Biochimica et Biophysica Acta, 555 (1979) 210—220

BBA 78454

MEMBRANES OF *RHODOPSEUDOMONAS SPHAEROIDES*

VI. ISOLATION OF A FRACTION ENRICHED IN NEWLY SYNTHESIZED BACTERIOCHLOROPHYLL *a*-PROTEIN COMPLEXES *

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(Received December 21st, 1978)

Key words: Membrane synthesis; Chromatophore; Bacteriochlorophyll-protein complex; (Rps. sphaeroides)

Summary

Radioactivity eventually destined for the chromatophore membrane of *Rhodopseudomonas sphaeroides* was shown in pulse-chase studies to appear first in a distinct pigmented fraction. This material formed an upper pigmented band which sedimented more slowly than chromatophores when cell-free extracts were subjected directly to rate-zone sedimentation on sucrose density gradients. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the purified fraction contained polypeptide bands of the same mobility as light-harvesting bacteriochlorophyll *a* and reaction center-associated protein components of chromatophores; these were superimposed upon cytoplasmic membrane polypeptides. The pulse-chase relation was confined mainly to the polypeptide components of these pigment-protein complexes. It is suggested that the isolated fraction may be derived from sites at which new membrane invagination is initiated.

Introduction

Recent studies in eukaryotic cells have provided considerable insight into the mechanisms by which proteins are secreted across [1—4] or inserted into intra-

* Paper V in this series is Ref. 25.

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Abbreviations: B-850 and B-875, light-harvesting bateriochlorophyll complexes with absorption maxima near 850 and 875 nm, respectively; SDH, succinate dehydrogenase.

cellular membranes [5]. Some aspects of these mechanisms are also involved in the transfer of specific proteins across bacterial cytoplasmic membranes [6,7] and their appearance in the periplasmic space [8,9] or outer membrane [10—13]. In contrast, very little is known of mechanisms by which integral proteins are inserted into energy-transducing membranes. Past investigations have focused mostly upon mitochondria [14,15] and chloroplasts [16,17]. The prokaryotic photosynthetic apparatus (the chromatophore membrane [18]) as elaborated by the faculative photoheterotrophic bacterium *Rhodopseudomonas sphaeroides* provides a structurally and genetically less intricate experimental system for such studies. This structure can account for as much as 30—50% of cellular protein [19]; and gives rise to uniform vesicular structures (chromatophores) that can be easily purified [18,20]. The major integral chromatophore membrane proteins are associated with the photochemical reaction center [21,22] and light-harvesting bacteriochlorophyll *a* [23,24] and can account for up to 70% of their total protein. These protein components are not found in other cellular membranes [25] and are specific to the chromatophore membrane [23,26,27]. It has recently been reported that chromatophore-specific polypeptides are synthesized continuously in synchronously dividing cell populations and it was suggested that they are incorporated into preexisting membranes [19].

The results of pulse-chase studies in phototrophically growing *Rp. sphaeroides* have suggested that radioactivity eventually destined for major chromatophore membrane proteins appears first in small membrane fragments isolated by differential and rate-zone sedimentation [28]. The rapidly labeled fraction consisted mainly of fragments of bacteriochlorophyll *a*-depleted cytoplasmic membrane together with pigmented material [29]. It was not known if this labeling was confined to protein components of the peripheral cytoplasmic membrane or a pigmented fraction. With recently developed techniques for the isolation of relatively unpigmented portions of the cytoplasmic membrane from phototrophically growing cells [25], it was possible to test these alternatives directly. Evidence presented here suggests that the proteins of this membrane are not involved in a pulse-chase relation with chromatophores. Instead, pulse labeling was confined mainly to bacteriochlorophyll-associated polypeptides localized within a pigmented fraction. The isolated material appears to be derived from specific sites at which new membrane invagination is initiated.

