National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canadä

SYNTHESIS AND USE OF A MEMBRANE-IMPERMEABLE GUANIDINATING REAGENT, 2-S-[^{14}C]THIURONIUM ETHANE SULFONATE FOR THE LABELING OF LIGHT-HARVESTING PROTEINS IN *RHODOBACTER SPHAEROIDES*

by

BHUPINDER S. HUNDLE

B.Sc. (Hons.), NORTH EAST LONDON POLYTECHNIC, LONDON,

U.K., 1980

M.Sc., LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY, LOUGHBOROUGH,

U.K., 1981

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

CHEMISTRY

© BHUPINDER S. HUNDLE 1988

SIMON FRASER UNIVERSITY

Nov 1988

All rights reserved. This work may not be
reproduced in whole or in part, by photocopy
or other means, without permission of the author.

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-48755-0

APPROVAL

Name: BHUPINDER S. HUNDLE

Degree: DOCTOR OF PHILOSOPHY

Title of thesis: **SYNTHESIS AND USE OF A MEMBRANE-IMPERMEABLE GUANIDINATING REAGENT, 2-S-[^{14}C]THIURONIUM ETHANE SULFONATE FOR THE LABELING OF LIGHT-HARVESTING PROTEINS IN *RHODOBACTER SPHAEROIDES***

Examining Committee:

Chairman: Dr. P. W. Percival

Dr. W. R. Richards
Senior Supervisor

Dr. B. L. Funt

Dr. R. Cornell

Dr. A. H. Burr
Department of Biological Sciences

Dr. G. Gingras
External Examiner
Department of Biochemistry
University of Montreal

Date Approved: nov 4, 1988

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/~~Project~~/~~Extended Essay~~

SYNTHESIS AND USE OF A MEMBRANE-IMPERMEABLE GUANIDINATING REAGENT, 2-S-[^{14}C]THIURONIUM ETHANE SULFONATE FOR THE LABELING OF LIGHT-HARVESTING PROTEINS IN *RHODOBACTER SPHAEROIDES*

Author:

(signature)

BHUPINDER SINGH HUNDLE

(name)

15th Dec, 1988

(date)

ABSTRACT

A new radioactively labeled guanidinating reagent has been synthesized by a single-step reaction using [^{14}C]thiourea and 2-bromoethane sulfonate. The reagent, 2-S-[^{14}C]thiuronium ethane sulfonate, reacts with membrane proteins under relatively mild labeling conditions, converting the accessible ϵ-amino groups of lysine residues to labeled homoarginine residues. The resulting homoarginine residues act as new cleavage sites for trypsin, but at a rather slow rate of hyrolysis. Model studies have shown that the reagent also reacts with the N-terminal amino group and any cysteine sulfhydryl groups, but not with tyrosine, histidine, or serine residues. The cysteine residues are converted to thiuronium ions, thereby labilizing the adjacent peptide bond towards acid cleavage. Hence, the reagent may also be used as a cysteine-specific protein cleavage reagent. The reagent has been shown to be impermeable to the intracytoplasmic membranes of *Rhodobacter sphaeroides*: when cytoplasmic-side-out chromatophores were treated with the reagent, it reacted with all four of the light-harvesting proteins, all of which have one or more lysine residue(s) on the N-terminal sides of their hydrophobic regions. However, when periplasmic-side-out vesicles, purified by cytochrome c affinity chromatography, were treated with the guanidinating reagent, three of the light-harvesting proteins (B850α, B850β, and B870β) were not

labeled. The only light-harvesting protein to be labeled (B870α) is the only one of the four to have a lysine residue on the C-terminal side of its hydrophobic region. The labeled B870α polypeptides from the treatment of both the cytoplasmic-side-out chromatophores and the periplasmic-side-out membrane vesicles have been purified, digested with trypsin, and the resulting peptide fragments have been analyzed for radioactivity. The results have confirmed the asymmetric orientation of the light-harvesting proteins of *R. sphaeroides*, with their N-terminal residues only on the cytoplasmic side and C-terminal residues only on the periplasmic side of the intracytoplasmic membrane. They have also demonstrated the effectiveness of the labeling reagent for future membrane-protein orientation studies.

ACKNOWLEDGEMENTS

I would like to extend my gratitude to Dr. W. R. Richards for his valuable guidance, constant encouragement, patience and financial support throughout this research project. I also thank Dr. B. L. Funt, Dr. M. J. Gresser and Dr. Rosemary Cornell for their editorial comments and advice.

I wish to thank Mr. M. Yang for introducing me to various lab techniques, Dr. Gurmukh Johal for computer aid and for offering his endless tid bits of biochemical knowledge. The typing and proofreading skills of Shinder Purewal and Sarbjit Hundle greatly helped in putting this manuscript together.

For their emotional and economic support, many thanks to R. S. Mahil and Hundle families. Faith, help and encouragement of Kartar S. Sanghera and Narinder Sandhu made a dream come true. Finally, I am greatly indebted to my wife Amarjit and daughters Kiranjit and Munpreet for their patience and endless support through the years.

DEDICATION

To my beloved Grandmother

"Bibi Jiwi"

and

My parents

TABLE OF CONTENTS

Approval ii

Abstract iii

Acknowledgements v

Dedication vi

List of Tables x

List of Figures xi

Abbreviations xiii

1. INTRODUCTION 1

1.1 Membrane-Protein Labeling Reagents 1

1.2 Labeling the Intracytoplasmic Membranes of *Rhodobacter sphaeroides* 6

1.2.1 Introduction 6

1.2.2 Reaction Center Polypeptides 9

1.2.3 Transverse Topography of Reaction Center Polypeptides 10

1.2.4 Light-Harvesting Polypeptides 12

1.2.5 Transverse Topography of Light-Harvesting Polypeptides 16

2. MATERIALS AND METHODS 23

2.1 Materials 23

2.2 Methods 24

2.2.1 Synthesis of N-Acetyl-S-(2-sulfoethyl)-L-cysteine 24

2.2.2 Synthesis of N,N'-Diacetyl-L-cystine 25

2.2.3 Synthesis of N-Amidinoglycine 25

2.2.4 Synthesis of N^{α}-t-BOC-L-homoarginine 26

2.2.5 Synthesis of the Guanidinating Reagent ... 26

2.2.6 Reaction of the Guanidinating Reagent with Poly(L-lysine) 30

2.2.7 Reaction of the Guanidinating Reagent with Derivatives of Amino Acids 31

2.2.8 Reaction of the Guanidinating Reagent with the α-amino Group of Glycylglycine and Glycine 33

2.2.9 Homoarginine as a Potential Cleavage Site of Trypsin 37

2.2.10 Reaction of the Guanidinating Reagent with Whole Cells of R. sphaeroides 38

2.2.11 Reaction of the Guanidinating Reagent with Purified Membrane Fractions of R. sphaeroides 40

2.2.12 Preparation of Spheroplast Derived Vesicles (SDV) 41

2.2.13 Purification of SDV using cytochrome c-linked Sepharose 4B 43

2.2.14 Labeling of Chromatophores and SDV 44

2.2.15 Purification of Light-Harvesting Proteins 45

2.2.16 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis 50

2.2.17 High Performance Liquid Chromatography of Tryptic Fragments of B870α-polypeptides Purified from Radiolabeled Chromatophores and SDV 51

3. RESULTS 54

3.0.1 Reaction of the Guanidinating Reagent with Poly(L-lysine) 54

3.0.2 Reaction of the Guanidinating Reagent with amino acid Functional Groups. 54

3.0.3 Reaction of the Guanidinating Reagent with an α-amino Group. 62

3.0.4 Homoarginine as a Potential Cleavage Site for Trypsin 62

3.0.5 Reaction of the Guanidinating Reagent with Membranes of R. sphaeroides 65

3.0.6 Purification of Spheroplast Derived Vesicles using Affinity Chromatography ... 70

3.0.7 The Near-Infrared Spectrum of Membranes .. 70

3.0.8 SDS-PAGE of Membranes Demonstrating Impermiability to the Guanidinating Reagent 72

3.0.9 SDS-PAGE of LIght-Harvesting Proteins purified from Labeled Chromatophores 74

3.0.10 SDS-PAGE of Light-Harvesting Proteins Purified from Labeled SDV 77

3.0.11 High Performance Liquid Chromatograhpic Results of the Trypsin Digest of B870α from Labeled Chromatophores and SDV 77

4. Discussion 81

REFERENCES 102

LIST OF TABLES

Table		Page
1	Thin-Layer Chromatography of the Products of the Guanidination of N-t-BOC-lysine with 2-S-Thiuronium Ethane Sulfonate	57
2	Thin-Layer Chromatography of the Products of the Guanidination of N-Acetyl-L-cysteine with 2-S-Thiuronium Ethane Sulfonate	57
3	Thin-Layer Chromatography of the Products of the Guanidination of N-amidinoglycylglycine with 2-S-Thiuronium Ethane Sulfonate	64
4	Thin-Layer Chromatography of the Trypsin hydrolysis products of 2-S-Thiuronium Ethane Sulfonate Guanidinated Pentapeptide	67
5	Specific Activities of Purified Light-Harvesting Proteins from Labeled Chromatophores and SDV.	74
6	Radioactivity of Trypsin Fragments of B870α purified from Labeled chromatophores and SDV	79

LIST OF FIGURES

Figure		Page
1	Primary structure of the light-harvesting polypeptides from *Rhodobacter sphaeroides*	15
2	Hydropathy plots of Bchl-binding polypeptides of the light-harvesting Complexes from *R. capsulatus*	18
3	^{13}C NMR spectra of 2-S-thiuronium ethane sulfonate	28
4	^{1}H NMR spectra of 2-S-thiuronium ethane sulfonate	29
5	^{13}C NMR Spectrum of guanidinated N-acetyl-L-cysteine	34
6	^{13}C NMR Spectrum of N-acetyl-L-cysteine	35
7	^{1}H NMR Spectrum of guanidinated N-acetyl-L-cysteine	36
8	Fractionation of organic solvent extract of SDV on preprative LH-60 column.	46
9	Separation of light-harvesting polypeptides by DEAE-cellulose column	47
10	Concentration of peak I from DEAE-celllose column chromatography on a CM-cellulose column	48
11	Separation of B870α and B850β, using Sephadex LH 60 high resolution column	49
12	Products of acid hydrolysis separated using Beckman Model-116 amino acid analyzer with a PA 28 ion exchange column.	55
13	Elution profile of N-amidinoglycylglycine using a Sephadex G-10 column.	63
14	Elution profile of guanidinated pentapeptide **Arg-Lys-Asp-Val-Tyr** on a Sephadex G-10 column.	66
15	Effect of varying incubation conditions on labeling of chromatophores	69
16	Separation of "cytoplasmic-side-out" and "periplasmic-side-out" vesicles from a prepration of SDV using a cytochrome c column.	71

17 Near-infrared spectrum of purified chromatophores and vesicles. 73

18 SDS-PAGE results of Chromatophores and SDV. 75

19 SDS-PAGE results of light harvesting proteins purified from radiolabeled chromatophores. 76

20 SDS-PAGE results of light harvesting proteins purified from radiolabeled SDV. 78

21 HPLC of tryptic digests of 870α protein purified from radiolabeled chromatophores and SDV. 80

22 Schematic diagram showing cleavage of coupled guanidinated cytochrom c to inert solid support with trypsin 92

23 Sites of Action of Trypsin on B870α. 99

ABBREVIATIONS

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
ICM	intracytoplasmic membrane
RC	reaction center complex
H, M, & L	heavy, medium, & light subunits of the reaction center complex
LH	light-harvesting complex
Bchl	bacteriochlorophyll
SDV	spheroplast derived vesicles
TLC	thin layer chromatography
t-BOC	*tert*-butoxycarbonyl
BOC-ON	N-(*tert*-butoxycarbonyl)oximino-2-phenyl-acetonitrile
EDTA	ethylenediaminetetraacetic acid
DNAase	deoxyribonuclease
RNAase	ribonuclease
C/M/NH_4OAc	chloroform/methanol (1:1, v/v) containing 0.1 M ammonium acetate.
2-S-TES	2-S-thiuronium ethane sulfonate
HPLC	high performance liquid chromatography
PMSF	phenylmethylsulfonyl fluoride

CHAPTER 1

INTRODUCTION

1.1 Membrane-Protein Labeling Reagents

The use of covalent labeling procedures, which are specific to various amino acid side chains of proteins which are exposed on the external cell surface, has yielded valuable information on the arrangement of proteins within cells. The covalent bond enables the protein to remain labelled during subsequent analysis under strong dissociating conditions such as SDS-PAGE.

The usual experimental approach in a surface labeling study is to react intact cells or sealed membrane vesicles (with homogenous single-sided orientation) with a radioactive labeling reagent and remove excess reagent by washing. The membrane is then dissolved in SDS, and proteins are separated using SDS-PAGE. Gels are then analysed for radioactivity (by sectioning and counting, or by autoradiography) and stained for protein. The protein bands coinciding with the labeled peaks are then (presumably) the only proteins which have accessible reactive amino acid side chains exposed at the outer surface of the membrane prepration under study.

