated with 0.15 M *n*-butanol could be restored to their original respiratory behavior if washed with cell-free medium. Ethylene glycol also inhibited the dark respiration, but the ratio of the dark to light respiration was never less than 1.7, the value reached at 2.5 M. (This maximal value varied from case to case; the highest concentration of ethylene glycol tested was 40%, Fig. 5.)

The action of sucrose was complex. No effect was found with either light or dark respiration from 10^{-5} to 10^{-2} M. At greater concentrations, both types of respiration were increased, but the ratio of dark to light fell, which indicated a preferential increase in the light-insensitive respiration.

Respiration of Chromatophores.—The chromato-

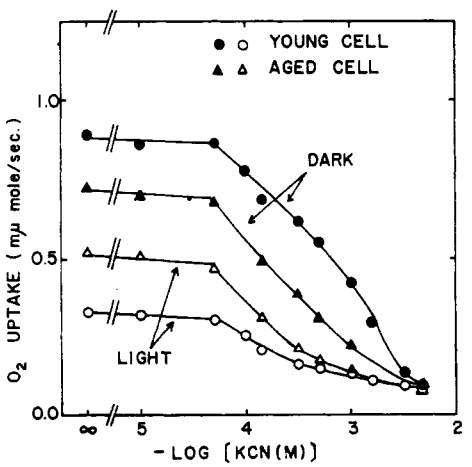

FIG. 2.—Effect of cyanide on oxygen consumption by wild-type light-grown *R. rubrum* cells. Young and aged cells were used with and without potassium cyanide; other experimental conditions were the same as for Fig. 1.

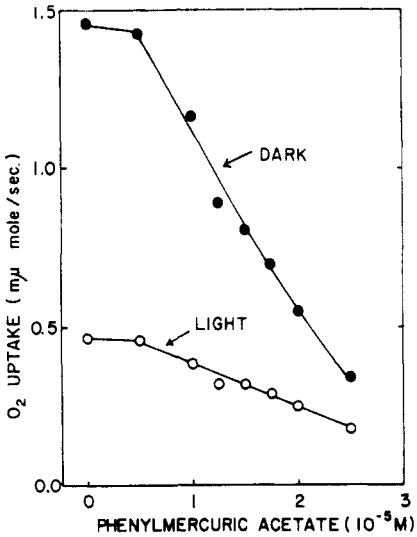

FIG. 3.—Effect of phenylmercuric acetate on oxygen consumption by wild-type light-grown *R. rubrum* cells. Experimental conditions were the same as for Fig. 1.

phore suspensions consumed oxygen at rates comparable to those found for intact cells. Oxygen uptake was *stimulated* by illumination at room temperature, in contrast with the behavior of light-grown cells (Fig. 6). On the other hand, oxygen uptake at the temperature of an ice-water bath was *inhibited* by illumination.

The chromatophore suspension, when heated at 70° for 5 minutes, coagulated and exhibited oxygen uptake under illumination, but none in darkness. In Figure 6, typical responses of this type are shown after treatment at 95° for 5 minutes. Observations were made over the range 40–95°. The oxygen uptake of the heated chromatophore could be inhibited by cyanide, but the inhibition appeared to be much less than for the untreated chromatophores and whole cells. This type of chromatophore respiration

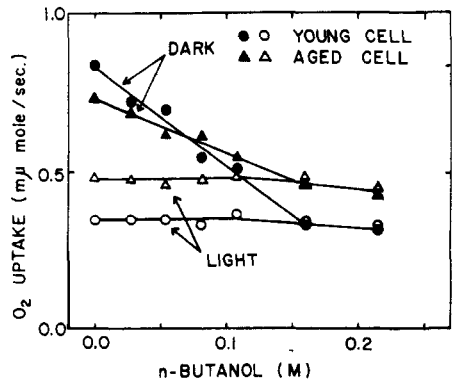

FIG. 4.—Effect of *n*-butanol on oxygen consumption by wild-type light-grown *R. rubrum* cells. Experimental conditions were the same as for Fig. 1.

stimulated by light was, in all likelihood, a photooxidation of some reducing substances catalyzed by bacteriochlorophyll, analogous to the photooxidation reactions described by Vernon and Kamen (1953).

Low Temperature Absorption Spectra of RHP

Previous work (Horio and Kamen, 1961) had shown that RHP was a di-heme protein. In connection with its apparent intimate involvement in the respiratory activity of dark-grown cells and its position as a key catalyst, presumably as an oxidase in the light-insensitive respiration (Horio *et al.*, 1962) we measured its low temperature spectra. We examined these spectra for the reduced forms of RHP in the presence and absence of carbon monoxide. In agreement with inferences from previous work, the low temperature spectrum revealed two completely resolved γ-absorption peaks at 436 mμ and 422 mμ. We

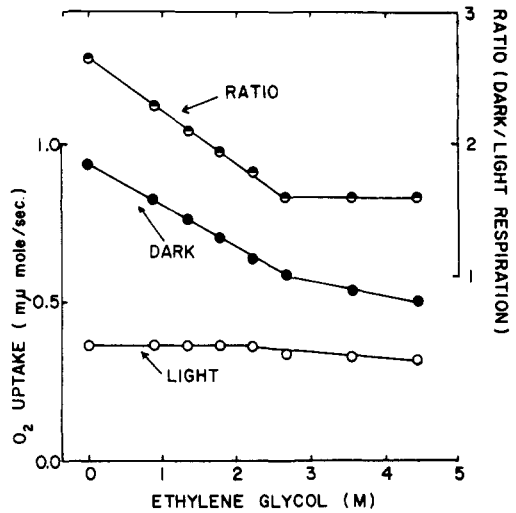

FIG. 5.—Effect of ethylene glycol on oxygen consumption by wild-type light-grown *R. rubrum* cells. Experimental conditions were the same as for Fig. 1.

had hoped also to see resolution of the α peaks of the RHP, but there was no evidence of better resolution in the visible region of the spectrum at liquid nitrogen temperature than at room temperature (Fig. 7).

Spectra observed at *p*H 8 differed from those seen at *p*H 7 in that the peak for CO-reduced RHP at 416 mμ was substantially lower at *p*H 7; in addition, a peak noted at 434 mμ at *p*H 7 appeared to exist only as a shoulder on the main peak at *p*H 8. The absorbancy of this shoulder was smaller than that for the 436 mμ peak in the absorption spectrum of reduced RHP at *p*H 8. We assumed that these differences indicated changes in affinity for CO at these two *p*H values.