Materials and Methods

Pulse chase procedures. Rp. sphaeroides NCIB 8253 was grown phototrophically in a defined medium [30,31] at 1800 lux under 95% nitrogen/5% CO_2. Exponentially growing cells were pulsed 4—5 min with L-[G-^{3}H]phenylalanine (Amersham/Searle, 0.88 mCi/μmol). Except where indicated otherwise, the pulse was terminated by a chase with a 10^3—10^4-fold excess of unlabeled phenylalanine. Culture aliquots were poured over crushed ice in the presence of chloramphenicol (0.8 g and 10—50 μg/ml, respectively). They were combined with fixed amounts of ^{14}C-labeled cells grown with L-[U-^{14}C]phenylalanine (ICN, 400 Ci/mol, 0.06 μCi and 10 μg unlabeled phenylalanine/ml). Specific radioactivities were calculated directly from ^{3}H/^{14}C since ^{14}C provided a mea-

sure of total protein. Harvested cells were washed in 1.0 mM Tris-HCl buffer (pH 7.5). Crude extracts were prepared in a French press at 20 000 lb/inch^2 and centrifuged at 10 000 × *g* for 10 min. Other isolation procedures are described in the respective figure legends. Radioactivity of gradient fractions was determined on filter paper discs as described previously [28] except that hot trichloroacetate treatment was omitted. At least 99% of the radioactivity in chromatophores purified from cells grown in the presence of [^{3}H]phenylalanine was found in the hot trichloroacetate precipitate after chloroform/methanol (2 : 1, v/v) extraction (Broglie, R.M. and Niederman, R.A., unpublished observations). In the experiment shown in Fig. 1, specific radioactivities were corrected by dividing values for the individual fractions by the ^{3}H/^{14}C values for the respective crude extracts [32]. When plotted in this manner, a fraction which serves as a precursor of proteins and exports label to other cellular fractions would have a corrected ^{3}H/^{14}C of greater than 1.0 at the beginning of the chase with a negative slope thereafter. A product fraction which imports label would have an initial ratio of less than 1.0 at the start of the chase and a positive slope thereafter. In the phenylalanine-chased cultures (Fig. 1, upper panel), the membrane fragment and chromatophore fractions exhibited the respective pulse-chase behavior consistent with these predictions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The slab gel procedure employed was essentially that described by Laemmli and Favre [33]. Samples were heated in 62.5 mM Tris-HCl buffer (pH 6.8) at 100° C for 60 s and 100 μg of sample protein (determined as described previously [25]) were applied. The fixation, staining, and destaining procedures have also been described [25]. For liquid scintillation counting, the gel slabs were fixed and sliced into individual sample lanes and then at right angles into 1—2-mm slices that were treated as described previously [29]. Radioactivity was determined in a Beckman LS-230 liquid scintillation counter. A computer program corrected for reciprocal cross-over of radioactivity; quenching was not corrected as it was essentially equivalent in all vials.

Results

Before definitive pulse-chase studies could be conducted, it was necessary to distinguish if the apparent precursor-product relations observed previously [28] reflected the continued synthesis and insertion of membrane proteins rather than exchange of labeled proteins by lateral diffusion from peripheral to intracytoplasmic regions of a fluid membrane continuum. Since protein exchange could be expected to continue in the absence of further synthesis, this possibility was tested by chasing with antibiotic inhibitors of protein biosynthesis. After a 4-min pulse with [^{3}H]phenylalanine, the apparent precursor-product relation between small membrane fragments and chromatophores revealed by chasing with excess unlabeled phenylalanine (Fig. 1, upper panel) was essentially obliterated by chloramphenicol (middle panel) or tetracycline (lower panel). Thus, the observed relation is dependent upon continued synthesis and integration of new polypeptides and does not result from the exchange of preexisting proteins. In Fig. 1, it is also seen that no significant breakdown of labeled protein occurred in the membrane fractions in the presence of the antibiotics.