In the case of intact cells, membrane proteins which react with the label are, by definition, exposed at least partly on

the "outside" surface of the cell membrane, while proteins which fail to react are exposed only on the inside surface or are buried within the membrane. It is quite possible, however, that certain proteins which are on the outer surface fail to react with the label because of their intrinsic properties. Therefore, only positive results can be interpreted correctly.

There are several ways by which proteins can be "tagged" by radioactive surface labels. Proteins can be labeled either enzymatically or non-enzymatically. Radioiodination of tyrosine or histidine residues of the membrane proteins of red blood cells is one such example of enzymatic modification. Phillips and Morrison (85) introduced the technique of iodinating cell surfaces with lactoperoxidase. Two iodine radionucleotides, ^{125}I and ^{131}I, are available (at low cost) and thus allow high-specific-activity labeling. Hydrogen peroxide is required when lactoperoxidase is used. Its concentration must be kept minimal since peroxides may cause cellular damage. The major disadvantage of enzyme-mediated labeling derives from the large size of the catalyst; only those reactive tyrosine and histidine residues accessible to a 30-40 nm diameter enzyme will be labeled (43). Thus the extent of labeling will depend upon the relative exposure of reactive residues on the external surface, with less-accessible or sterically hindered residues being missed although still located at the suface. However, the large size of the enzyme

has one advantage: it is less likely to be permeable to the membrane and thus only labeling the external surface of the membrane.

The most common non-enzymatic modification occurs with unprotonated amino groups and many of the resulting derivatives constitute the most stable covalent bonds. Side reaction with sulfhydryl, imidazole or tyrosine phenol residues may also accur, but the bonds thus formed are less stable and may hydrolyze even at pH 7.4 (68). The list of amino acid modifying reagents is almost endless; Hubbard and Cohn (43) have reviewed it in great detail and many more have been developed since 1976. Only reagents used to label lysine side chains will be emphasized in the following discussion.

Guanidination was used by Greenstein in 1935 to modify lysine (as a free amino acid) to homoarginine by the use of O-methylisourea (37). O-Methylisourea, when used to label human serum albumin, gave a crystallizable derivative of greatly reduced solubility (44). Over the ensuing 50 years, the guanidination of a large number of proteins has been accomplished with this reagent almost exclusively. S-Methylisothiourea has also been used as a guanidinating reagent (95), but it is much less reactive than O-methylisourea (44). Although O-methylisourea is fairly specific, and reacts with the ϵ-amino groups of lysyl residues (17), it is rather unstable in aqueous systems. Other

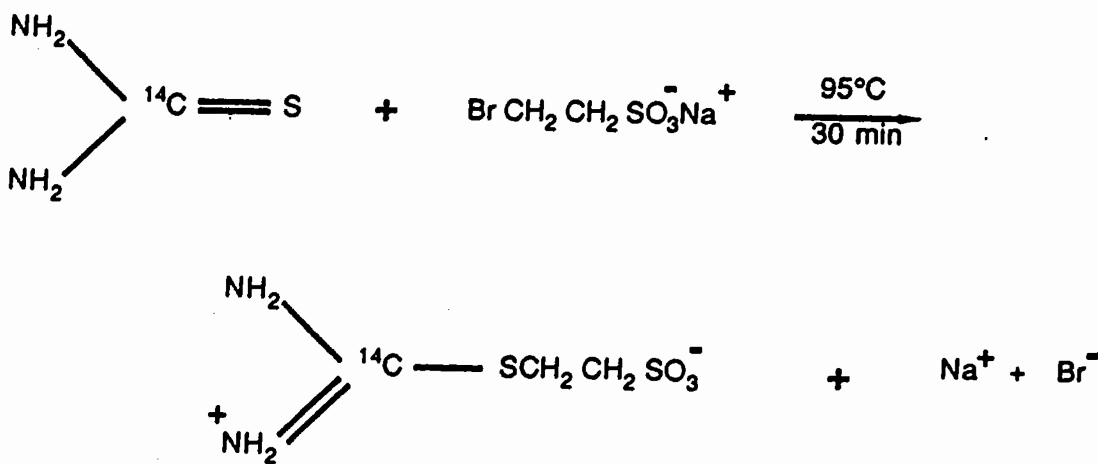
disadvantages are that it requires longer incubation times (4-6 days at 0-4°C) and a high pH of 10-11 (55,68).

Recently, O-methyl-[^{13}C]isourea has been prepared and used to [^{13}C]guanidinate cytochrome c from various sources. The extent of reaction was monitored using ^{13}C-NMR (53,100). Amidination using N-ethyl acetamidate (128) has also been used to modify lysine residues of proteins to ϵ-acetamido-derivatives. Although the reaction requires much less time than O-methylisourea and may be carried out at a lower pH of 8-9, the product is partially destroyed during acid hydrolysis. Furthermore, the reagent is permeant to membranes and, therefore, has limited use as a surface labeling reagent.

Whitely and Berg synthesized both ^{3}H- and ^{14}C-labeled isethionyl acetamidate and N-ethyl acetamidate imidoesters and used them to study the localization of membrane proteins in human erythrocyte membranes (124). While N-ethyl acetamidate labels both surfaces of red blood cell membrane, isethionyl acetamidate, being a zwitterion, is impermeable to membranes and only labels the outer surface.

A variety of other lysine-specific labeling reagents have been employed for the modification of soluble proteins (65), or for the localization of membrane proteins (43). In an earlier report from our laboratory, pyridoxal phosphate plus

KB^3H_4 was used to study the localization of membrane proteins in the intracytoplasmic membrane of *Rhodobacter sphaeroides* (32). We have now synthesized an effective lysine guanidinating reagent,

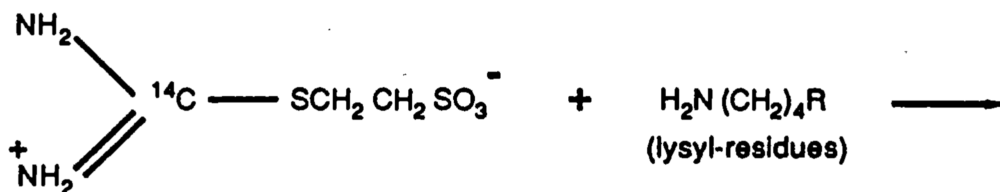


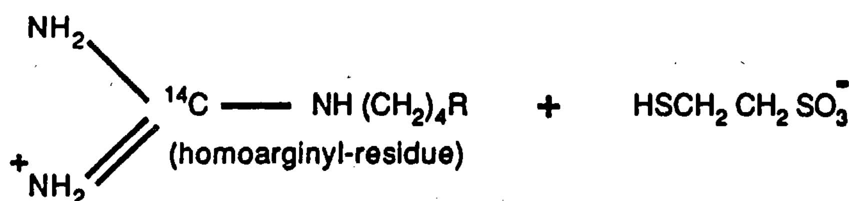
2-S-[^{14}C]thiuronium ethane sulfonate, which is synthesized in a single step reaction (cf. Equation 1) from which the product is easily crystallized. The reagent combines the advantage of O-methylisourea, by yielding the more stable product, homoarginine (cf. Equation 2), with those of N-ethyl acetamidate, by reacting in much shorter time (1-2h) and at milder conditions (pH 9.6 and temp. 30-37°C) (cf. Equation 2).

It is also charged like isethionyl acetamidate and is thus impermeable to biomembranes and can be used for membrane protein localization studies.

In addition to reacting with lysyl residues, the reagent also reacts with cysteinyl residues,

Equation #2





converting them to thiouronium derivatives rather than leading to alkylation as with O-methylisourea (45).

1.2 Labeling the Intracytoplasmic Membranes of *Rhodobacter sphaeroides*.

1.2.1 Introduction

The non-sulfur purple photosynthetic bacterium *R. sphaeroides* provides a model system to study photosynthesis. The gram negative bacterium can be grown both phototrophically and chemoheterotrophically. In the presence of oxygen, growth is by aerobic respiration. When oxygen is removed, a series of events is triggered which, through a process of invagination,

results in the differentiation of the cytoplasmic membrane into a specialized domain, constituting the photosynthetic intracytoplasmic membrane system (ICM). The inducible ICM is physically continuous with (39,78), but structurally and functionally distinct from the cytoplasmic membrane. This membrane system houses all of the components of the photosynthetic apparatus (79). Although its continuity with the cell membrane has been questioned (42,58), a consensus remains in favour of a continuous model. The invaginations formed under low light intensities can be separated from the cytoplasmic membrane by mechanical breakage of the cells with a high pressure cell such as a French press (79). The resulting vesicles (termed "chromatophores") have an "inside-out" (i.e. cytoplasmic-side-out) orientation. Vesicles of an orientation opposite to that of chromatophores (110) (i.e. "right-side-out" or periplasmic-side-out orientation) are prepared by lysis of spheroplasts obtained from bacteria grown under high light intensity (39). The ability to obtain vesicles of either orientation make these bacteria ideal models for a variety of biochemical localization techniques, such as enzyme digesion or labeling studies.

Photosynthesis in *R. sphaeroides* begins with the absorption of light energy (photons) by Bchl and carotenoid pigment molecules bound to polypeptides within the antenna or light-harvesting complex (116). The effect is the creation of

mobile electronic singlet states, called "excitons" (57). The photosynthetic apparatus is organized such that these excitons are channeled down to the reaction center complex. The process of the chemical trapping of the light energy is a result of the rapid formation of separated charged radicals. A special Bchl pair donates an electron to a bacteriopheophytin molecule (passing a voyeur Bchl molecule in the process) and leaving a positive charge on the special pair. From the bacteriopheophytin, the electron transfers to a quinone (Q_A) molecule (76). From Q_A the electron passes to the second quinone molecule (Q_B) at the end of another chain of prosthetic molecules (a chain along which electrons are not conducted). The charge on the special pair is neutralised by electron donation from a cytochrome c_2 molecule. The entire process of electron flow is repeated once again, resulting in Q_B acquiring another electron and the Bchl pair accepting another electron from a second cytochrome c_2 molecule. Thus, a positive charge is created on the periplasmic side and a negative charge on the cytoplasmic side. This charge separation represents stored energy which may be used to produce ATP, or to supply the energy for the reduction of NAD' by succinate.

The antenna and reaction center complexes are multimolecular systems present within the photosythetic membrane; together they constitute a photosynthetic unit. The

pigments are maintained in their correct position, orientation and separation within the membrane by specific binding by the polypeptides. The complexes can thus attain the specific environments which determine their particular spectral and photochemical properties.

1.2.2 Reaction Center Polypeptides

The reaction center contains three polypeptides referred to as the H (heavy), M (medium), and L (light) subunits, having apparent M_r's of 28,000, 24,000 and 21,000 daltons (Da) respectively, as determined by SDS-PAGE (81). The N-terminal sequence of reaction center subunits M and L of *Rhodospirillum rubrum* (111), was determined by protein sequencing. The complete amino acid sequence of M and L subunits of *R. rubrum* (7) and *R. sphaeroides* (125,126,127) and of H, M and L subunits of *Rhodobacter capsulatus* (129) and *Rhodopseudomonas viridis* (71,72) have been determined from their DNA sequences. From the derived amino acid sequence, it was concluded that the H subunit is a fairly hydrophilic protein with a molecular weight of 28,534 Da. The L and M subunits are both very hydrophobic proteins with calculated molecular weights of 31,565 and 34,440 Da. The anomalous SDS-PAGE electrophoretic mobility behavior no doubt stems from their hydrophobic characteristics.

Only the L and M subunits are essential in *R. sphaeroides* for reaction center activity; the H subunit binds no pigments and is not required for electron transfer (30); however, it greatly accelerates electron transfer from Q_A to Q_B (24,25). The H subunit might serve as the structural anchor protein maintaining the proper association of L and M subunits within the membrane (18). The M and L subunits together bind 4 molecules of Bchl, 2 molecules of bacteriopheophytin, 1 molecule of carotenoid, 2 molecules of ubiquinone and 1 atom of iron.

1.2.3 Transverse Topography of Reaction Center Polypeptides

Topographic studies on the RC polypeptides have been done by hydrophilic and hydrophobic photoaffinity reagents, as well as digestion of proteins exposed on membrane surfaces by specific and non-specific proteases. Ferritin-coupled antibodies prepared against the purified reaction center complex of *R. sphaeroides* were used to show the exposure of reaction centers in EDTA-washed chromatophores (88). The presence of reaction centers on the chromatophore surface has also been confirmed using antiserum precipitation (99) and absorption studies (22). Antibodies prepared against the purified H subunit of *R. sphaeroides R-26* complexed with chromatophores (119), while antibodies to the L-M complex reacted on both sides of the membrane (31). Antibodies raised

against purified H, M and L polypeptides demonstrated the presence of H and M subunits on both surfaces, while antibodies to L labelled only the periplasmic surface of the membrane (51,118).