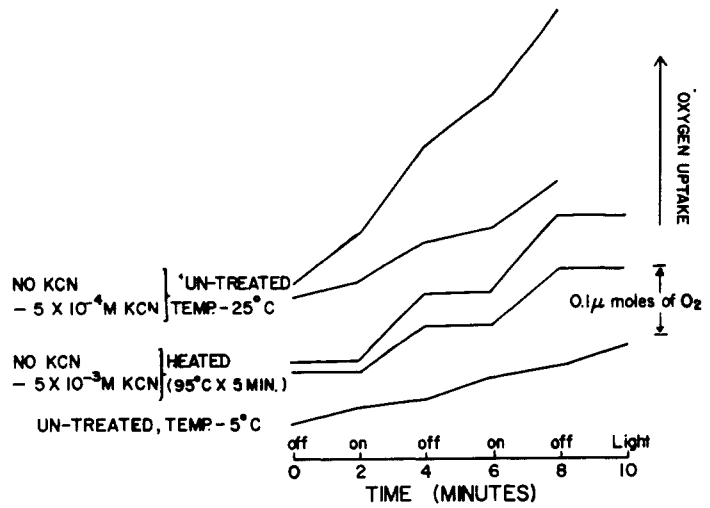

FIG. 6.—Time course of oxygen consumption by chromatophores of wild-type light-grown *R. rubrum* cells. A fixed concentration of chromatophore suspension was added (total absorbancy at 880 mμ per ml suspension, approximately 10). Reactions proceeded either at room temperature (approximately 25°) or at approximately 5°. Other experimental conditions were the same as for Fig. 1.

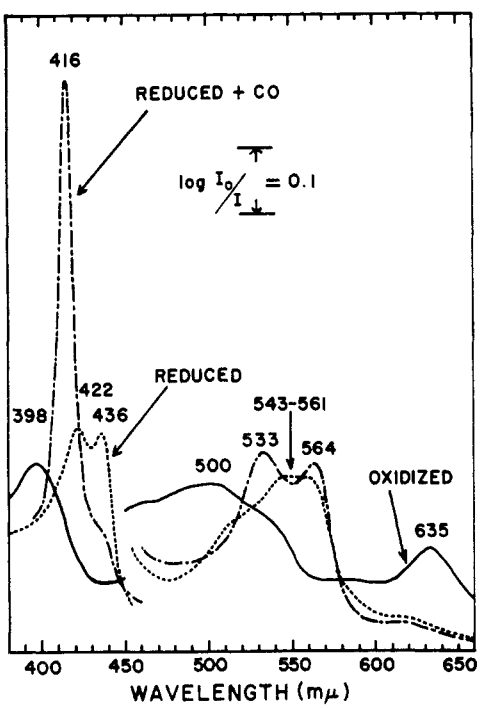

FIG. 7.—Low temperature spectra of RHP. For explanation of procedures, see text.

Observations on the Heme Protein Reductase(s)

Previous researches (Horio and Kamen, 1960) indicated a key role for this enzyme(s) in the electron-transport systems of *R. rubrum*, so we prosecuted further studies of the samples we had obtained by our improved procedures.

Physicochemical Properties of Heme Protein Reductase

SEDIMENTATION ASSAY.—The enzyme preparation was essentially homogeneous. Ultracentrifugal sedimentation revealed one major component with a symmetrical sharp peak, and a very small amount of a light impurity which appeared to account for less than 5% of the total protein. The sedimentation constant ($s_{20,w}$) was 3.3 *S*, which indicated a molecular weight of approximately 35,000. Runs with three different protein concentrations gave values in good agreement (±4%); thus, sedimentation velocities were independent of protein concentrations in the range studied.

ABSORPTION SPECTRUM ASSAY.—The enzyme absorption spectrum was that of a *c*-type cytochrome (Fig. 8).

The absorption maxima of the sample reduced by dithionite differed slightly but appreciably from those of reduced cytochrome c_2. The shifts noted could be ascribed either to changes induced by binding of cytochrome c_2 to enzyme, or to the presence of a *c*-type heme different from that of cytochrome c_2.

Physiologic Properties

REQUIREMENT OF FLAVIN AS CO-FACTOR FOR ENZYME ACTIVITY.—If the extract from step 1 in the purification procedure (refer to Table I) was exposed to high concentrations of salts [NaCl, Na_2SO_4, or $(NH_4)_2SO_4$] and dialyzed against water, the resulting dialyzed sample lost activity, although it had retained activity upon dialysis before such treatment. The inactivated enzyme was completely reactivated by the addition of

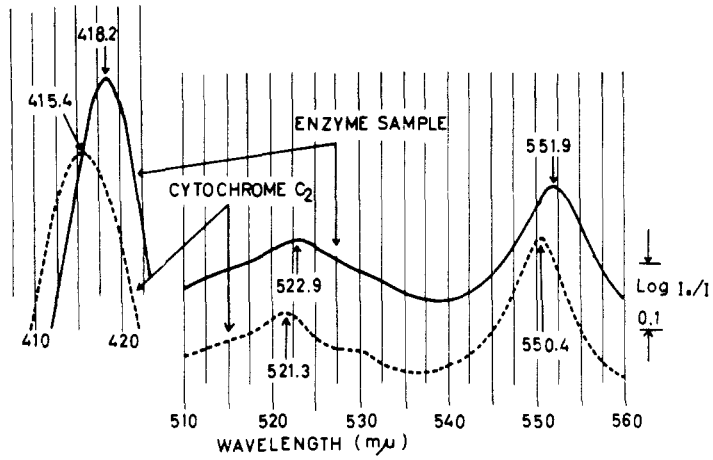

FIG. 8.—Comparison of absorption peaks of cytochrome c_2 and DPNH-heme protein reductase in dithionite-reduced forms, at 24° in 0.04 M acetate buffer (*p*H 5.8).

flavins (FAD, FMN, or riboflavin). The higher the ionic strength of salt used, the greater was the ensuing degree of reactivation. A moderate value for ionic strength, such as 0.2 in NaCl, was effective in producing this inactivation over the *p*H range tested (*p*H 3–9), which included the most stable *p*H range of the enzyme (*p*H 5–6).

The extract, which had been thoroughly dialyzed without serious inactivation (see Table I), was treated with saturated ammonium sulfate at *p*H 5–6, followed by centrifugation to separate the precipitate which contained inactivated enzyme. Then, the supernatant fluid was extracted with phenol in the usual manner for preparation of flavins from tissue extract. The flavins so obtained were found to include riboflavin, FMN, and FAD, together with some unknown flavin(s). This flavin mixture was very effective for reactivation of the enzyme present in the precipitate.

As purification proceeded, the enzyme was progressively inactivated; its DPNH – 2,6-dichlorophenolindophenol reducing activity was increasingly restored by the addition of flavins. The enzyme obtained in the final purification step was found to show less than 5% of the activity observed after reactivation by addition of FMN (see Table I).

The enzyme, after partial inactivation by salt treatment, was inhibited somewhat by maleate buffer and much more so by boric-borax buffer. It appeared that the enzyme inactivated by these buffers was reactivated by FAD to the same extent (Table III). The effect of FMN was not tested.

All of these results are consistent with a requirement for bound flavin. However, determination of the nature of the flavin originally bound to the active protein must await development of a procedure which does not dissociate flavin in the course of purification.

We noted that the enzyme activity evoked by added flavins was very easily inhibited by the addition of salts. The DPNH – 2,6 - dichlorophenolindophenol reducing activity after reactivation by FMN was inhibited 50% in the presence of as little as 3.5% ammonium sulfate.