Physicochemical characterization of the rapidly labeled fraction suggested that it consisted of a mixture of pigmented material and non-pigmented fragments of peripheral cytoplasmic membrane [29]. With the methods developed recently for the isolation of relatively unpigmented fragments of cytoplasmic membrane [25], it was possible to test directly whether the precursor polypeptides were localized in this fraction. In Fig. 2A, it is seen that after a 5 min pulse with [^{3}H]phenylalanine, the $^3H/^{14}C$ ratio of cytoplasmic membrane was lower than that of chromatophores and did not change appreciably during the chase; in contrast, a two-fold increase was observed for chromatophores. Thus, in phototrophically growing cells, the conserved peripheral cytoplasmic membrane [25] does not contain detectable polypeptide components that are chased into chromatophores. It thus appeared that the pigmented material within the unresolved fraction contained the rapidly labeled polypeptide components.

Reconstitution experiments had shown that about 65% of the bacteriochlorophyll in crude membrane fragments could be accounted for by contaminating chromatophores carried over during differential centrifugation [29]. To assure that pigmented material containing the rapidly labeled polypeptides was separated adequately from the vast excess of chromatophores, crude extracts were applied directly to sucrose gradients (Fig. 3A). After rate-zone sedimentation, the A_{850nm} profile (absorbance maximum of the bulk B-850 bacteriochlorophyll-protein complex) revealed an upper pigmented band (fractions 3—6). Although it accounted for only 6% of the bacteriochlorophyll, it was resolved satisfactorily from chromatophores (fractions 12—15). Ultraviolet absorbance indicated that the upper pigmented fraction had cosedimented with ribosomal material. In addition, the distribution of succinate dehydrogenase (succinate:phenazine methosulfate oxidoreductase, EC 1.3.99.1) activity suggested that the upper fraction had also cosedimented with cytoplasmic membrane. This suggestion is based upon the much higher specific activity of the cytoplasmic relative to chromatophore membrane [20,29] and the expected banding of small fragments of relatively unpigmented cytoplasmic membrane [25] in this position in the gradient. The distribution of this activity also indicates that some cytoplasmic membrane has remained with cell envelope (fractions 20 and 21). The upper pigmented band was separated from ribosomal material after prolonged sedimentation and a second rate-zone centrifugation (Fig. 3B). Further pulse-chase studies indicated that the upper pigmented band contained the rapidly labeled polypeptide components that were chased into major chromatophore proteins (Fig. 2B). A reciprocal relation in the $^3H/^{14}C$ ratio for the respective fractions was observed.

The polypeptide profile of the upper pigmented band was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared to that of chromatophore and cytoplasmic membranes (Fig. 4). In stained gels (Fig. 4A), the upper pigmented fraction contained polypeptide bands of the same mobility as the light-harvesting bacteriochlorophyll and reaction center-associated protein components [21—24] prominent in chromatophores. These were superimposed upon many polypeptide bands characteristic of the cytoplasmic membrane with only a few differences in their relative amounts. The gradient gel procedure separated the light-harvesting bacteriochlorophyll com-