Lactoperoxidase-catalysed ^{131}I (96) and ^{125}I (133,80) iodination and KB^3H_4/pyridoxal phosphate labeling studies (32) showed the exposure of the H subunit on the cytoplasmic side of the membrane. Using ^{125}I-iodination (83) alone and in combination with enzymatic cleavage (3), H, M, and L polypeptides were shown to be exposed asymmetrically on both sides of the membrane. Trypsin and proteinase K digested the H subunit much faster than both the M and L subunits (38). Tadros *et al.* (106) used proteinase K to show that while only 26-28 N-terminal amino acid residues of the L and M peptides were removed from the chromatophore surface, the H subunit was completely digested. Hoger and Kaplan (41), using cross-linking reagents, showed that H, M and L are in close proximity to one another and the cross linking of cytochrome c_2 with L or M subunits indicated that they are both present on the periplasmic surface.

The polypeptide part embedded in the hydrophobic core of the membrane has been investigated. Fluorescamine and dicyclohexylcarbodiimide reacted specifically with subunit M of the reaction center (4,132). With iodonaphthyl-1-azide all three subunits of the reaction center were labeled (77).

3-Trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine and naphthyldiazotrifluoropropionate, two less specific reagents, only labeled the two more hydrophobic subunits of the reaction center, L and M (69).

The analysis of the sequence of the L and M subunits according to their hydropathy index suggests that both L and M span the membrane five times while H only has one membrane-spanning region (125). While the L and M subunits are both soluble in organic solvents (e.g: chloroform-methanol) the H subunit is not, due to its less-hydrophobic character. So from the above results, it can be concluded that all three subunits span the membrane and are exposed at both membrane surfaces, but that the majority of the H subunit is on the cytoplasmic surface. The X-ray crystal structure of the reaction center has been determined for both *R. viridis* (26) and *R. sphaeroides* (2) which has confirmed the above findings.

1.2.4 Light-Harvesting Polypeptides

The antenna or light-harvesting systems of various photosynthetic bacteria are composed of pigment-protein complexes with different absorption maxima (9,116), which is the basis for heterogenous energy transfer. On the basis of their near-infrared absorption maxima, purple photosynthetic bacteria are classified into three groups (116):

a) Group 1 (*e.g. R. rubrum,* and *R. viridis*) has one

complex: B880 (for Bchl a) or
B1015 (for Bchl b).

b) Group 2 (*e.g. R. sphaeroides and R. capsulatus*) has two complexes: B870 and B800-850.

c) Group 3 (*e.g. Chromatinum vinosum* and *Rhodobacter acidophila*) has three complexes: B870, B800-820 and B800-850.

The efficiency of the light-harvesting antenna varies according to the number of B800-850 complexes, as determined by environmental conditions (eg. light intensity). In low light intensity number of B800-850 complexes is higher, as opposed to high light intensity. By combining more than one type of complex, a specific energy transfer system is formed within the whole antenna
(eg. carotenoid ---> B800-850 ---> B870 ---> RC), in case of *R. sphaeroides*).

Each of these antenna complexes usually contains two different types of small polypeptides termed the α- and β-subunits. They are the smallest structural units of the antenna complexes and non-covalently bind either 1 Bchl molecule (α-subunits) or 1-2 Bchl molecules (β-subunits). The antenna proteins have been isolated as Bchl-protein complexes called holochromes (86,92) by the use of detergents or as pigment free proteins by the use of organic solvents (115) The primary structure of the α- and β-subunits of many of the

purple bacteria has been determined *eg*. *R. rubrum* (35), *R. rubrum G-9*⁺ (15,16), *R. rubrum* S1 (16) *R. viridis* (14), *R. capsulatus* (102,103), *R. sphaeroides R26.1* (115), *R. sphaeroides* (113,114) and *R. acidophila* (8). The genes for B880α and β polypeptides of *R. rubrum* S1 (6), *R. sphaeroides* B800-850α and β polypeptides (54), *R. capsulatus* B870α andβ (129), and B850α and β polypeptides (130) have also been cloned and their amino acid sequences deduced. The apparent molecular weights of these polypeptides as determined by SDS-PAGE are 8,000 to 12,000 Da, but were found to have much smaller molecular weights of 5,000 to 9,000 Da as determined by their amino acid sequences (27). They are all composed of 50-60 amino acid residues.

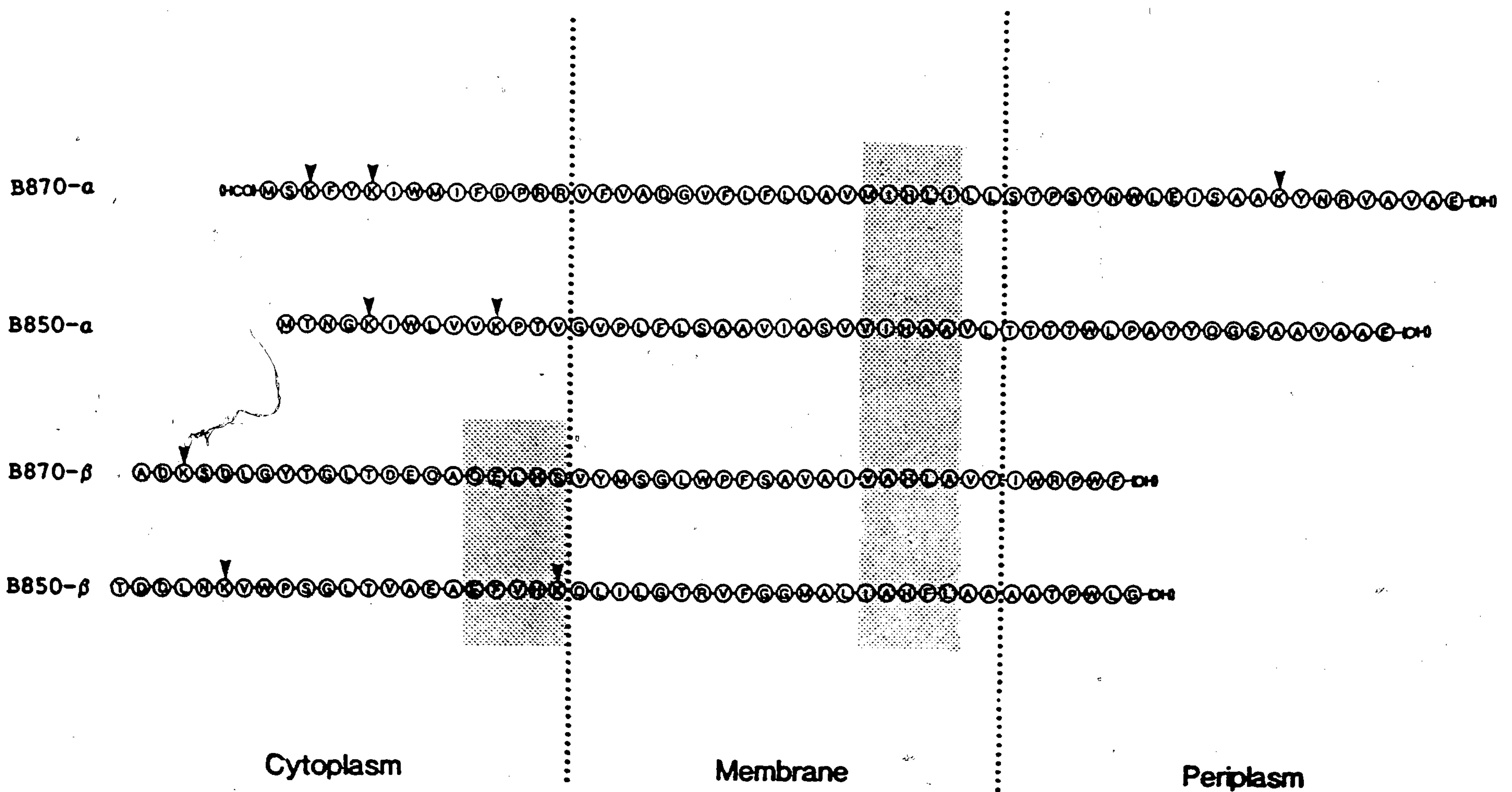
Both α- and β-subunits have a typical three domain structure in all of the proteins sequenced so far (cf. Figure 1).

a) A polar, charged N-terminal domain (α: 12-19 residues, β: 20-22 residues) on the cytoplasmic side of the membrane.

b) A hydrophobic, central domain (α: 21 residues, β: 23 residues)

c) A polar, charged C-terminal domain (α: 19-25 residues, β: 3-10 residues) on the periplasmic side of the membrane.

Figure 1: Primary structures of the light-harvesting polypeptides from *Rhodobacter sphaeroides*; Shaded areas represent potential Bchl-binding sites. The position of the lysine residues is highlighted by the arrows. The N-terminus of the B870α peptide is blocked, perhaps by a formyl group.


B870-α
B850-α
B870-β
B850-β
Cytoplasm
Membrane
Periplasm

The central hydrophobic domain of both the α- and β-subunits has a single conserved histidine residue which is believed to interact with Bchl as a fifth Mg ligand (131). The β-subunit has a second conserved histidine residue in the N-terminal domain which is also believed to interact with Bchl in *R. sphaeroides* and *R. capsulatus*, but not in *R. rubrum*. Other amino acids in the proteins may serve as hydrogen bond donors to Bchl or bind it via hydrophobic or aromatic π-π interactions, e.g. a conserved Phe residue three amino acid residues from the hydrophobic domain His.

Circular dichromism, polarized infrared spectroscopy and hydropathy plots of the amino acid sequences (cf. Figure 2) all indicate that the central hydrophobic stretch of amino acids of the light-harvesting polypeptides has an α helical region (27), and that it spans the membrane (assuming a thickness of the photosynthetic membrane of between 4-6 nm). This means that the N- and C-terminal domains should be found at the polar head region of the membrane surface.

1.2.5 Transverse Topography of Light-Harvesting Polypeptides

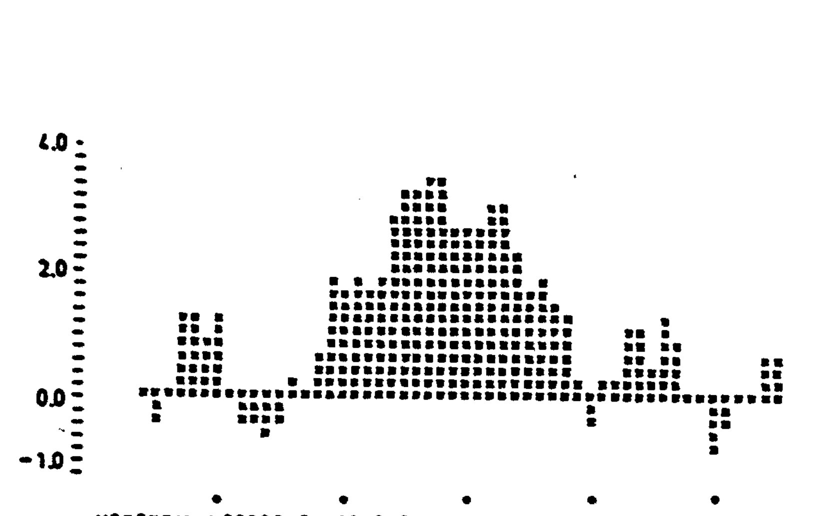
The transverse topography of light-harvesting proteins has been investigated by similar techniques to those used for reaction centers. Earlier studies, using radioiodination of chromatophores, indicated that a single 14,000 Da polypeptide

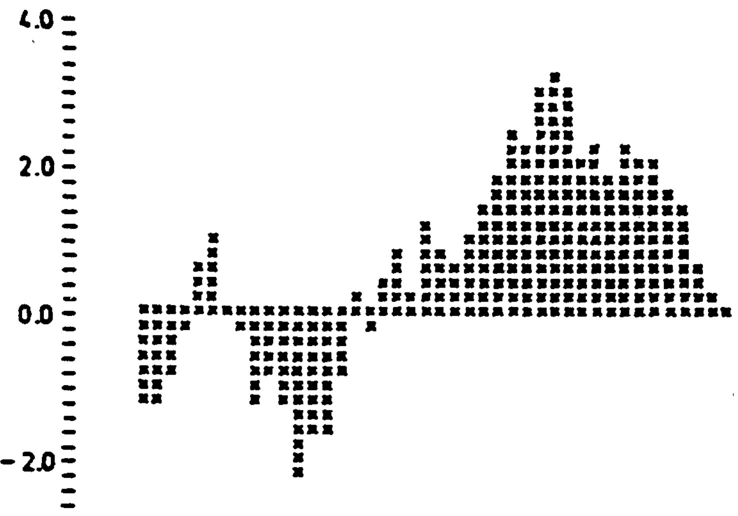
was partialy exposed on the chromatophore surface of a carotenoid-less mutant (G-9⁺) of *R. rubrum* (23). The B890-complex of *R. rubrum* is in fact composed of two subunits, B890α (M_r6079 Da) and B890β (M_r 6101 Da) was first shown by Cogdell *et al.* (19) and by Picorel *et al.* (86). Brunisholz *et al.*, by using a combination of mild protease treatment and immunoprecipitation with antibodies prepared against the B890-complex and individual apoproteins, observed that both the α- and β-apoproteins were exposed on the chromatophore surface of *R. rubrum G-9⁺* (13,16). In the carotenoid-containing strain S1, the α-apoprotein was insensitive to digestion by proteinase K from the N-terminus (13). Meister *et al.* used three hydrophobic photolabeling reagents to label the B890α-apoprotein of *R. rubrum G-9⁺* (70):