DPNH–CYTOCHROME c_2 REDUCING ACTIVITIES.—As shown previously (Horio and Kamen, 1960), the enzyme could reduce cytochrome c_2 in the presence of either DPNH or TPNH. After final purification of the enzyme, DPNH was approximately 8-fold more effective as an electron-donor for 2,6-dichlorophenolindophenol than was TPNH, in agreement with our previous findings for a cruder enzyme preparation.

The purified enzyme was active in DPNH–cytochrome c_2 reduction only when flavins were added. All three flavins tested (FAD, FMN, and riboflavin) were effective as activators. In the presence of FMN, the DPNH–cytochrome c_2 reducing activity was optimal at *p*H 6 (Fig. 9). FAD and riboflavin gave results similar to those for FMN. If the extract from step 1 in the purification procedure, or an extremely high concentration of the purified enzyme, was used, both could reduce cytochrome c_2 by DPNH without addition of flavins. Their *p*H-activity curves were the same as for pure enzyme reactivated with FMN; this provided presumptive evidence that the natures of the activities, original and reactivated, were identical.

In the presence of DPNH, the purified enzyme could reduce cytochrome c_2 at almost the same rate in the presence of varied concentrations of riboflavin and FMN, whereas the rate was much less in the presence of FAD (Fig. 10). In the presence of a fixed concentration of DPNH (0.25 mM), K_m values for riboflavin and FMN were roughly the same, *i.e.*, approximately 6×10^{-8} M. K_m for FAD appeared to be greater than 10^{-6} M. The relatively large value of K_m for FAD might be ascribed to steric hindrance caused by the adenosine group of FAD.

Reactivation by these flavins vanished at approximately 5×10^{-9} M. If this concentra-

TABLE III

EFFECTS OF VARIATION IN BUFFER COMPOSITION ON ACTIVITY OF ENZYME

Experimental conditions: FAD was used instead of FMN, various kinds of buffer solutions (0.50 ml of 0.1 M, *p*H 8.0) were employed, and the extract from step I in the purification procedure was used, after treatment with 2% NaCl and dialysis against water to inactivate the enzyme partially. In some experiments, 0.20 ml of 0.1 M sodium citrate (*p*H 8.0) was added. Otherwise, conditions were the same as for the experiments of Table I.

Buffer	Additions	$-\Delta E_{600}^{DCPI}$/min.	Relative Activity[a] (%)	Ratio of Activities With/Without FAD
Glycylglycine-NaOH	—	0.216	67	1.49
	FAD	0.321	(100)	
Tris-HCl	—	0.210	65	1.48
	FAD	0.309	96	
Tris-maleate	—	0.184	57	1.48
	FAD	0.270	84	
Boric acid-borate	—	0.088	27	1.48
	FAD	0.130	40	
Na_2HPO_4-NaH_2PO_4	—	0.192	60	1.53
	FAD	0.294	92	
	citrate	0.150	47	1.50
	FAD + citrate	0.225	70	

[a] Activity with FAD in glycyl-glycine buffer was taken as 100%.

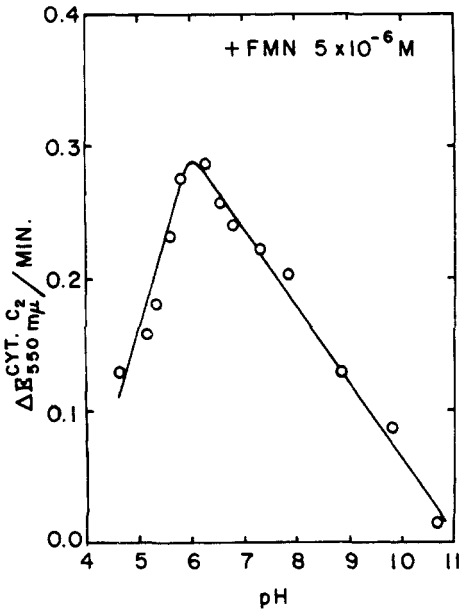

FIG. 9.—Activity-*p*H curve for DPNH-cytochrome c_2 reduction by enzyme in the presence of FMN. The reaction components were as follows: 0.50 ml 0.1 M sodium phosphate buffer of various *p*H's, 0.20 mM oxidized cytochrome c_2, 0.10 ml 60 μM FMN, 0.10 ml purified enzyme appropriately diluted, 0.10 ml 3.0 mM DPNH, and 0.20 ml distilled water to make total volume of 1.20 ml. Reactions were started by final addition of DPNH. *p*H was measured after completion of activity assay.

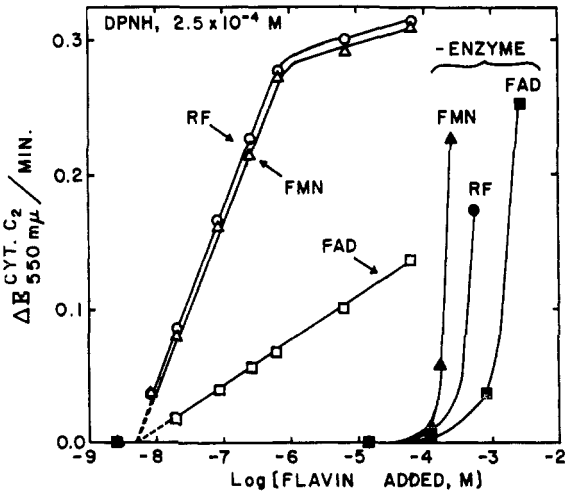

FIG. 10.—Effect of flavins on DPNH-cytochrome c_2 reduction. The experimental conditions were the same as for Fig. 9, except that sodium acetate buffer (*p*H 5.9) and varied concentrations of flavins were present. In the control assays, reactions were carried out without the addition of enzyme. Open circles, addition of riboflavin; open triangles, addition of FMN; open squares, addition of FAD; closed symbols, with no enzyme.

tion was taken to represent a minimum concentration of the enzyme present, the turnover number in reduction of cytochrome c_2 by DPNH could be estimated as approximately 2,000 at 24°. The turnover number was approximately 5,000, based upon the dry weight of the enzyme and the assumed molecular weight of 35,000, as indicated by sedimentation analysis.

As noted originally by Singer and Kearney (1950), cytochrome c_2 was rapidly reduced by DPNH in the absence of the enzyme if flavins were present in concentrations higher than 10^{-4} M. Thus, the presence of flavins in such high concentration could introduce artifacts of a non-physiologic nature in the assay of enzymic reduction of cytochrome c_2. At a low concentration of flavin,

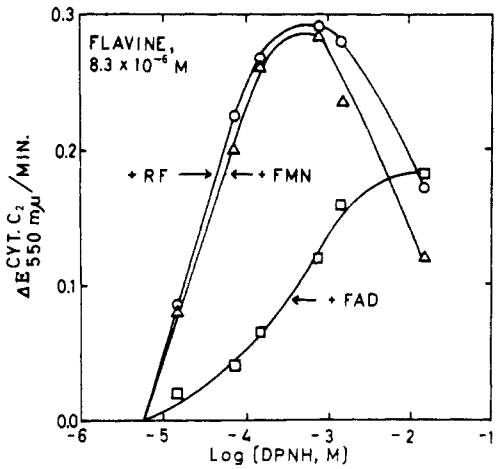

FIG. 11.—Effect of DPNH concentration on DPNH-cytochrome c_2 reduction activity. The experimental conditions were the same as for Fig. 9, except that a fixed concentration of riboflavin, FMN, and FAD (8.3 μM in final concentration) and varied concentrations of DPNH were used.