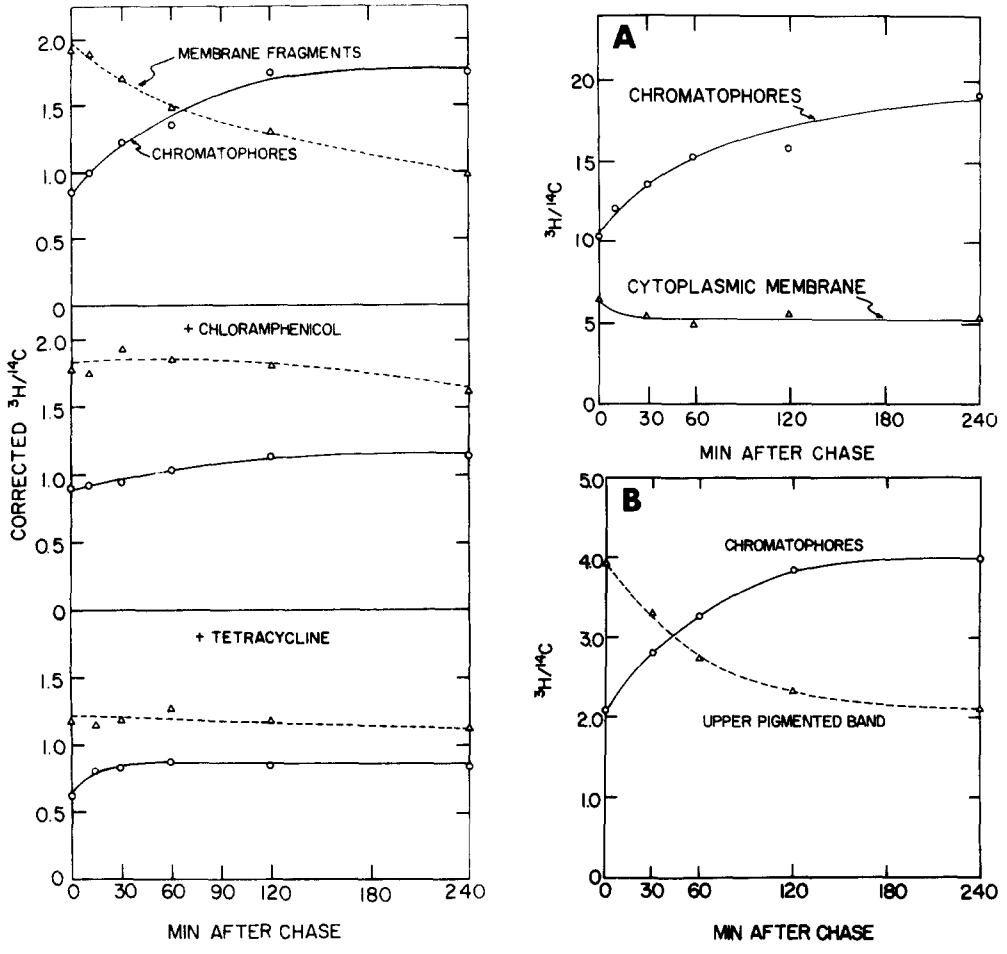

Fig. 1. Effects of antibiotics on pulse-chase relations. Cultures were grown in 500-ml gas-washing bottles for 17—20 h until an A of 0.90 was reached as measured at 680 nm (1-cm light path) on a Gilford spectrophotometer. The cultures were divided into 250-ml portions and each was pulsed with 30 μCi of [^{3}H]-phenylalanine. After 4 min, one portion was chased 4 h with excess unlabeled phenylalanine (upper panel), a second was chased with 100 μg of chloramphenicol/ml (middle panel), while the third was chased with 2.0 μg of tetracycline-HCl/ml (lower panel). These antibiotic concentrations completely inhibited protein synthesis over the duration of the chase period as measured by [^{3}H]phenylalanine incorporation into trichloroacetate-precipitable cellular material. Cell-free extracts (10 000 $\times$ g supernatants) prepared from aliquots of the cultures removed at various intervals during the chase were subjected to the differential centrifugation procedure described previously [29]. Chromatophore and crude small membrane fragments (peripheral cytoplasmic membrane plus pigmented material) [29] were isolated by rate-zone sedimentation procedures. A 5—35% (wt./wt.) sucrose gradient prepared over a 0.5 ml cushion of 60% (wt./wt.) sucrose was used for the isolation of chromatophore and cell envelope fractions, whereas the reaggregated membrane fragments were isolated on a 5—60% (wt./wt.) sucrose gradient. The gradients were prepared in 1.0 mM Tris-HCl buffer (pH 7.5) and centrifuged in a Beckman SW50.1 rotor at 40 000 rev./min (150 000 $\times$ g) for 90 and 105 min, respectively. Individual points were obtained by summing the radioactivity of the most highly labeled fractions in the appropriate bands. The specific radioactivities ($^3H/^{14}C$) were corrected as described in the text. All cultures were incubated at 30°C.