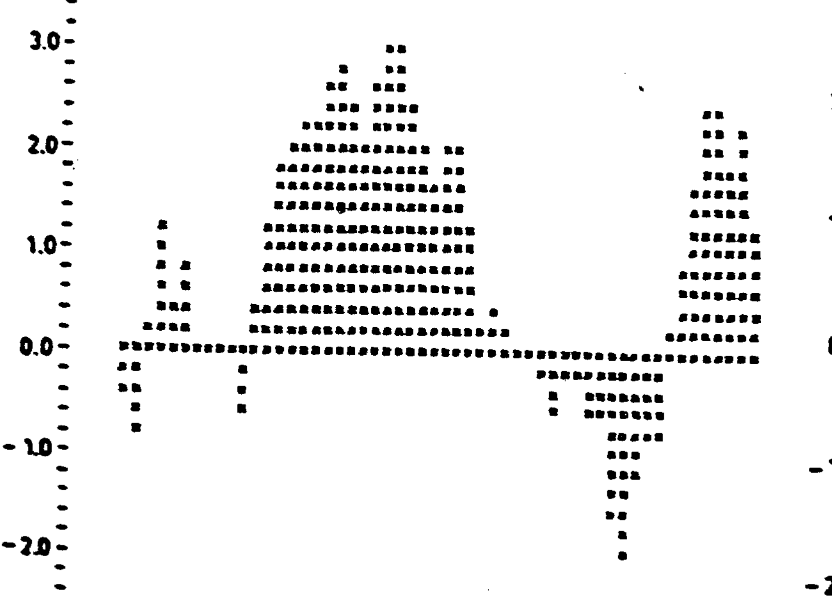
1) 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine, a highly lipid-soluble compound.
2) 11-[4-{(trifluoromethyl)diazirinyl}-phenyl]-[10-^{3}H]-9-oxaundecanoic acid, an analogue of a fatty acid.
3) 1-palmitoyl-2-[11-{(trifluoromethyl)diazirinyl}-phenyl]-[10-^{3}H]-9-oxaundecanoyl-*sn*-glycero-3-phosphorylcholine, a lecithin analogue.

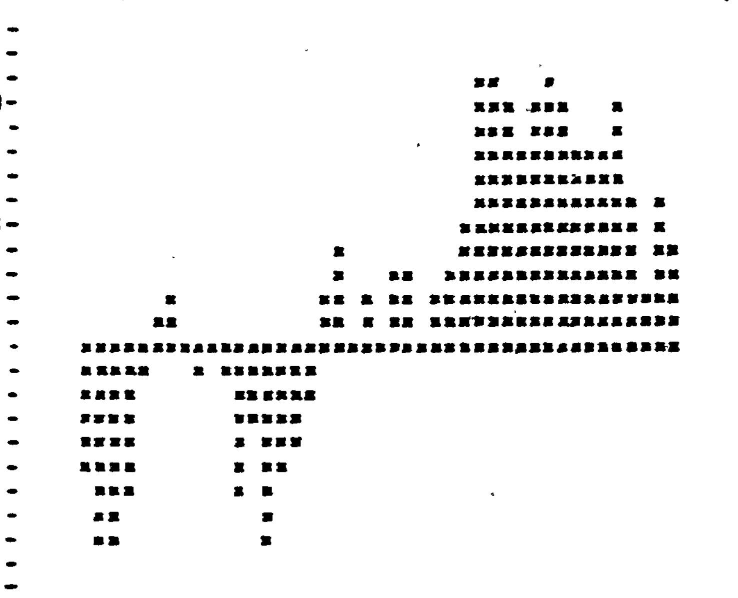
The distribution of all three labels was uniform in the hydrophobic segment of the peptide, but striking differences were observed with the N-terminal portion. Meister *et al.* (70) suggested a tight association of the transmembrane helix of

Figure 2: Hydropathy plots of Bchl-binding polypeptides of the light-harvesting complexes. a) B870α, b) B870β, c) B850α, and d) B850β from *Rhodobacter capsulatus* (27).









18b

B870α with that of the B870β chain. They also suggested that the N-terminal segment forms an amphipathic helix which interacts with the water-lipid interface of the membrane.

R. capsulatus also has two types of complexes: LH I or B870 and LH II or B800-850. The B870-complex (LH I) is composed of two subunits, B870α [M_r 6538 Da (105)] and B870β [M_r 5341 Da (103)]. The B800-850-complex (LH II) is composed of two subunits B850α [M_r 7322 Da (102)] and B850-β [M_r 4597 Da (104]. Another ca. 14,000 Da polypeptide, which does not bind any pigments (B850-γ), is also presumed to be associated with part of the LH II complex. Upon radioiodination of *R. capsulatus* chromatophores, the α-polypeptide of B870 and the β-polypeptide of B850 were labeled (121). Peters and Drews, using 1,3,4,6,-tetrachloro-3α,6α-diphenylglycouril catalyzed radioiodination (^{125}I), showed that the B850β and -γ, and B870β polypeptides are exposed on either surface of the membrane (83). Protease digestion studies indicated that the N-terminal groups of all β-apoproteins are present on the cytoplasmic side of the membrane, but the N-terminals of the α-apoproteins are protected in wild type *R. capsulatus* (108). With mutant strains Y5 (RC^- and $B870^-$) and Ala^+ ($B850^-$) of *R. capsulatus*, N-terminals of both B850α and -β (in the case of Y5) and both B870α and -β (in the case of Ala^+) were exposed on the cytoplasmic surface of chromatophores (107,106).

R. viridis has two polypeptides (B1015α, and -β) with M_r of 6848 Da and 6138 Da, respectively (14). Another polypeptide -γ is also believed to be associated with the B1015-complex because all three polypeptides are present in a 1:1:1 ratio in the thylakoid membrane. The γ-polypeptide consists of 36 amino acid residues, and exhibits a high hydrophobicity and aromatic amino acid content. It is postulated that the B1015γ polypeptide is involved in the formation of regular arrays of light-harvesting complexes (14). Jacob and Miller, using trypsin and pronase, did not observe digestion of light-harvesting polypeptides (47); however, monoclonal antibodies raised against B1015α, -β and -γ polypeptides complexed with chromatophores, showing the presence of all three on the chromatophore surface (50).

R. sphaeroides has two sets of apoproteins: B850α (M_r 5599 Da) and -β [M_r 5969 Da (113)], and B870α (M_r 6809 Da) and -β [M_r 5457 Da (114)]. Cogdell *et al.*, using a ferritin antibody labeling technique, observed antigenic sites on both surfaces of the membrane to anti-B850-complex antibodies (20). Earlier, Francis & Richards, using KB^3H_4 and pyridoxal-phosphate, had observed that two light-harvesting bands (B870α and a mixture of B870β and B850α and -β) were labeled from the cytoplasmic side whereas only B870α was labeled from the periplasmic side (32). Bachmann *et al.* using radioiodination observed both light-harvesting bands to be labeled from both sides (3). The

reagent used by Francis and Richards is specific for ε-amino groups of lysine and only the B870α-polypeptide has a lysine on its C-terminal side (cf. Figure 1). That may be why the C-terminals of no other light-harvesting polypeptides were labeled. Radioiodination can label any of two amino acids (Tyr and His) available on the membrane surfaces (59). Recently, by using a combination of immunochemical detection and protein digestion and sequencing methods, Takemoto *et al.* have shown that B870α of *R. sphaeroides* has its N-terminal end exposed at the cytoplasmic surface and the C-terminal end exposed on the periplasmic surface (109). It seems that, as in the case of wild type strains of *R. rubrum,* and *R. capsulatus*, when both carotenoid, and the B850-complex are present, the α-apoproteins of *R. sphaeroides* are protected from protease digestion.

The emerging theme from the studies done so far seems to be that all the light-harvesting apoproteins are transmembrane proteins with N-terminal ends exposed on the cytoplasmic surface and C-terminal ends exposed on the periplasmic surface. Although there is no X-ray analysis data yet available on the tertiary structure of any light-harvesting protein; these proteins will nevertheless serve as a good model system in demonstrating the usefulness of the labeling reagent. Only B870α has one lysine on the C-terminal side, while all four (B870α, B870β, B850α and B850β) have one or

more lysyl residues on the N-terminal side (cf. Figure 1) Therefore, all four subunits should be labeled when the reagent is added to the cytoplasmic side whereas only B870α should be labeled when the reagent is added to the periplasmic side. The amino acid sequence of B870α (cf. Figure 1) shows that it has two lysine residues at its N-terminal end, and one lysine residue at its C-terminal end. A positive labeling result from both feprations would not preclude either a symmetrical or an asymmetrical orientation of the B870α polypeptide in the membrane. An asymmetric orientation, however, can be confirmed by analysis of the labeling patterns of peptides formed during trypsin digestion of the purified polypeptide recovered from both chromatophore and spheroplast derived vesicle preparations. If the polypeptide has an asymmetrical orientation, different peptide fragments should demonstrate radioactivity when analyzed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The following materials were purchased from the sources indicated: [^{14}C]thiourea (2.15 G Bq/mmol) from Amersham Canada Ltd., Oakville, Ontario; Aquasol and En^{3}Hance, from NEN Products, DuPont Canada, Inc., Lachine, Quebec; BOC-ON, Poly-(L-lysine), Arg-Lys-Asp-Val-Tyr, Sephadex LH-60, glycylglycine, cytochrome c, CNBr-linked Sepharose 4B, TCPK treated trypsin, Acrylamide, Bis-acrylamide and protein molecular weight standards from Sigma Chemical Co., St. Louis, MO; Glycine was from Matheson Coleman & Bell, Norwood, OH; MN-Polygram Sil G-22 (Silica gel G) TLC sheets from Brinkmann Instruments Canada Ltd., Rexdale, Ontario; Merck silica gel $60F_{254}$ TLC sheets from BDH Chemicals Canada Ltd., Vancouver, B.C; J. T. Baker Si 250 PA (19C) TLC plates from American Hospital Supplies Richmond, B.C; and Kodak XRP film from Kodak Canada Inc., Toronto, Ontario. All other chemical reagents were of reagent grade. CM-Cellulose (Cm 52) DEAE-Cellulose (DE 32) were products of Whatmann Co., Kent, England purchased from Terochem Laboratories Ltd., Edmonton, Alberta; Sephadex G-10 was from Pharmacia Fine Chemicals, Lachine, Quebec.

2.2 Methods

2.2.1 Synthesis of N-Acetyl-S-(2-sulfoethyl)-L-cysteine

N-Acetyl-S-(2-sulfoethyl)-L-cysteine was synthesized by the method of Nikety *et al.* (75). N-acetyl-L-cysteine (4 mmol, 652.8 mg) and sodium 2-bromoethane sulfonate (4 mmol, 844 mg) were dissolved in 8 ml of 0.5 M NH_4HCO_3 and the pH was adjusted to 8.6 with concentrated ammonia. The solution was flushed thoroughly with nitrogen and left under a positive pressure of nitrogen at room temperature. The pH was adjusted periodicaly to 8.6 with concentrated ammonia. After 60 h the sample was lyophilized. In order to remove ammonia comletely the sample was redissolved in water and lyophilized several times. Analysis of the product mixture on TLC system B using Silica gel $60F_{254}$ sheets developed in 2-propanol/acetic acid/water (4:2:1 v/v) gave a spot at R_f 0.35 (assumed to be N-acetyl-S-(2-sulfoethyl)-L-cysteine) which was well separated from authentic N-acetyl-L-cysteine (R_f 0.51). Spots were visualised by the KI/starch spray reagent of Rydon and Smith (91) as follows: following development, TLC plates were dried in the fume hood overnight and exposed for 10 min in a glass chamber saturated with chlorine gas. Plates were removed and air dried in a fumehood for at least 30 min and finally sprayed with a 1% starch-1% potassium iodide solution.

2.2.2 Synthesis of N,N'-Diacetyl-L-cystine

N-Acetyl-L-cysteine (1 mmol, 163.2 mg) and cystamine (0.5 mmol, 76 mg) were dissolved in 0.1 M phosphate buffer (10 ml) at pH 7.4. The reaction was allowed to proceed at 20°C for 16 h. The desired product, N,N'-diacetyl-L-cystine, had an R_f value of 0.38 when analyzed on Silica gel $60F_{254}$ sheets developed in 2-propanol/acetic acid/H_2O (4:2:1 v/v) and visualised by KI/starch spray following treatment with chlorine gas.