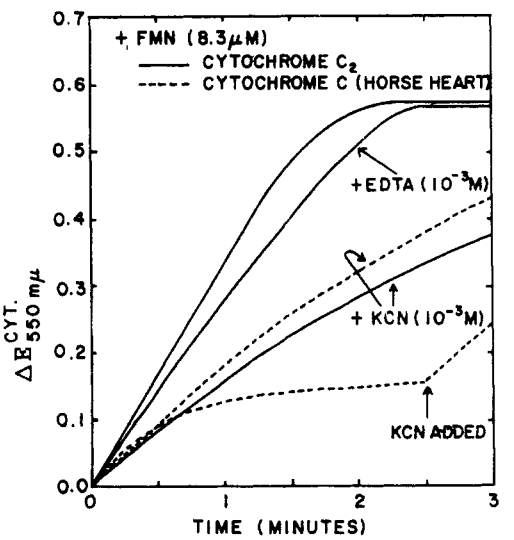

FIG. 12.—Effect of cyanide and EDTA on DPNH-cytochrome c_2 and DPNH-cytochrome c reduction. The experimental conditions were the same as for Fig. 9, except that in addition to cytochrome c_2 horse heart cytochrome c (0.20 ml approximately 0.2 mM) a fixed amount of DPNH (0.10 ml 3 mM), cyanide, and EDTA were used.

increasing concentrations of DPNH brought about increased cytochrome c_2 reduction, but at high concentrations of DPNH there was a marked inhibition (Fig. 11). In the presence of flavins, the purified enzyme could reduce all of the cytochrome c_2 added if an excess of DPNH was present.

The reduction was inhibited in the presence of cyanide (Fig. 12). However, inhibition was incomplete (50–60%) even at a high concentration of cyanide (10^{-3} M). EDTA (10^{-3} M) appeared to be somewhat inhibitory. Carbon monoxide (80%), with 20% N_2 or O_2, had no influence.

The purified enzyme could catalyze reduction of horse heart cytochrome c by DPNH, but much more slowly than for cytochrome c_2, if flavins were present (Fig. 12). Preincubation of the enzyme with cytochrome c before the start of enzymic reduction by the addition of DPNH caused rapid inactivation of the enzyme. Even when the enzymic reaction was started by final addition of the enzyme, inactivation of the enzyme took place within one minute. In contrast, the same pretreatment of the enzyme with cytochrome c_2 did not cause any inactivation; the reduction of cytochrome c_2 continued at an almost linear rate for the whole experimental period. If compared in the steady state (lapse of 1–2 minutes), the reduction rate of cytochrome c_2 by DPNH was some 20-fold faster than that of cytochrome c.

Although cyanide inhibited cytochrome c_2 reduction, it markedly accelerated cytochrome c reduction (Fig. 12). The addition of cyanide abolished inactivation caused by preincubation with cytochrome c. The cytochrome c reduction reactivated by cyanide appeared to be almost as fast as initial reduction of cytochrome c in the absence of cyanide, and also of cyanide-inhibited cytochrome c_2 reduction.

The enzymic reduction rate of cytochrome c by DPNH was the same whether air was present or not (*i.e.*, in helium).

DPNH–RHP REDUCING ACTIVITY.—If flavins were added with excess DPNH under anaerobic conditions, it was found that the purified enzyme catalyzed a rapid reduction of all the RHP added. Under aerobic conditions no reduction occurred (Fig. 13). Reduction rates with RHP as acceptor were exceedingly sensitive to trace amounts of oxygen still present after the sequence of gas changes to produce anaerobiosis. Residual traces of oxygen caused a lag in RHP reduction until they were removed completely.

The flavin requirement in DPNH–RHP reduction by the purified enzyme appeared to be somewhat different from that found for the DPNH-cytochrome c_2 reduction. FMN was the most effective of the three flavins. The RHP reduction rate with FMN was calculated to be 44% that of the cytochrome c_2 reduction per molecule. Since it had been established that cytochrome c_2 and RHP possessed one and two hemes per molecule, respectively (Horio and Kamen, 1961), it followed that the enzyme, supplemented by FMN, could reduce both heme proteins with DPNH at almost the same rate per heme under anaerobic conditions.

The reduction of RHP was partially inhibited by 100% carbon monoxide (Fig. 13). The initial rate in the presence of carbon monoxide was 68% of that in its absence. This value corresponded to 57% of the rate of the cytochrome c_2 reduction in the absence of CO (CO had no effect on cytochrome c_2 reduction). Furthermore, the time

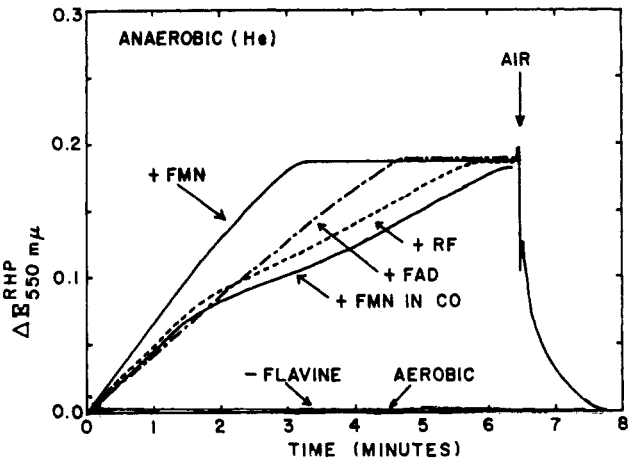

FIG. 13.—Effect of flavins on DPNH-RHP reduction. The reaction components were as follows: 1.00 ml 1.0 M acetate buffer (*p*H 6.0), 0.20 ml 10^{-4} M riboflavin, FMN, or FAD, 0.20 ml 0.2 mM RHP, 0.20 ml of purified enzyme, 0.20 ml 3 mM DPNH, and distilled water to make a total volume of 2.40 ml. Reactions, at 24° in Thunberg-type cuvet under anaerobic conditions (He or CO), or in air, were started by the final addition of DPNH from the side-compartment. In some experiments, air was flushed into the anaerobic cuvet at the end of the RHP reduction.

course of the CO-inhibited RHP reduction with FMN exhibited a marked drop just halfway to full reduction of the RHP added. This phenomenon was also seen with riboflavin as the cofactor of the enzyme, even in the absence of carbon monoxide. On the other hand, with FAD, only a continuously lower rate was observed (61% of the rate with FMN).

Introduction of air into the Thunberg cuvet containing completely reduced RHP and enzyme caused immediate oxidation (Fig. 13). This indicated that the auto-oxidizability of RHP in its reduced form was very rapid and much faster than the enzymic reduction of RHP.