Fig. 2. Pulse-chase activity of non-pigmented and pigmented fractions. (A) Bacteriochlorophyll-depleted cytoplasmic membrane. A 3-l phototrophic culture (A_{680} = 1.9) was pulsed with 0.5 mCi of [^{3}H]phenylalanine 5 min and chased 4 h with excess unlabeled phenylalanine. Cytoplasmic membrane and chromatophores were purified as described [25]. Specific radioactivities ($^3H/^{14}C$) are expressed in arbitrary units. (B) Upper pigmented band. A 500-ml culture (A_{680} = 1.0) was pulsed with 80 μCi [^{3}H]phenylalanine for 4 min and chased as above. The isolation of the upper pigmented band is described in the legend of Fig. 3.

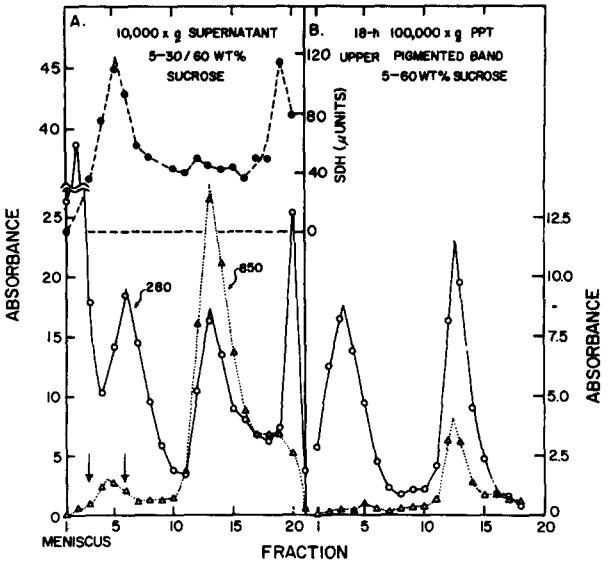

Fig. 3. Isolation of pigmented membrane fragments. (A) Crude extracts from phototrophically grown cells were placed directly on the indicated sucrose gradient prepared in 1.0 mM Tris-HCl buffer (pH 7.5) and centrifuged in a Beckman SW 27 rotor at 27 000 rev./min (96 000 X *g*) for 230 min. The absorbance of diluted fractions was determined at 280 (○) and 850 nm (△) and succinate dehydrogenase (SDH, ●) activity was assayed as described previously [20]. One unit of SDH activity catalyzed the reduction of 1.0 μmol of 2,6-dichlorophenol indophenol/min. The arrows denote that portion of the upper pigmented band that was pooled and sedimented at 100 000 X *g* for 18 h. (B) The pellet was resuspended in 1.0 mM Tris-HCl buffer (pH 7.5), layered on the indicated sucrose gradient, and centrifuged at 40 000 rev./min (200 000 X *g*) for 105 min in a Beckman SW 40Ti rotor.

plexes into multiple polypeptide components, consistent with results reported for *Rhodopseudomonas capsulata* [34,35]. Differences in their relative amounts between chromatophores and upper pigmented band are evident. After fluororadiography, apparent reaction center and light-harvesting components comprised the major radioactive polypeptides in pulse-labeled upper pigmented band (Fig. 4B). The H subunit of the reaction center has not yet become labeled detectably in pulsed chromatophores; most of the small amount of radioactivity present was observed in light-harvesting protein components, the other reaction center subunits, and two higher molecular weight bands. With regard to the latter components, the band of lower molecular weight was labeled only transiently; a small concentration of this component was suggested by the low amount of stain at this position in the chromatophore gel (Fig. 4A). After coelectrophoresis of [^{3}H]phenylalanine-pulsed upper pigmented band with [^{14}C]phenylalanine steady-state-labeled chromatophores, all major pulse-labeled polypeptide bands of the former have comigrated with proteins associated with the bacteriochlorophyll complexes of the chromatophores (Fig. 4C).