2.2.3 Synthesis of N-Amidinoglycine

Glycine (0.05 mmol, 3.75 mg) was reacted with 1 mmol of 2-S-thiuronium ethane sulfonate in 0.1 M phosphate buffer (5 ml), pH 9.5, for 2 h at 37°C. The reaction mixture was freeze-dried and analyzed by TLC system A using silica gel G sheets in chloroform/methanol/9 M ammonia (2:2:1 v/v) A spot visible with alkaline ferricyanide/nitroprusside spray (120) at R_f 0.38 due to N-amidinoglycine was well-separated from glycine (R_f 0.27), which was visible only with ninhydrin. The alkaline ferricyanide/nitroprusside spray reagent consists of a mixture of 2.5% sodium hydroxide, 2.5% potassium ferricyanide and 2.5% sodium nitroprusside. Samples as low as 20 µg can be detected which appear as distinct colored spots.

2.2.4 Synthesis of N^{α}-t-BOC-L-homoarginine

To a solution of L-homoarginine (5 mmol, 1.12 g) and triethylamine (7.5 mmol, 1.05 ml) in 50% aqueous dioxane (6 ml), BOC-ON (5.5 mmol, 1.35 g) was added with stirring at room temperature. After stirring for 16 h, water (7.5 ml) and ethyl acetate (10 ml) were added to the mixture. The aqueous phase was washed with ethyl acetate (10 ml) to remove the by-product.

Water was removed by freeze-drying and the product was analyzed by TLC in system B [silica gel $60F_{254}$ sheets {or Si 250 PA (19C) plates when used with the alkaline ferricyanide/nitroprusside spray (120)} in 2-propanol/acetic acid/water (4:2:1 v/v)]. The product had an R_f value of 0.75 well separated from authentic L-homoarginine (R_f 0.13).

2.2.5 Synthesis of the Guanidinating Reagent

2-S-Thiuronium ethane sulfonate was synthesized from sodium 2-bromoethane sulfonate (25 mmol, 5.25 g) and thiourea (25 mmol, 1.903 g) in 10 ml of water (cf. Equation 1). The reaction mixture was heated to 95°C for 30 min, after which time hard white crystals began to appear rapidly upon cooling. The product was recrystallized from water; the yield was 78%.

2-S-Thiuronium ethane sulfonate does not melt but decomposes upon heating to 265°C.

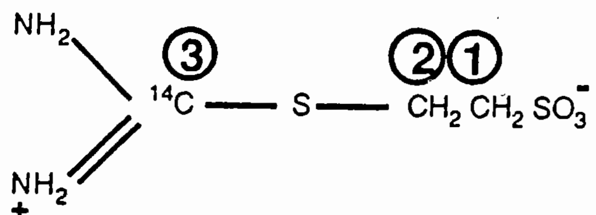
Chemical analysis for $C_3H_8N_2O_3S_2$:

Calcd: C, 19.55; H, 4.37; N, 15.21

Found: C, 19.39; H, 4.52; N, 14.99

The ^{13}C NMR spectrum showed three resonances (cf. Figure: 3); A, C and D, represent ethylene C1 (26.711 ppm), ethylene C2 (50.066 ppm), and thiuronium C3 (170.598 ppm), respectively (cf. Structure 1). Peak B represents the solvent (dimethyl sulfoxide) peak.

Structure #1



The ^{1}H NMR spectrum showed a multiplet (cf. Figure 4) due to ethylene C1 and C2 protons (3.1976-3.5765 ppm).

2-S-[^{14}]Thiuronium ethane sulfonate was synthesized in a similar manner from 1 mmol each of sodium 2-bromoethane sulfonate and [^{14}C]thiourea (37.7 GBq/mol). The product was used without recrystallization or diluted with unlabeled 2-S-thiuronium ethane sulfonate as required.

Figure 3. ^{13}C NMR spectra of 2-S-thiuronium ethane sulfonate (recorded on a Bruker Model WM 400) in dimethyl sulfoxide.

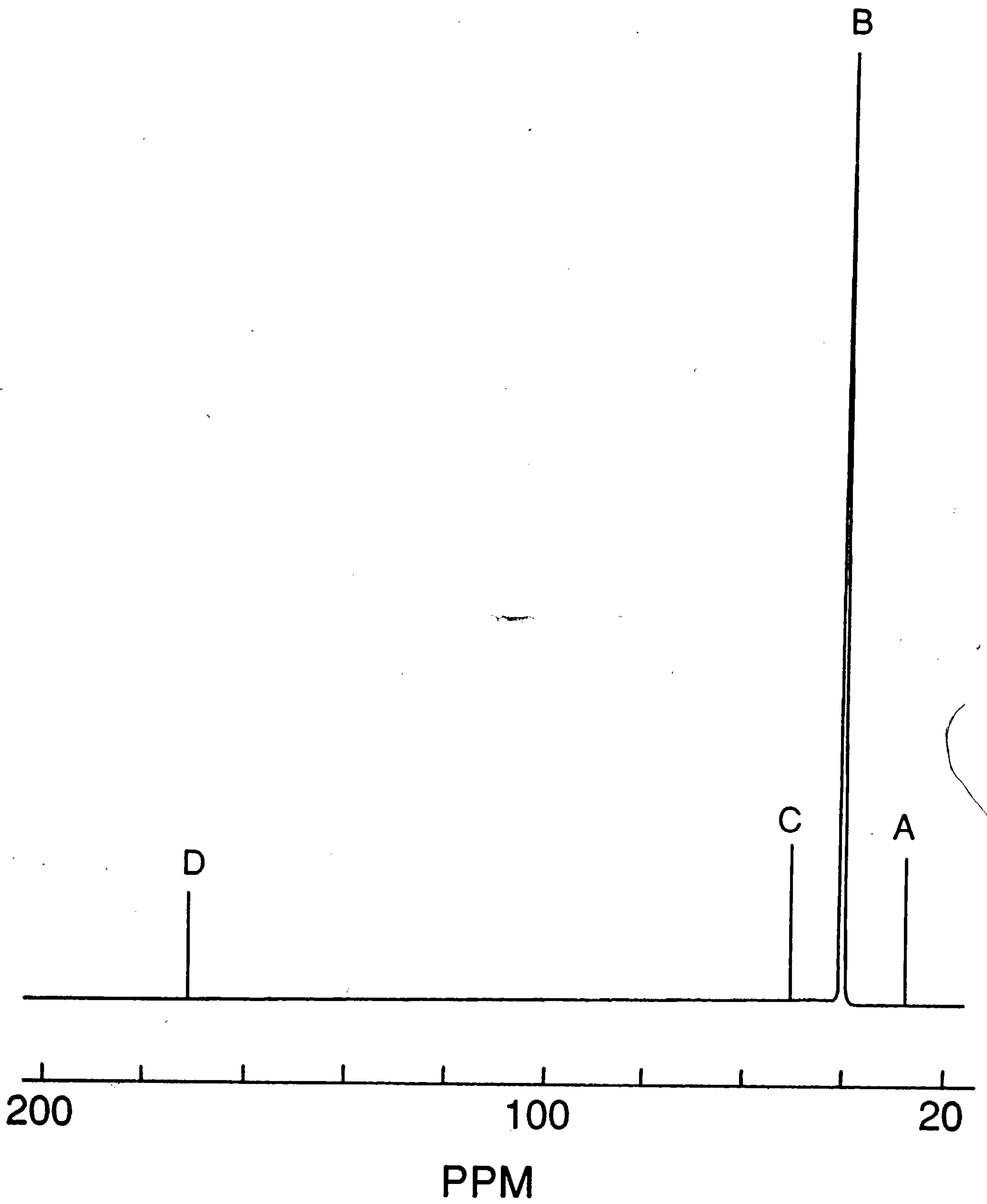
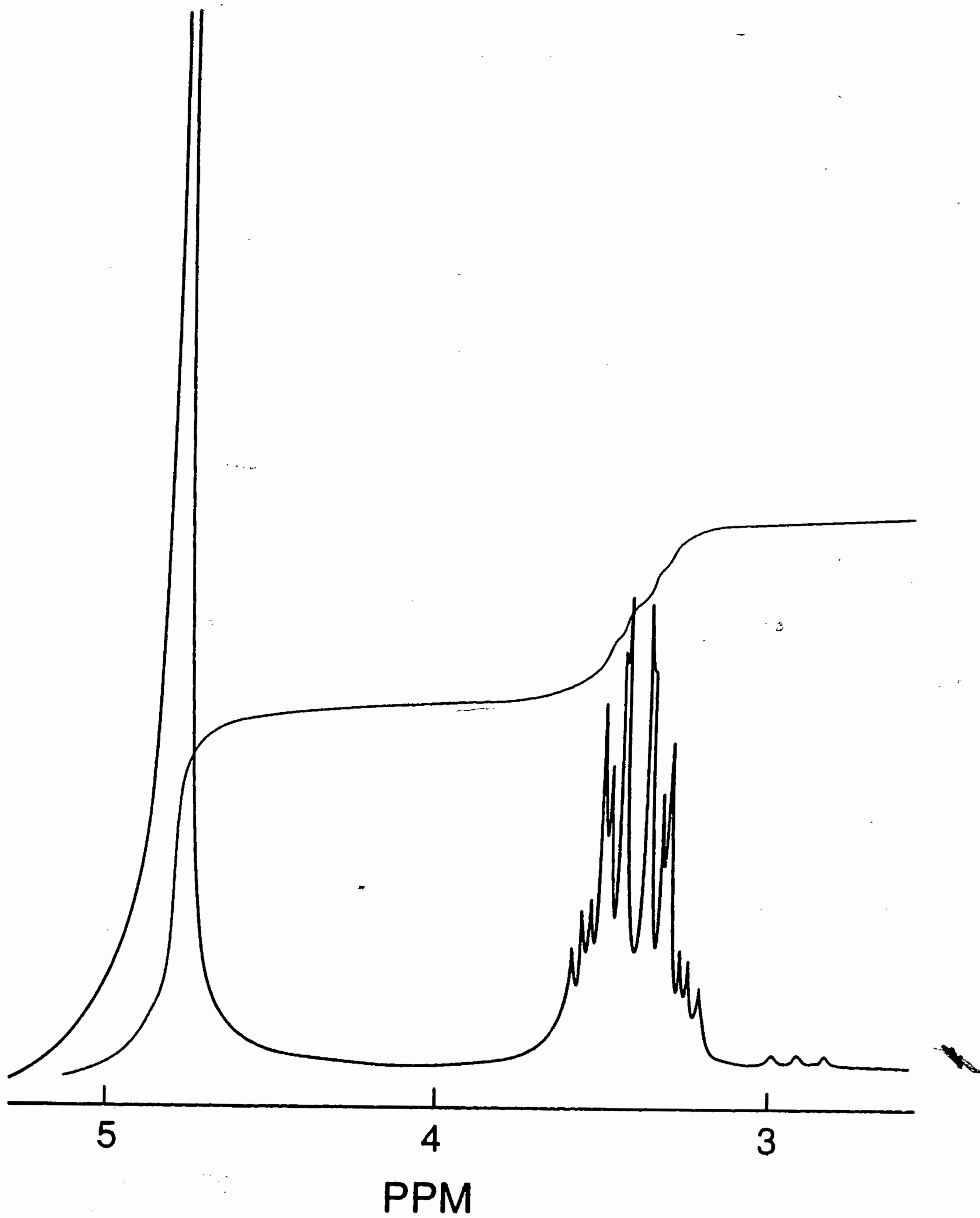

B
C
A
D
200
100
20
PPM

Figure 4. ^{1}H NMR spectra of 2-S-thiuronium ethane sulfonate (recorded on a Bruker Model SY 100) in D_2O.


5
4
3
PPM

Although the same compound was synthesized by Schramm *et al*. (94), along with other mercaptoalkane sulfonic acids, its use as a labeling reagent has never been previously exploited.

2.2.6 Reaction of the Guanidinating Reagent with Poly(L-lysine)

Poly(L-lysine) (10 mg; equivalent to 78 μmol of lysine residues) was reacted with a 50-fold molar excess of unlabeled 2-S-thiuronium ethane sulfonate in 0.1 M potassium phosphate buffer (10 ml), pH 10.5, for 1 h at 37°C. After dialysis against distilled water, the solution was concentrated to 1 ml *in vacuo* and an equal volume of 12 M HCl added. The sample was hydrolyzed for 16 h at 110°C in a sealed tube under vacuum. The hydrolysate was analyzed by TLC on silica gel G sheets in chloroform/methanol/9 M ammonia (2:2:1 v/v) using a ninhydrin spray, and by a Beckman Model-116 amino acid analyzer with a PA 28 ion-exchange column (29 x 0.9 cm). Sodium citrate buffer (0.127 M, pH 4.25) was used initialy at a flow rate of 50 ml/h at 32.5°C and 210 psi. After 185 minutes the temperature was increased to 62.5°C and sodium citrate buffer (0.117 M) of higher pH (5.36) was used to elute the basic amino acids.