DPNH-OXIDIZING ACTIVITY.—The purified enzyme could catalyze oxidation of DPNH by air if flavins were present, without the addition of oxidation mediators such as cytochrome c_2, RHP, and 2,6-dichlorophenolindophenol. In the presence of FMN, the DPNH-oxidizing activity curve was symmetrical around a maximum at *p*H 6. Similar results were obtained with riboflavin and FAD. The flavin requirement for DPNH-oxidation was quite similar to that for DPNH-cytochrome c_2 reduction (Fig. 14; compare with Fig. 16). Thus, addition of FMN or riboflavin stimulated activity to a greater extent than did the addition of FAD. The oxidation rate of DPNH with FMN or riboflavin was approximately 26% that for the reduction rate of cytochrome c_2 with FMN or riboflavin, and approximately 59% that for the reduction rate of RHP with FMN, calculated per mole DPNH (molar extinction coefficient of DPNH at 340 mμ, 6.22 $\times$ 10^6 cm^2/mole, as given by Horecker and Kornberg, 1948). The enzymic DPNH-oxidation was not inhibited in the presence of cyanide (10^{-3} M) regardless of which flavins were employed for reactivation (Fig. 15). EDTA appeared to be weakly stimulatory or ineffective.

DPNH-FLAVIN REDUCTION.—The purified enzyme, when tested under anaerobic conditions, rapidly reduced all three flavins. The reduction rate was almost the same for FMN and riboflavin and less with FAD, the relationship being much the same as for the DPNH oxidation. In the presence of an excess amount of DPNH, the

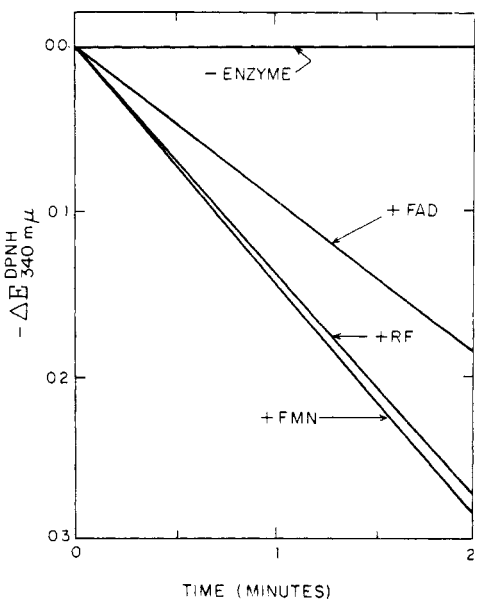

FIG. 14.—Effect of flavins on enzymic DPNH-oxidation. The experimental conditions were the same as for Fig. 9, except that riboflavin and FAD, as well as FMN, were added at a final concentration of 8.3 μM, and acetate buffer (*p*H 5.9) was used.

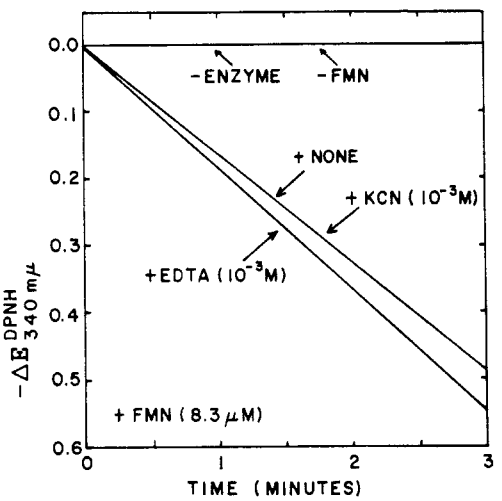

FIG. 15.—Effect of cyanide and EDTA on DPNH-oxidizing activity. The experimental conditions were the same as for Fig. 14, except that cyanide and EDTA were present in a final concentration of 10^{-3} M.

purified enzyme reduced all of the flavins present. With FMN as H-acceptor, the rate of the flavin reduction by DPNH was nearly the same (approximately 92%) as that for the DPNH oxidation in air (Fig. 16). Again, as in the DPNH oxidation, cyanide and carbon monoxide had no influence on the DPNH-flavin reduction rate. Under aerobic conditions, the flavins added were not reduced; however, the DPNH oxidation by air proceeded at almost the same rate as for the anaerobic DPNH-flavin reduction.

DISCUSSION

Before we attempt to rationalize the results of our present researches, it will be helpful to present briefly some remarks, as well as some speculations, about the nature of the systems involved. Chromatophores have been conceived as similar to mitochondria, in that they are held to include a linear sequence of electron carriers grading in oxidation-reduction potential from that at the substrate end to that of the terminal oxidase. However, in the chromatophore, as in the green plant chloroplast, no classical cytochrome oxidase is present (Kamen, 1961a). Following the original suggestion of Hill (1955), we may assume the place of oxidase to be filled by the photoactive pigment—bacteriochlorophyll (BChl)—which may function in its light-activated form either as photoreductant or "photo-oxidase" (Kamen, 1961b).

Duysens (1958) has depicted the action of light as changing the "effective" oxidation potential of ground-state (unexcited) BChl from a high oxidizing (positive) mid-point potential to a very high reduction (negative) mid-point potential, corresponding to BChl in an excited state. Kamen (1961b) has proposed that the end result of the photochemical action is production of a "semichlorinogen" species of BChl in which the pigment is stabilized in a partially-reduced form, BChlH. Such a product is more likely to possess a definable mid-point potential than a molecule in an excited state, because a reversible electrode process can be specified usually only for a reactant in an unexcited state.

Duysens (1956) and Goedheer (1958) have deduced from differential spectra obtained in *in vivo* systems treated with a variety of oxidants (ferricyanide, permanganate, *etc.*), that BChl has an "effective" mid-point potential of 400 to 500 mv. The electrode reaction with which this potential is associated is not specified, but may correspond to the process: $BChl^+ + \epsilon^- \rightleftharpoons BChl$. The reaction for BChlH production by reductive de-excitation after light absorption may be written: $BChlH \rightleftharpoons BChl + \epsilon^- + H^+$; a high negative (reduction) potential can be assigned to this reaction (approximately −800 to −900 mv). The basis for this value has been discussed elsewhere (Kamen, 1961a).

With these preliminaries, we invite the reader's attention to Figure 17, which shows in skeletal form the situation as we conceive it in the bacterial chromatophore electron-transport system. This very simple diagram is much the same as the scheme to be found in discussions by most workers in this field (see Geller, 1961, for a review). It differs in that it emphasizes the central role of the photoactive pigment (BChl) as the connecting link in a respiratory chain which functions in cyclic fashion when photoactivation occurs. Also, it assigns specifically to RHP the terminal oxidase function in a conventional linear open-ended chain for dark respiration. BChl, by virtue of its normal oxidation potential in the dark, can be placed at the end of the chain. On illumination, it shifts in potential to the beginning of the chain, which thus is tied together in a "loop." The energy for this shift in potential is supplied by the absorbed quantum (greater than 1.3 volts per electron). The scheme shown in Figure 17 is intended to visualize in a crude way this process of promotion of BChl from one end of the chain to the other.