Pulsed and chased fractions were also electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. The labeling kinetics of the chromatophore polypeptides (Fig. 5, upper panel) confirmed the specific radioactivity profile

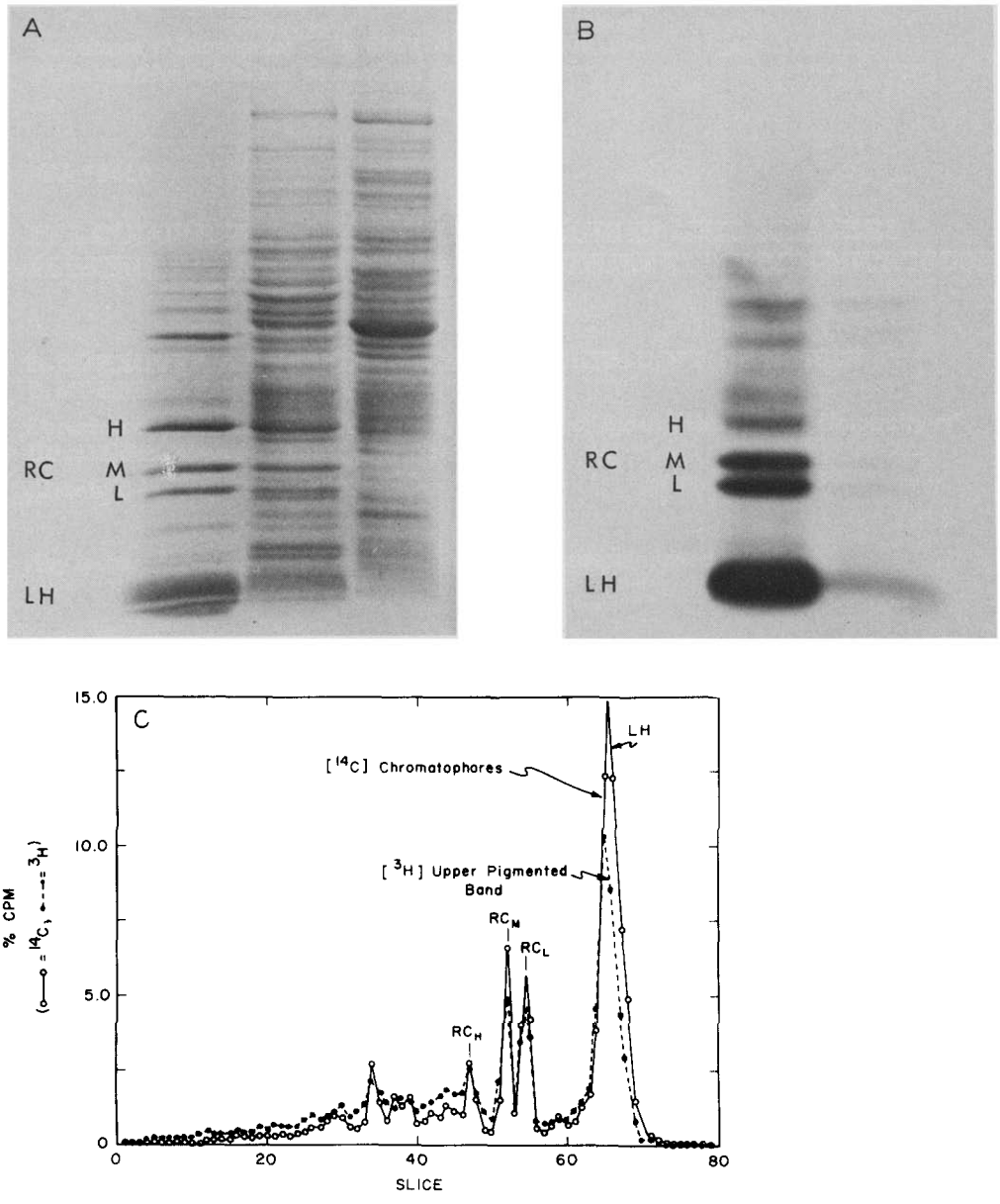

Fig. 4. Electrophoresis of membrane fractions on sodium dodecyl sulfate-polyacrylamide gradient gel slabs. (A) Electrophoresis of (from left to right) chromatophores, upper pigmented band and phototrophic cytoplasmic membrane [25] on a 10—14% polyacrylamide gradient. The gel was stained with Coomassie brilliant blue. The chromatophore and upper pigmented fractions were purified from [^{3}H]-phenylalanine-pulsed cells. RC$_{H,M,}$ and $_{L}$ refer to the three reaction center polypeptide subunits of M_r 28 000, 24 000 and 21 000, respectively [21,22]; LH, apparent light-harvesting bacteriochlorophyll associated polypeptides ($M_r \approx$ 8000—14 000) [23,24,34,35]. (B) Fluororadiographic analysis [36] of the gel slab; upper pigmented band (left), chromatophores (right). (C) Coelectrophoresis of [^{3}H]phenylalanine-pulse-labeled upper pigmented band and [^{14}C]phenylalanine-steady-state labeled chromatophores on an 8—12% polyacrylamide gradient. The mobility of the putative light-harvesting protein precursor is slightly retarded and is thought to be comprised primarily of the higher molecular weight B-875-associated component; this is consistent with near-infrared absorption spectra in which a two-fold enrichment in the B-875 component was observed in the upper pigmented fraction [37].

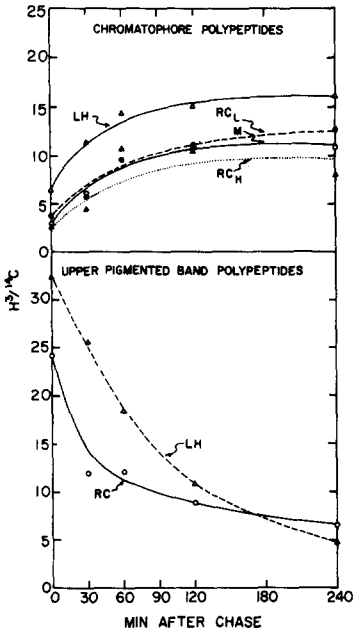