2.2.7 Reaction of the Guanidinating Reagent with Derivatives of Amino Acids

The N^{α}-t-BOC derivatives of L-lysine (4.93 mg), L-tyrosine (5.63 mg), L-histidine (5.07 mg), and L-serine (4.10 mg) plus N-acetyl-L-cysteine (3.26 mg), (20 μmol each) were reacted with a 20-fold molar excess of 2-S-thiuronium ethane sulfonate (0.4 mmol, 73.6 mg) in 0.1 M potassium phosphate buffer (2 ml), pH 9.0 for 2 h at 37°C. The reaction was stopped and water was removed by freeze-drying. The t-BOC moiety was removed by treatment with dry concentrated trifluoroacetic acid (distilled over P_2O_5) for 30 min at 20°C. The volatile trifluoroacetic acid was removed by a steady stream of nitrogen gas and traces of the acid were removed *in vacuo*. The samples were analyzed by TLC in Silica gel G or $60F_{254}$ sheets with 2-propanol/acetic acid/water (4:2:1 v/v). Spots were detected using ninhydrin, ferricyanide/nitroprusside, or KI/starch spray reagents.

In order to identify the product, a large scale run was carried out. N-Acetyl-L-cysteine (30.6 mmol, 5 g) was reacted with the guanidinating reagent (68.5 mmol, 12.6 g) in 0.1 M potassium phosphate buffer (340 ml, pH 9.0) for 3 h at 37°C. The product mixture was freeze-dried to completely remove the water, and the residue was extracted three times with 75 ml acetone. The combined acetone fractions were filtered and evaporated to dryness. The residue was re-extracted three

times with 25 ml 2-propanol. The 2-propanol extract was filtered and its volume was reduced to 4 ml. One-half of the 2-propanol extract was then applied onto a Kieselgel 60 silica (230-400 mesh) column (2.8 x 36 cm) and developed with 2-propanol at a flow rate of 6 ml/min. Fractions of 3 ml were collected. The fraction eluted nearest the solvent front exhibited only a single component at R_f 0.60 when analysed by TLC on Si 250 PA (19C) plates in 2-propanol/acetic acid/water (4:2:1 v/v) (cf. Chapter 3). Spots were detected using the KI/starch spray reagent. The solvent was completely removed *in vacuo* for 48 h. The ^{13}C NMR spectrum of the product (in D_2O) showed six resonances (cf. Figure 5) assigned as follows: acetylmethyl (24.610 ppm); cysteine C3 (36.568 ppm), shifted from 27.952 ppm in N-acetyl-L-cysteine; cysteine C2 (56.850 ppm); thiuronium C (174.122 ppm); acetylcarboxyl and cysteine C1 (176.955 and 178.022 ppm, found at 176.664 and 177.301 ppm in N-acetyl-L-cysteine) (cf Figure 6). The ^{1}H NMR spectrum (in D_2O) showed three resonences (cf. Figure 7) assigned as follows: acetylmethyl (singlet at 2.040 ppm); cysteine C3 (octet centered at 3.513 ppm, shifted from doublet centered at 2.974 ppm in N-acetyl-L-cysteine); cysteine C2 (triplet centered at 4.532 ppm).

Both N^{α}-t-BOC-L-lysine (10 μmol, 2.46 mg) and N-acetyl-L-cysteine (10 μmol, 1.63 mg) were reacted with 2-S-[^{14}C]thiuronium ethane sulfonate (0.2 mmol, 36.9 mg)

(diluted 25-fold) in 0.1 M potassium phosphate buffer, pH 9.0, at 37°C and analyzed by TLC on Silica gel G sheets in 2-propanol/acetic acid/water (4:2:1 v/v) before and after treatment with trifluoroacetic acid. Spots were detected with the KI/starch spray reagent, scraped from the TLC plates, eluted with developing solvent, and counted in 10 ml of Aquasol on an LKB Wallac Model 1217 RackBeta liquid scintillation counter.

2.2.8 Reaction of the Guanidinating Reagent with the α-amino Group of Glycylglycine and Glycine

Glycylglycine (0.04 mmol, 5.28 mg) was reacted with a 20-fold molar excess of 2-S-thiuronium ethane sulfonate (0.8 mmol, 147 mg) at 37°C for 2 h in 0.1 M potassium phosphate buffer (4 ml), pH 9.5. The sample was freeze-dried and was applied directly to a Sephadex G-10 column (1 x 28 cm); the elution was performed with water at a flow rate of 75 ml/h. N-Amidinoglycine was prepared by treating glycine (0.05 mmol, 3.75 mg) with 2-S-thiuronium ethane sulfonate (1 mmol, 184 mg) at 37°C for 2 h in 0.1 M phosphate buffer, pH 9.5. The product mixture was analyzed by TLC on Silica gel $60F_{254}$ sheets developed in chloroform/methanol/9 M ammonia. Both nitroprusside and ninhydrin spray reagents were used to visualize spots on two TLC plates, which were run simultaneously.

Figure 5. ^{13}C NMR Spectrum of guanidinated N-acetyl-L-cysteine (recorded on a Bruker Model WM 400) in D_2O.

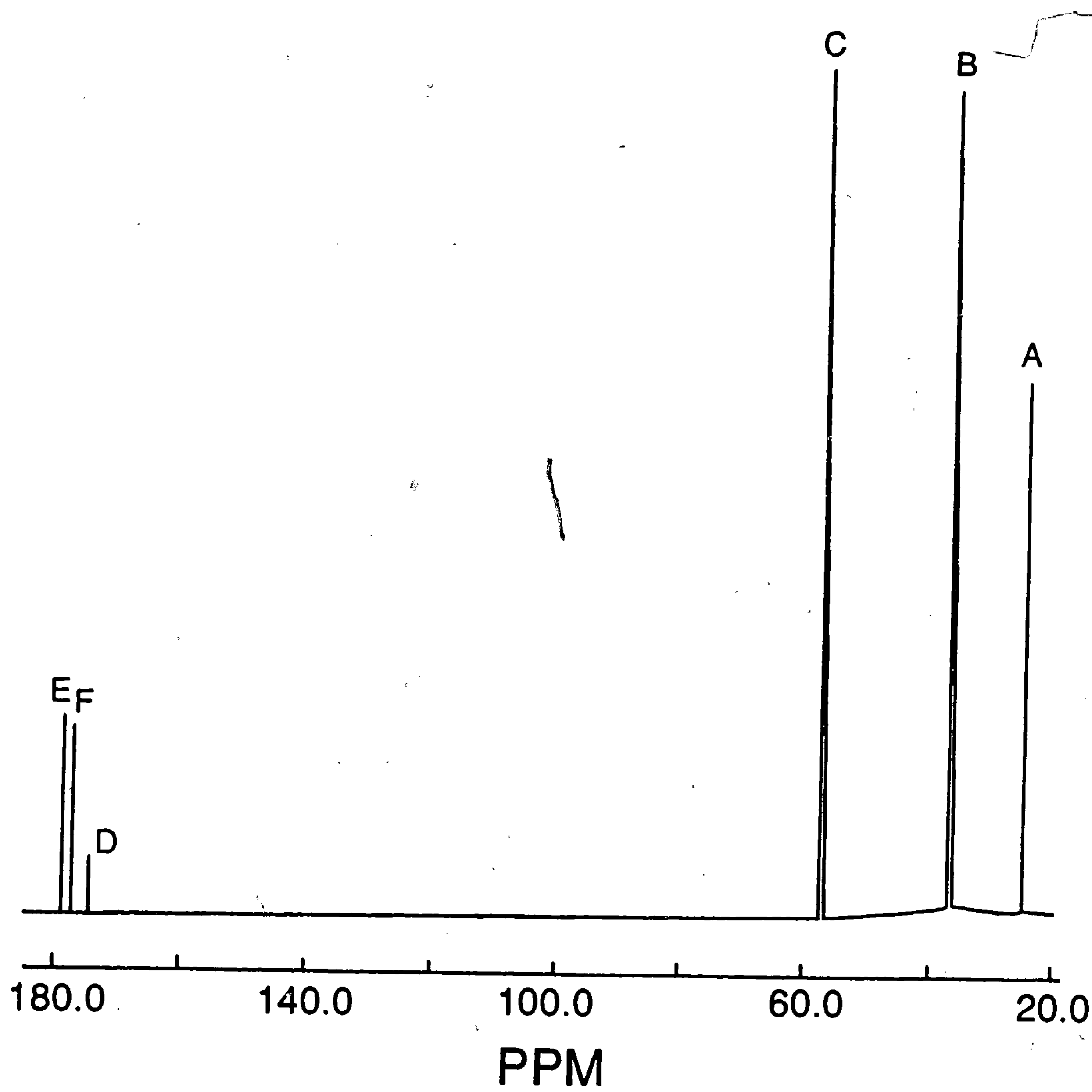

C
B
A
E
F
D
180.0
140.0
100.0
60.0
20.0
PPM

Figure 6. ^{13}C NMR Spectrum of N-acetyl-L-cysteine (recorded on a Bruker Model WM 400) in D_2O.