The site of light suppression of respiration is in the chromatophore, as established not only from the previous observations of Katoh (1961) but also from our present results. All evidence available indicates that photoactivative processes in bacterial photosynthesis are localized in the chromatophore (Frenkel, 1958). Very little is known about the organelles which presumably exist and support respiration in dark-grown cells. However, Horio *et al.* (1962) have found that the action spectrum for relief of CO inhibition of respiration is closely identical with the difference spectrum obtained for (CO, reduced)-minus-(reduced) RHP. Moreover, Chance and Smith showed some time ago (1955) that light-grown cells contain a reversibly oxidized component which, when reduced, forms a light-dissociable CO-complex with a peak in the difference spectrum

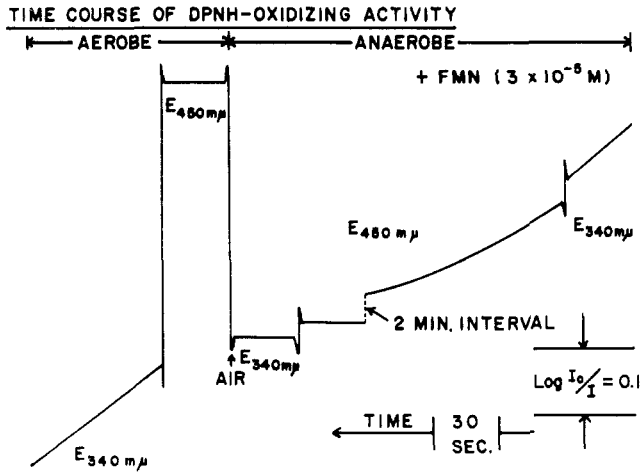

FIG. 16.—Time course of DPNH-oxidation activity. The experimental conditions were the same as for Fig. 14, except that the total volume was doubled for the reaction components in the Thunberg-type cuvet and FMN concentration was 3×10^{-5} M. Readings were made at 340 mμ and at 450 mμ under both anaerobic and aerobic conditions. The time course of the experiment is read from right to left. Beginning at the right, the drop in absorbancy at 340 mμ (DPNH oxidation) is followed for 30 seconds, then the Cary setting is changed quickly to 450 mμ to follow flavin reduction until a steady-state reduction of flavin and oxidation of DPNH has been established. Then, air is admitted, whereupon the reduced flavin is oxidized immediately (30 sec. observation), and the oxidation of residual DPNH resumes.

for (CO, reduced)-minus-(reduced) at 415 mμ, as does RHP. They also noted, as did Duysens originally (1954), that the dark anaerobic-minus-aerobic difference spectrum exhibited a peak at 428 mμ, also like RHP. These observations, extended by the recent researches of Horio *et al.* (1962), appear to indicate that in dark-grown cells RHP may be identical with the terminal oxidase—called "cytochrome O" by Castor and Chance (1959)—and that it also serves an oxidase function in chromatophores of light-grown cells. However, it is unlikely that it is the only pathway to oxygen, as can be seen from the present observations on the purified heme protein reductase(s).

It is plausible to assign RHP a function in electron transport to the chlorophyll terminus of the chain through cytochrome c_1. This seems to be very likely in the chromatophore of the obligate photoanaerobe, *Chromatium*, as Olson and Chance (1960) have shown on the basis of differential spectrophotometry. Chromatium contains large amounts of RHP (Bartsch and Kamen, 1960), and, as a strict anaerobe, has no requirement for an oxidase, although it shows weak oxidase activity in cell-free extracts (Kamen and Vernon, 1954). Horio and Kamen (1962) have shown that in *R. rubrum* chromatophores RHP acts as an intermediate in phosphorylation coupled to the photoactivated transport of electrons.

The place assigned cytochrome c_2—between RHP or flavin-mediated RHP-reductase and bacteriochlorophyll—is based on its high oxidation potential (midpoint potential, *p*H 7, approximately 330 mv)—and evidence based on our studies of photophosphorylation (Horio and Kamen, 1962) which show clearly it is at the terminal end of the electron transport chain coupled to photophosphorylation.

We propose, in brief, that in chromatophores the respiratory chain involves reduction of oxygen *via* RHP, and not *via* cytochrome c_2. There may be alternative pathways which bypass RHP and lead directly from flavin to oxygen (as we indicate by the dotted arrow in Figure 17), because cyanide only partially inhibits the reduction of either RHP or cytochrome c_2 catalyzed by the heme protein reductase, and does not inhibit the DPNH-oxidase function.

Although the reductase is approximately 500-fold purified and essentially homogeneous by physiochemical criteria, it must be emphasized that it retains all ten activities of the initial crude extract (*i.e.*, the DPNH- and TPNH-linked reductions of flavin, RHP, cytochrome c_2, 2,6-

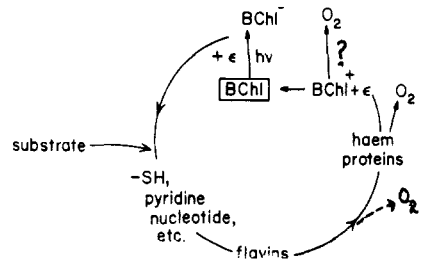

FIG. 17.—Reaction scheme for catalysis of light-sensitive and light-insensitive respiration. For explanation, see text. The placement of the "—SH" and "flavin" components is arbitrary.

dichlorophenolindophenol, and DPNH and TPNH oxidase functions). Hence, it would be premature to interpret oxidative mechanisms in chromatophores in other than the most general terms; nor is it appropriate to dwell at this time on the possible significance of the quantitative relation found between DPNH oxidase rates and the various reductase rates, with and without inhibitors. Further uncertainty comes from our neglect of ubiquinones, which undoubtedly are also involved (Rudney, 1961). Further progress must await development of procedures which enable isolation of the reductase without cleavage of the flavin bound in the native state.

On the positive side, we can see from the observed values of Michaelis-Menten constants that the prosthetic flavin is most likely to be riboflavin or FMN, or some flavin like them, rather than FAD. The binding constant for DPNH indicates that the interposition in the respiratory chain of pyridine nucleotide bound to the reductase is plausible. Our data show that flavin bound to the apoenzyme is reduced at a rate which can be increased two-fold by the presence of RHP or cytochrome c_2, which presumably are bound to the apoenzyme near the site of interaction between flavin and DPNH. It is significant that cytochrome *c* differs dramatically from cytochrome c_2 in that it acts as a substrate inhibitor. Its inhibitory action probably arises because, in consequence of its very basic isoelectric point, it is charged in the opposite sense to cytochrome c_2. Most likely, it is bound so firmly that it cannot dissociate after reduction. Hence, because of steric hindrance which is a consequence of the large protein bound to the active site, other substrate cytochromes cannot reach the active site and enzyme action halts. It is important to note, also, that cytochrome *c* is effective as an accelerator of photophosphorylation by *R. rubrum* chromatophores only up to concentrations of approximately 10^{-5} M, whereas cytochrome c_2 activates photophosphorylation in a linear manner at concentrations as high as can be tested (Horio and Kamen, 1962).