Fig. 5. Specific radioactivities of polypeptides from chromatophores and upper pigmented band from Fig. 2B separated by electrophoresis on 12% gel. The points for reaction center components of the upper pigmented fraction were obtained by summing radioactivity of all three bands. Specific radioactivities are expressed in arbitrary units.

observed in the isolated fraction (Fig. 2); however, the elevated $^{3}H/^{14}C$ ratio (specific radioactivity) for the light-harvesting bacteriochlorophyll-associated polypeptides indicated that they were inserted more rapidly than the reaction center polypeptides. The low initial $^{3}H/^{14}C$ ratio of the H subunit of the reaction center was consistent with the fluorographic result which also suggested that little radioactivity was pulsed into this polypeptide (Fig. 4B). The decay in the specific radioactivity of the apparent polypeptide components of the bacteriochlorophyll-protein complexes within the upper pigmented band (Fig. 5, lower panel) confirmed the pulse-chase behavior observed in the overall fraction; these combined polypeptides rather than those of the cytoplasmic membrane accounted for the majority of the radioactivity chased into chromatophores (cf. Fig. 2A). The initial $^{3}H/^{14}C$ ratio of these polypeptides was much higher than that ultimately reached by their chromatophore membrane counterparts. This suggests a small pool of these components within the upper pigmented band relative to that in chromatophores. The presence of cytoplasmic membrane protein within the upper pigmented band has lowered the overall $^{3}H/^{14}C$ ratio in the total fraction.