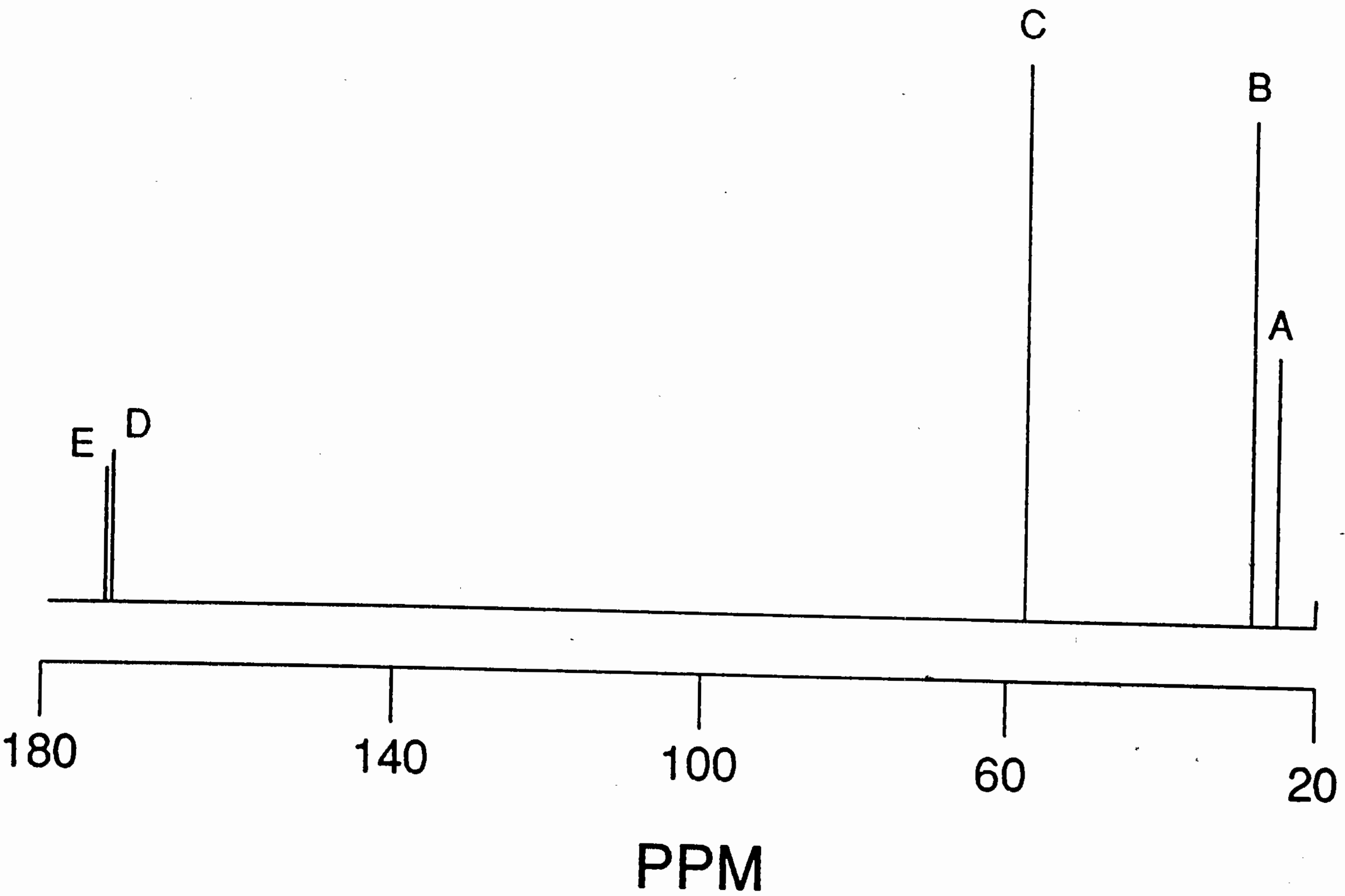
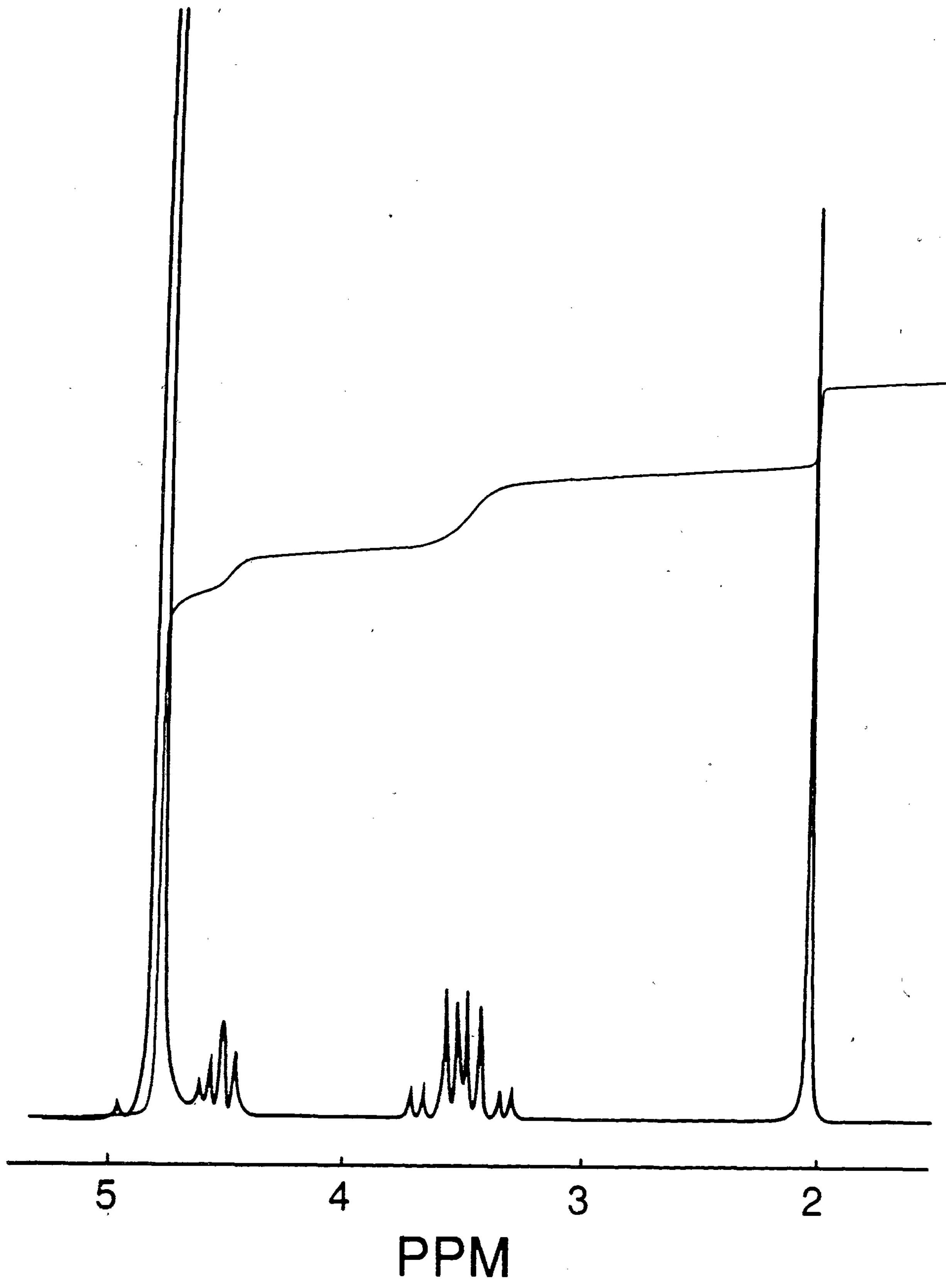

C
B
A
E
D
180
140
100
60
20
PPM

Figure 7. ^1H NMR Spectrum of guanidinated N-acetyl-L-cysteine (recorded on a Bruker Model SY 100) in D_2O.


5
4
3
2
PPM

2.2.9 Homoarginine as a Potential Cleavage Site of Trypsin

A pentapeptide (1.47 mmol, 1 mg) with the sequence **Arg-Lys-Asp-Val-Tyr** was dissolved in 0.05 M NH_4HCO_3 buffer (0.97 ml), pH 8.0. To this solution, 0.5 ml of the above buffer containing 25 μg of trypsin [1/40 the amount (w/w) of pentapeptide] was added and the mixture was incubated at 37°C for 0.5 h. The reaction was stopped by boiling for 90 sec.

The pentapeptide, (2.94 mmol, 2 mg) was also incubated with 2-S-thiuronium ethane sulfonate (1 mmol, 184 mg) at 37°C for 2 h in 0.1 M potassium phosphate buffer (5 ml), pH 9.5. After freeze-drying, the sample was redissolved in water (5 ml) and applied to a Sephadex G-10 column (1 x 28 cm) at a flow rate of 8 ml/h. The fractions collected during the elution of the first peak, representing the guanidinated peptide, were pooled. Following freeze-drying, the guanidinated pentapeptide was resuspended in 0.05 M NH_4HCO_3 buffer (0.97 ml), pH 8.0, and hydrolyzed for 0.5 h with trypsin as described above. One half of the sample was removed for TLC analysis and the remainder was allowed to react for total of 3 h. TLC analysis was carried out on 0.1 mm cellulose sheets using 1-butanol/pyridine/acetic acid/water (50:33:1:40 v/v) as the developing solvent. Samples were visualised using a fluorescamine spray reagent (101). The dried plates were evenly sprayed with 0.025% (w/v) fluorescamine in acetone. The plates were allowed to dry for a few minutes and sprayed with

5% triethylamine in acetone. Both the fluorescamine and the stabilizer were prepared fresh using reagent grade solvents. Spots were observed using long wavelength ultraviolet light.

2.2.10 Reaction of the Guanidinating Reagent with Whole Cells of R. sphaeroides

R. sphaeroides NCIB 8253 was grown phototrophically at 30°C by stirring magnetically in the MG medium of Lascelles (61), as described by Francis and Richards (32). Bacteria were harvested by centrifuging at 7000 rpm in a Beckman GS-3 rotor and washed twice with 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM EDTA and 5 mM β-mercaptoethanol. Washed cells were resuspended in 0.1 M potassium phosphate buffer, pH 9.0, at a concentration of 0.5 g/ml. Cells (0.5 g, wet weight) were incubated with various concentration of 2-S-[^{14}C]thiuronium ethane sulfonate (diluted 8-fold) for 2 h at 35°C. Following the labeling reaction, the cell suspension was washed 4 times with 0.05 M potassium phosphate buffer, pH 7.4. The cells were incubated 3 times with 15% (w/v) sucrose and phosphate buffer alternatively for 20 min each. The cells were kept overnight in sucrose solution and washed with buffer a further four times until no more radioactivity was detected in the supernatant. This procedure was performed to osmotically shock the guanidinating reagent out of the periplasm of the bacteria. Bacteria were resuspended in 5 ml

of 50 mM potassium phosphate buffer containing 10 mM EDTA, 5 mM β-mercaptoethanol and 0.1 mM PMSF, pH 7.4, and sonicated 5 times for 1-min pulses at a setting of 50 on a Bronwill Biosonik III ultrasonicator. The unbroken cells and cell debris was removed by centrifugation at 15,000 x g for 20 min. The resulting supernatant was centrifuged at 100,000 x g in a Beckman Ti 50.2 rotor to sediment the chromatophore fraction. The chromatophore pellet was resuspended in 0.1 M potassium phosphate buffer (1 ml) and purified over a discontinous sucrose density gradient of 10:20:45% sucrose (w/w) in a 1:2:1 ratio, by the method of Francis and Richards (32). The centrifugation in discontinuous sucrose density gradients was done in a Beckman Model L5-75 ultracentrifuge using an SW 40 rotor for 10 h at 21,000 rpm. The chromatophores were finally resuspended in 1% aqueous SDS (0.05 ml). Protein content was determined by the method of Lowry *et al.* (64) using 0.5 ml of the above suspension, and bovine serum albumin as a protein standard. Radioactivity was determined by boiling the remaining 0.45 ml of the chromatophore suspension with 0.225 ml of 10% aqueous SDS for 1 min and adding 10 ml of Aquasol for scintillation counting.

2.2.11 Reaction of the Guanidinating Reagent with Purified Membrane Fractions of R. sphaeroides

R. sphaeroides was grown phototrophicaly by the method of Francis and Richards (32) under normal light intensities (4500 Lx). Cells (15 g, wet weight) were harvested by centrifuging at 7,000 rpm for 16 min, and washed once with 0.1 M potassium phosphate buffer, pH 7.4. Cells were resuspended in 0.05 M potassium phosphate buffer (150 ml), pH 7.0, containing 10 mM EDTA, 5 mM β-mercaptoethanol and 0.1 mM PMSF and were subjected to French press disruption at 15,000 psi. The broken-cell suspension was incubated with DNAase (15 mg) and RNAase (15 mg) for 30 min. Unbroken cells and cell debris were removed by centrifugation at 15,000 x g for 20 min. Chromatophores were sedimented at 100,000 x g and purified by 10:20:45% (w/w) discontinuous sucrose density gradient centrifugation by the method of Francis and Richards (32) using an SW 27 rotor for 16 h at 20,000 rpm. The dark red band which formed a layer at the 20:45% interface was removed and dialyzed extensively at 4°C against 0.1 M potassium phosphate buffer, pH 7.4. Chromatophores were sedimented at 100,000 x g for 30 min and the pellet was finally resuspended in the same buffer at a concentration of 4 mg of protein/ml. Aliquots of 0.2 ml were added to enough solid 2-S-[^{14}C]thiuronium ethane sulfonate (diluted 36-fold) to give the concentrations desired. In order to determine optimum conditions for labeling

the membranes, the concentration of the guanidinating reagent, the time of reaction, the pH of the buffer, and the temperature were varied as indicated in Figure 15. When the effect of one variable was studied, the others were kept at the standard conditions of 0.2 M 2-S-[^{14}C]thiuronium ethane sulfonate incubated for 2 h at 30°C and pH 9.0. When the concentration of the guanidinating reagent was varied, however, the 2-S-[^{14}C]thiuronium ethane sulfonate was diluted only 10-fold. After labeling, the chromatophores were sedimented by centrifugation at 100,000 x g and washed several times with 0.1 M potassium phosphate buffer, pH 7.4 until no more radioactivity was detected in the supernatant. The chromatophore pellet was then dissolved in 0.5 ml of 1% aqueous SDS and analyzed for protein content and radioactivity as described above.

2.2.12 Preparation of Spheroplast Derived Vesicles (SDV)

R. sphaeroides was grown in 5 liter flasks in high light intensity (32) (15,000 Lx) by placing two 150-Watt spot lamps on either side of the flask at 30°C. The cells were disrupted by the lysozyme/osmotic shock procedure of Michels and Konings (73). The cells (8 g, wet weight) were harvested by centrifugation at 7,000 rpm for 16 min and washed once with 0.05 M potassium phosphate buffer, pH 7.0, containing 10 mM EDTA. Cells were resuspended in 0.120 M potassium phosphate

buffer (150 ml), pH 8.0, containing 10 mM EDTA and 10 mM potassium ascorbate, and incubated for 10 min at room temperature with lysozyme (30 mg) at a concentration of 200 μg/ml. The cell suspension was diluted with 10 mM ascorbate buffer (150 ml), pH 6.0, and incubated for 30 min. This procedure should have facilitated the penetration of lysozyme into the cell wall. Spheroplasts were lysed by dilution of the suspension with 10 mM ascorbate buffer (900 ml), pH 6.0. DNAase (12 mg) and RNAase (12 mg) were added and the suspension was incubated for a further 15 min. The suspension was finaly treated with magnesium sulfate (1.479 g) for 15 min. The cells and unlysed spheroplasts were sedimented by centrifugation for 20 min at 2,500 rpm in a GSA rotor. The supernatant was decanted carefully and centrifuged at 15,000 x g for 30 min. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4, and the SDV were purified by centrifugation in continous 30-55% (w/w) sucrose gradients by the method of Takemoto and Backmann (110), using an SW 27 rotor for 16 h at 20,000 rpm. The SDV were dialyzed at 4°C in 0.1 M phosphate buffer, pH 7.4, and sedimented at 50,000 x g in a Ti 50.2 rotor for 30 min. The pelleted SDV were resuspended in 50 mM potassium phosphate buffer, pH 7.4, and stored in 50% (v/v) glycerol at -20°C until required for further use. All incubations were carried out in the dark under nitrogen atmospheres at room temperature.