The effect of cyanide in inhibiting respiration of *R. rubrum* has been difficult to understand in the past on the basis of RHP as oxidase because RHP does not combine with cyanide (Vernon and Kamen, 1954; Bartsch and Kamen, 1958). However, the demonstration that cyanide is an inhibitor of heme protein reduction by the reductase paves the way for at least a partial rationalization of the cyanide effect. We assume that the preferential inhibition of the light-insensitive respiration by cyanide arises from cyanide binding on the heme protein reductase which interferes with electron transport to RHP as oxidase.

Carbon monoxide should affect only light-insensitive respiration because it combines only with RHP among all the respiratory carriers present. This expectation is borne out experimentally. The action of phenylmercuric acetate as an inhibitor for both light-sensitive and light-insensitive respiration is easily explicable as owing to attack on −SH containing carriers in the chain on the substrate side of the reductase.

The observation that a heme group like that of cytochrome *c* is still attached to the reductase suggests that this enzyme may be another example of a heme-bearing flavoprotein like yeast lactate dehydrogenase (Appleby and Morton, 1954). However, we have not obtained sufficient data on either the stoichiometry or the fissionability of the heme moiety to warrant further discussion.

It is encouraging to see that the reductase after reactivation by FMN or riboflavin at relatively low concentrations (approximately 10^{-6} M) exhibits turnover numbers for heme protein reduction of approximately 5000; this high activity is equal to the diaphorase activity, whereas in our previous efforts the diaphorase activity was very much more in evidence (Horio and Kamen, 1960). The finding that concentrations of flavins in excess of 10^{-4} M cause a rapid nonenzymic reduction of heme proteins should introduce a note of caution for investigators who, in the past, have used such concentrations in reconstitution of photophosphorylation systems.

It is apparent that the major physiologic function of the enzyme can be assigned as electron carrier from reductant (DPNH, and eventually photoreductant) to RHP. The possibility that the reductase is still heterogeneous remains. Hence, the notion must be entertained, as remarked previously, that oxidase activities which by-pass the heme proteins may be called into play under conditions not as yet understood.

We may now consider the question of light supression of respiration on the basis of the carrier complex, visualized in Figure 17 and based on the data of the present researches. We assume that the chromatophores of light-grown cells, the organelles of dark-grown cells, and the photoactive subcellular bodies present in the blue-green mutants of *R. spheroides* all contain respiratory chains which include as common components pyridine nucleotide, flavin-mediated heme protein reductase, and the two bacterial heme proteins, RHP and cytochrome c_2. Cytochrome O (RHP?) is the only oxidase known to be present in the organelles of the dark-grown cells. These three types differ from each other in the detailed disposition of these respiratory agents. In the mature chromatophore, the respiratory chain is organized structurally in a manner determined by the photochemical function as represented by the great preponderance of bacteriochlorophyll and associated pigments. In the chromatophore of the blue-green mutant (which we assume to be incomplete by analogy with experience from many studies of chloroplasts in plant mutants), the oxidation chain may not be closely coupled to the photoactive pigments. In the dark organelle, structure is determined wholly by respiratory requirements. Newton (1960) has shown that the relatively simple procedure of vigorous aeration

of growing *R. rubrum* cell suspensions in the dark induces rapid structural alterations in the bacteriochlorophyll-bearing apparatus.

These notions lead us to expect that illumination of chromatophores will cause electrons to flow through, or around, RHP to cytochrome c_2 and thence to bacteriochlorophyll in the cyclic fashion shown in Figure 17. Hence oxygen uptake will be reduced progressively with increasing light absorption. The action spectrum for light inhibition will be that of absorption by the photoactive pigment system. This, in fact, recently has been found to be true by Horio *et al.* (1962). The amount of inhibition depends only partially on the relative rates of photo-oxidation and auto-oxidation of RHP, because oxidation pathways alternative to RHP as oxidase probably exist (see above). In the blue-green mutant, a looser coupling of the photochemical system to the respiratory chain seems plausible, and can be invoked to explain the absence of a light-sensitive respiration. In the dark organelle, we expect only the RHP-mediated respiration, as found experimentally (Horio *et al.*, 1962).

The remarkable effect of alcohols in abolishing the light-sensitive respiration is explicable in a number of ways. Probably the simplest is that the addition of alcohol disrupts the coupling between the photoactive pigments by alteration of structure. In this connection, it is important to note the observations of Teale (1961), who has demonstrated that, in micelles containing chlorophyll and other pigments (carotenoids, fucoxanthol, *etc.*) in concentrations approaching those effective in chloroplasts and chromatophores, small amounts of alcohol can inhibit resonance transfer of excitation energy between chlorophyll and the other pigments. An alternative explanation of a quite radical character is one based on the possibility that bacteriochlorophyll itself may act as an oxidase. No evidence is available in favor of this suggestion; indeed, certain observations of Krasnovsky and Voinovskaya (1957) contraindicate it. These workers found that cytochrome on which chlorophyll was absorbed could be photo-oxidized in air but was not appreciably oxidized by air in the absence of light.

No unique causes can be assigned to the differential effects of *p*H and temperature. Effects of *p*H could arise from differences in response to proton concentration characteristic of the various respiratory pathways possible at the flavin level. Elevation of temperature would be expected to cause structural alterations which could lead to uncoupling effects like those assumed to be obtained with alcohols. At very high temperatures, inactivation of the reductase, as well as other enzyme activities, could result; nonenzymic photo-oxidation would be expected and is, in fact, found to become dominant.

In summary, despite the complex of observations accumulated, it is unnecessary to postulate more than one oxidation chain in *R. rubrum* to account for the existence of light-sensitive and light-insensitive respirations. The members of this chain which are known already possess properties which can be correlated with the multiplicity of phenomena observed so far in photoactivated reactions of chromatophores. Further progress demands isolation of homogeneous subcellular particulates from both dark- and light-grown cells.

Acknowledgments

The researches were initiated, and performed in part, at the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass., with the aid of grants from the National Institutes of Health (C-3649) and the National Science Foundation (G-6441). Their continuation at the Johnson Foundation, University of Pennsylvania, and the University of California at San Diego, was made possible by grants from the same sources, C-5992 (NIH) and G-19642 (NSF). We are especially indebted to Professors B. Chance and Walter H. Bonner, who made available the facilities of the Johnson Foundation.

References

Appleby, C. A., and Morton, R. K. (1954), *Nature 173*, 749.

Bartsch, R. G., and Kamen, M. D. (1958), *J. Biol. Chem. 230*, 47.

Bartsch, R. G., and Kamen, M. D. (1960), *J. Biol. Chem. 235*, 825.

Castor, L. N., and Chance, B. (1959), *J. Biol. Chem. 234*, 1587.

Chance, B. (1951), *Rev. Sci. Instruments 22*, 634.

Chance, B., and Smith, L. (1955), *Nature 175*, 803.

Clayton, R. K. (1953), *Arch. Mikrobiol. 19*, 107.