Discussion

Further evidence that the rapidly labeled polypeptide components demonstrated here are the polypeptide subunits of the light-harvesting and reaction

center complexes is provided from a recent study of the photochemical activity of the upper pigmented band [37]. Significant levels of active light-harvesting and reaction center complexes were demonstrated in this fraction. It was enriched in the reaction center-associated B-875 complex which predominates early in chromatophore membrane development [38]. Fluorescence yield studies have indicated efficient energy transfer between the B-875 antenna component and the reaction centers present within the isolated upper pigmented band [39]; however, a less efficient coupling between the B-850 and B-875 complexes was observed which is consistent with energy transfer properties during the initial stages of development of the chromatophore membrane [40]. These results suggest that the structural organization essential to energy transfer and primary photochemical events has already become established within newly synthesized bacteriochlorophyll-protein complexes from those membrane regions that give rise to the upper pigmented band.

With regard to the cellular localization of the rapidly labeled fraction, it is possible that this material has arisen from membrane invaginations observed in electron micrographs [41] that are thought to be the sites at which new chromatophore membrane growth is initiated [42]. This interpretation is supported by recent cell fractionation studies in this laboratory which suggest that the upper pigmented fraction originates from the peripheral areas of spheroplast membranes rather than the internal membranes. The presence of polypeptide components characteristic of the peripheral cytoplasmic membrane within the overall upper pigmented fraction also supports such a cellular localization; however, radioactivity from relatively unpigmented regions of the cytoplasmic membrane was not chased into chromatophores which is also consistent with the possibility that the rapidly labeled material represents new invagination sites at which pigment-protein complexes are inserted.

The proposed cellular localization for the rapidly labeled fraction also provides an explanation for the unique pulse-chase relation observed in this study. The small pool of the apparent pigment-protein complexes within the upper pigmented band relative to that of the chromatophore membrane would be expected of a region at which new membrane growth is initiated. Since the specific radioactivity of the chromatophore polypeptides increases throughout the chase while that of the upper pigmented band decreases, it is suggested that the membrane regions from which the latter material is derived are converted continuously into the chromatophore membrane. In the chromatophore fraction, however, the specific radioactivity of the individual polypeptides at the start of the chase is not zero as predicted by an ideal pulse-chase relation but is instead approximately one-third that at the end. Thus, some of the newly synthesized material may also be inserted directly into the growing intracytoplasmic membrane. This suggests that the chromatophore membrane does not arise exclusively from new invagination sites, but as shown by density shift analyses new proteins are added at random over all regions [19,43]. A preliminary estimate of the distribution of pulse label, however, suggests that material within the upper pigmented band may represent preferred sites for the insertion of newly synthesized polypeptides destined eventually for the chromatophore membrane. Thus, the observed pulse-chase relation is interpreted to reflect the mode of membrane growth rather than a strict precursor-

product mechanism. This interpretation is also based upon the results of a freeze-etch study in which invagination sites were visualized in *Rhodospirillum rubrum* [44]. By quantitative assessment of such sites, it was concluded that the overall chromatophore membrane content of the cell is increased through both the elongation of existing invaginations and an increase in their number. A recent freeze-fracture examination of *Rp. sphaeroides* has also revealed such sites and they were thought to represent areas at which intracellular vesicules are forming or have already formed through cytoplasmic membrane invagination [45].

This explanation of the pulse-chase observations suggests that the French-press disrupts the overall cytoplasmic membrane system of phototrophically grown cells into at least three rather distinct membrane types: chromatophores which arise from the pigmented intracytoplasmic membrane; the relatively unpigmented portions of the cytoplasmic membrane thought to be localized at peripheral regions that have been isolated recently as small fragments [25]; and pigmented material that accounts for the rapidly labeled portion of the upper fraction isolated here. It is this third membrane type that may represent specific sites of new membrane invagination.

Acknowledgements

This work was supported by National Science Foundation grants PCM76-24142 and PCM74-18151 and Public Health Service grant GM20367-03 from the National Institute of General Medical Sciences. Institutional support was provided from Public Health Service Biomedical Research Support grant RR7058-12 and the Rutgers Research Council. R.A.N. is the recipient of Public Health Service Research Career Development Award GM00093-04 from the National Institute of General Medical Sciences. We thank Kenneth D. Gibson, Richard M. Broglie, and C. Neil Hunter for useful discussions and R.M. Broglie for assistance with fluororadiography.

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