Clayton, R. K. (1955), *Arch. Mikrobiol. 22*, 180, 195.

Cohen-Bazire, G., Sistrom, W. R., and Stanier, R. Y. (1951), *J. Cell. Comp. Physiol. 49*, 25.

Davies, P. W., and Brink, F. (1942), *Rev. Sci. Instruments 13*, 524.

Duysens, L. M. N. (1954), *Nature 173*, 692.

Duysens, L. M. N. (1956), *Ann. Rev. Plant Physiol. 7*, 25.

Duysens, L. M. N. (1957), *in* Research in Photosynthesis, Gaffron, H., *et al.*, editors, New York, Interscience Publishers, p. 164.

Duysens, L. M. N. (1958), *Brookhaven Symp. Biol.*, no. 11, p. 10.

Estabrook, R. W. (1956), *J. Biol. Chem. 223*, 781.

Frenkel, A. W. (1958), *Brookhaven Symp. Biol.*, No. 11, p. 276.

Geller, D. M. (1961), *in* The Bacteria, vol. II, Gunsalus, I. C., and Stanier, R. Y., editors, New York, Academic Press, Inc., p. 472.

Gest, H., and Kamen, M. D. (1960), *in* Handbuch der Pflanzen Physiologie, vol. V, Ruhland, W., editor, pt. 2, chap. IV, p. 604.

Gest, H., Kamen, M. D., and Bregoff, H. M. (1950), *J. Biol. Chem. 182*, 153.

Goedheer, J. C. (1958), *Brookhaven Symp. Biol.*, No. 11, p. 325.

Griffiths, M., Sistrom, W. R., Cohen-Bazire, G., and Stanier, R. Y. (1955), *Nature 176*, 1211.

Hill, R. (1955), *in* Proceedings of the Third International Congress of Biochemistry, Brussels, p. 255.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.
Horio, T., and Kamen, M. D, (1960), *Biochim. Biophys. Acta 43*, 382.
Horio, T., and Kamen, M. D. (1961), *Biochim. Biophys. Acta 48*, 266.
Horio, T., and Kamen, M. D. (1962), *Biochemistry 1*, 144.
Horio, T., Taylor, C. P. S., and Chance, B. (1962), in press.
Kamen, M. D. (1961a), *in* Structure and Function, Symposium of the International Union of Biochemists, Stockholm, 1960, London, Academic Press, Inc., p. 277.
Kamen, M. D. (1961b), *in* Light and Life, McElroy, W. D., and Glass, B., editors, Baltimore, Johns Hopkins Press, p. 483.
Kamen, M. D., and Vernon, L. P. (1953), *Biochim. Biophys. Acta 17*, 10.
Kamen, M. D., and Vernon, L. P. (1954), *J. Biol. Chem. 211*, 633.
Katoh, S. (1961), *J. Biochem.* (Japan) *49*, 126.
Krasnovsky, A. A., and Voinovskaya, K. K. (1957), *Doklady Akad. Nauk., S. S. S. R. 112*, 911.
Morita, S. (1955), *J. Biochem.* (Japan) *42*, 533.
Nakamura, H. (1937), *Acta Phytochim.* (Tokyo) *9*, 189.
Newton, J. W. (1960), *Biochim. Biophys. Acta 42*, 34.
Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K. (1957), *J. Biochem.* (Japan) *44*, 453.
Olson, J. M., and Chance, B. (1960), *Arch. Biophys. Biochem. 88*, 26, 40.
Rudney, H. (1961), *J. Biol. Chem. 236*, PC39.
Singer, T. P., and Kearney, E. B. (1950), *J. Biol. Chem. 183*, 409.
Smith, L., and Ramirez, J. (1959), *Arch. Biochem. Biophys. 79*, 233.
Smith, L., and Baltscheffsky, M. (1959), *J. Biol. Chem. 234*, 1575.
Teale, F. W., quoted by Weber, G. (1961), *in* Comparative Biochemistry of Photoreactive Systems, Allen, M. B., editor, New York, Academic Press, Inc., p. 386.
Van Niel, C. B. (1941), *Adv. Enzymol. 1*, 263.
Van Niel, C. B. (1944), *Bacteriol. Rev. 8*, 1.
Vernon, L. P., and Kamen, M. D. (1953), *Arch. Biochem. Biophys. 44*, 298.
Vernon, L. P., and Kamen, M. D. (1954), *J. Biol. Chem. 211*, 643.
Yagi, K., Matsuoka, Y., Koyama, S., and Tada, M. (1956), *J. Biochem.* (Japan) *43*, 93.
Yang, C. C., and Legallais, V. (1954), *Rev. Sci. Instruments 25*, 801.

2-Isopropylmalate and 3-Isopropylmalate as Intermediates in Leucine Biosynthesis*

JOSEPH M. CALVO,† M. G. KALYANPUR, AND CARL M. STEVENS

From the Department of Chemistry, Washington State University, Pullman, Washington

Received June 18, 1962

2-Isopropylmalic acid and 3-isopropylmalic acid have been synthesized and identified as excretion products of a leucine-requiring mutant of *Neurospora crassa*. Homogenates of *Escherichia coli* form 2-isopropylmalic acid from valine.

It is known that in some microorganisms the carbon skeleton of leucine is derived from acetate and the isobutyl moiety of valine (Strassman *et al.*, 1956; these authors also present a good review of the literature). A biosynthetic pathway to leucine was proposed by Strassman *et al.* involving the condensation of acetate with α-ketoisovalerate followed by a sequence of reactions, analogous to the Krebs cycle, leading to α-ketoisocaproate. The synthesis of 2-isopropylmalic acid and 3-isopropylmalic acid, two of the intermediates in this proposed pathway, and their identification in culture media of a mutant of *Neurospora crassa*, is described in this report. During the progress of this work, Jungwirth *et al.* (1961) have reported that extracts of *Salmonella typhimurium* form 2-isopropylmalic acid from valine and acetyl coenzyme A and that leucine-less mutants of this organism excrete 2-isopropylmalic acid into the culture medium. Also, Gross *et al.* (1962) have tentatively identified a *N. crassa* metabolite as 3-isopropylmalic acid by its neutralization equivalent, by elementary analysis, and by the fact that extracts of *S. typhimurium* and *N. crassa* convert it to α-ketoisocaproate.

* This work was supported in part by Research Grant E-1111 from the National Institutes of Health.

† National Science Foundation Fellow (1959–1961).

EXPERIMENTAL RESULTS

2-Isopropylmalic Acid.—The synthesis of 2-isopropylmalic acid, developed independently in this laboratory, differed from that reported by Yamashita (1958) in that ethyl isobutyrylacetate cyanohydrin was isolated from the reaction of ethyl isobutyrylacetate, NaCN, and $NaHSO_3$. Yield, 53%; b.p. 118–120°/10 mm.

Anal. Calcd. for $C_9H_{15}NO_3$: C, 58.36; H, 8.16; N, 7.56. Found: C, 58.45; H, 8.24; N, 7.46.

Acid hydrolysis of ethyl isobutyrylacetate cyanohydrin gave 2-isopropylmalic acid in the yield reported.