2.2.13 Purification of SDV using cytochrome c-linked Sepharose 4B

Cytochrome c was linked to Sepharose 4B by the method of Lotscher *et al*. (63). Freeze-dried CNBr-activated sepharose 4B (5 gm) was swollen for 15 minutes in 1 mM HCl (200 ml) and washed using a sintered glass G3 filter. A total of one liter of 1 mM HCl was added in several aliquots, supernatant being sucked off between successive additions. The gel was quickly washed twice with 0.1 M sodium bicarbonate buffer containing 0.5 M NaCl, pH 8.3, and suspended to a volume of 10 ml in the same buffer. Horse heart cytochrome c (100 mg) was added and the mixture was incubated for 16 h at 4°C with gentle end-to-end shaking. After washing with 0.1 M sodium bicarbonate buffer plus 0.5 M NaCl, pH 8.3, the remaining active groups were blocked with 1 M ethanolamine (10 ml) at 4°C for 16 h. The gel was then washed three times, alternately, with 0.1 M sodium acetate plus 1 M NaCl, pH 4.0, and 0.1 M sodium bicarbonate plus 1 M NaCl, pH 8.5.

A column (0.9 x 17 cm) was packed and equilibrated with 10 mM tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. SDV were washed with 0.15 M NaCl to remove any intrinsic cytochrome c. SDV (up to 8-10 mg in 1.2 ml equilibrium buffer) were applied to the equilibrated column and eluted with the same buffer containing 0.15 M KCl, followed by a gradient of 0-1.5 M KCl in equlibrium buffer (cf. Figure 16). In the other

runs, a fixed KCl concentration (0.75 M), as determined by gradient elution (cf. Figure 13), was used to elute the SDV, which were then dialyzed against 0.1 M phosphate buffer (pH 7.4) and stored in 50% glycerol (v/v) at -20°C until required. The column was re-equilibrated and repeatedly used with no noticeable loss of activity.

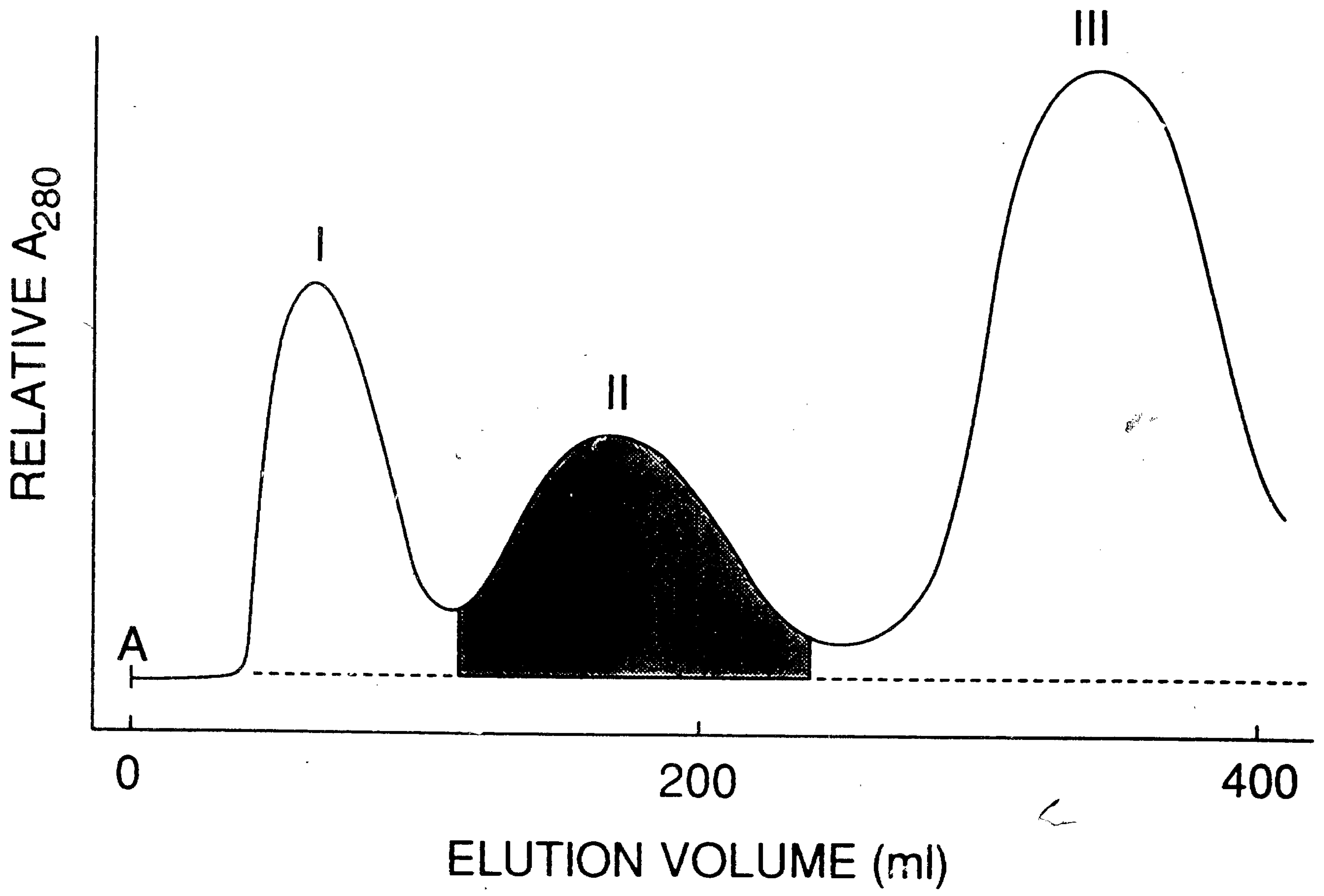
2.2.14 Labeling of Chromatophores and SDV

For labeling large amounts of chromatophores for the purpose of isolating the light-harvesting proteins, chromatophores from 15 g wet weight of *R. sphaeroides* were resuspended in 10 ml of 0.1 M phosphate buffer, pH 9.0. Membranes were then labeled with 0.2 M 2-S-[^{14}C]thiuronium ethane sulfonate (37.7 GBq/mol) at 37°C. SDV from 25 g wet weight of cells were also suspended in 10 ml of 0.1 M phosphate buffer at pH 9.0, and labeled with 0.2 M 2-S-[^{14}C]thiuronium ethane sulfonate (37.7 GBq/mol). Both the chromatophores and SDV were sedimented and washed with 0.1 M phosphate buffer, pH 7.4, until no radioactivity was detected in the supernatant. The membranes were then freeze-dried and stored at -20°C until required.

2.2.15 Purification of Light-Harvesting Proteins

The light-harvesting polypeptides were purified by the method of Theiler *et al.* (115). Freeze-dried chromatophores and SDV were repeatedly extracted with 32 ml of chloroform/methanol (1:1, v/v) containing 0.1 M ammonium acetate (C/M/NH_4OAc) in dim green light at 4°C. The resulting extract was fractionated at 4°C on a Sephadex LH-60 column (5.4 x 30 cm) in C/M/NH_4OAc with a flow rate of 180 ml/h. The eluate was monitored with an LKB UV analyzer. The fractions representing the middle peak, containing low molecular weight polypeptides, were pooled (cf. Figure 8). All subsequent purification steps were carried out at room temperature. The pooled fractions (containing low M_r polypeptides) were applied to a DEAE-cellulose (DE-32) column (2.1 x 40 cm) which had been equilibrated in C/M/NH_4OAc at a flow rate of 130 ml/h. Elution was performed at a flow rate of 30 ml/h with the same solvent system to remove any loosely-bound material. The tightly-bound material was eluted with 4% (v/v) acetic acid in C/M/NH_4OAc. Fractions of 6 ml were collected; fractions containing peaks II, III and IV were individualy pooled together (cf. Figure 9) and dialyzed extensively against distilled water, using Spectroporin dialysis tubing with a molecular weight cut off at 3500 Da. The precipitated polypeptides were recovered from the water/chloroform interface and freeze-dried.

Figure 8. Fractionation of the organic solvent extract of SDV purified from *R. sphaeroides* by preprative column chromatography on Sephadex LH-60. The shaded area represents the fraction containing low molecular mass polypeptides. This part of the eluent was pooled and used for subsequent chromatography on DEAE-cellulose (cf. Figure 9), (A) Elution with $C/M/NH_4OAc$ buffer.



RELATIVE A_{280}
I
II
III
A
0
200
400
ELUTION VOLUME (ml)

Figure 9. Separation of the light-harvesting polypeptides by DEAE-cellulose column chromatography in organic solvent. isocratic elution with (A) C/M/NH_4OAc, (B) elution with C/M/NH_4OAc containing 4% (v/v) acetic acid. Peaks I-IV represent; (I) mixture of B870α and B850α, (II) purified 850β, (III) residual 850β, and (IV) purified 870β polypeptides.

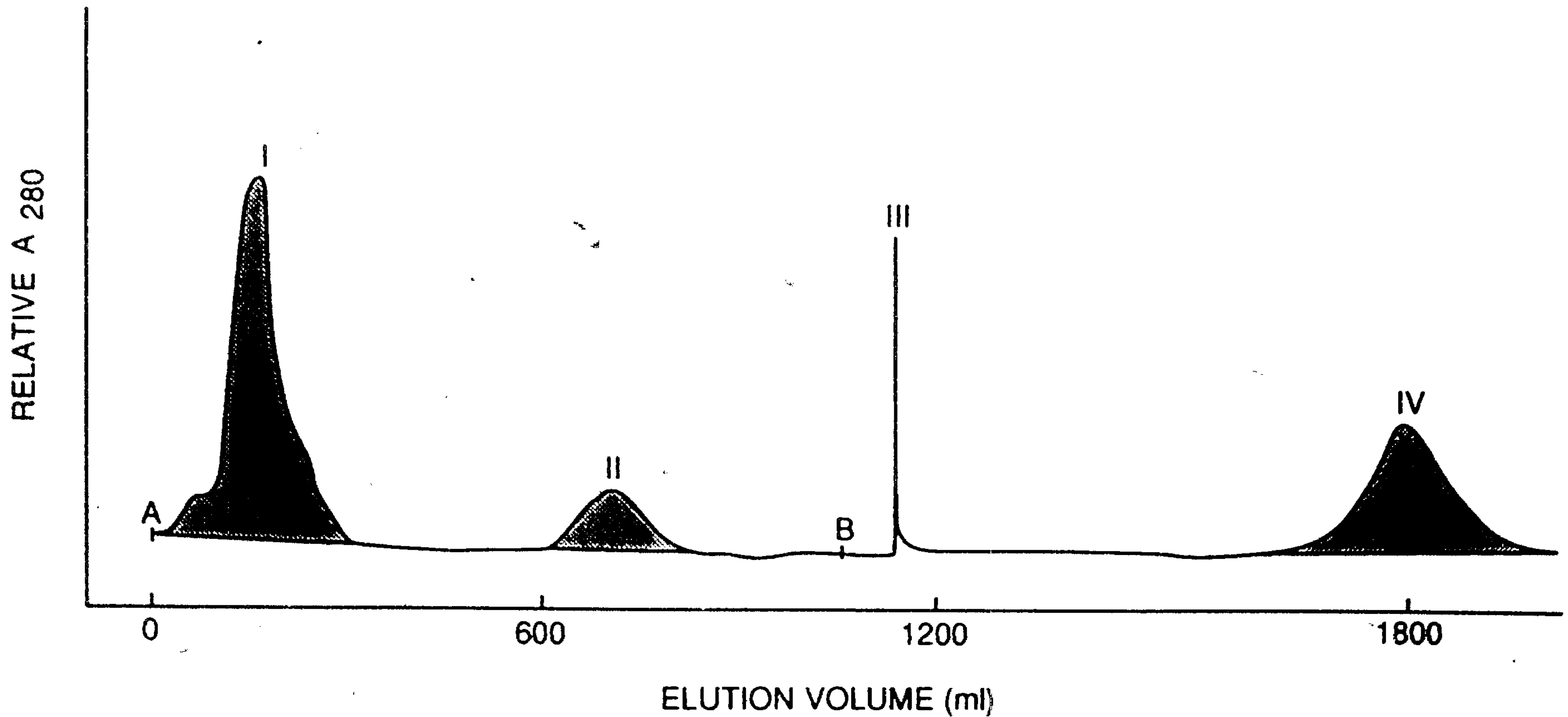

RELATIVE A 280
I
II
III
IV
A
B
0
600
1200
1800
ELUTION VOLUME (ml)

Figure 10. Concentration of peak I (ie. B870α & B850α polypeptides) from DEAE-cellulose column chromatography (cf. Figure 9) on a CM-cellulose column. (A) Elution with C/M/NH_4OAc buffer containing 20% (v/v) acetic acid.

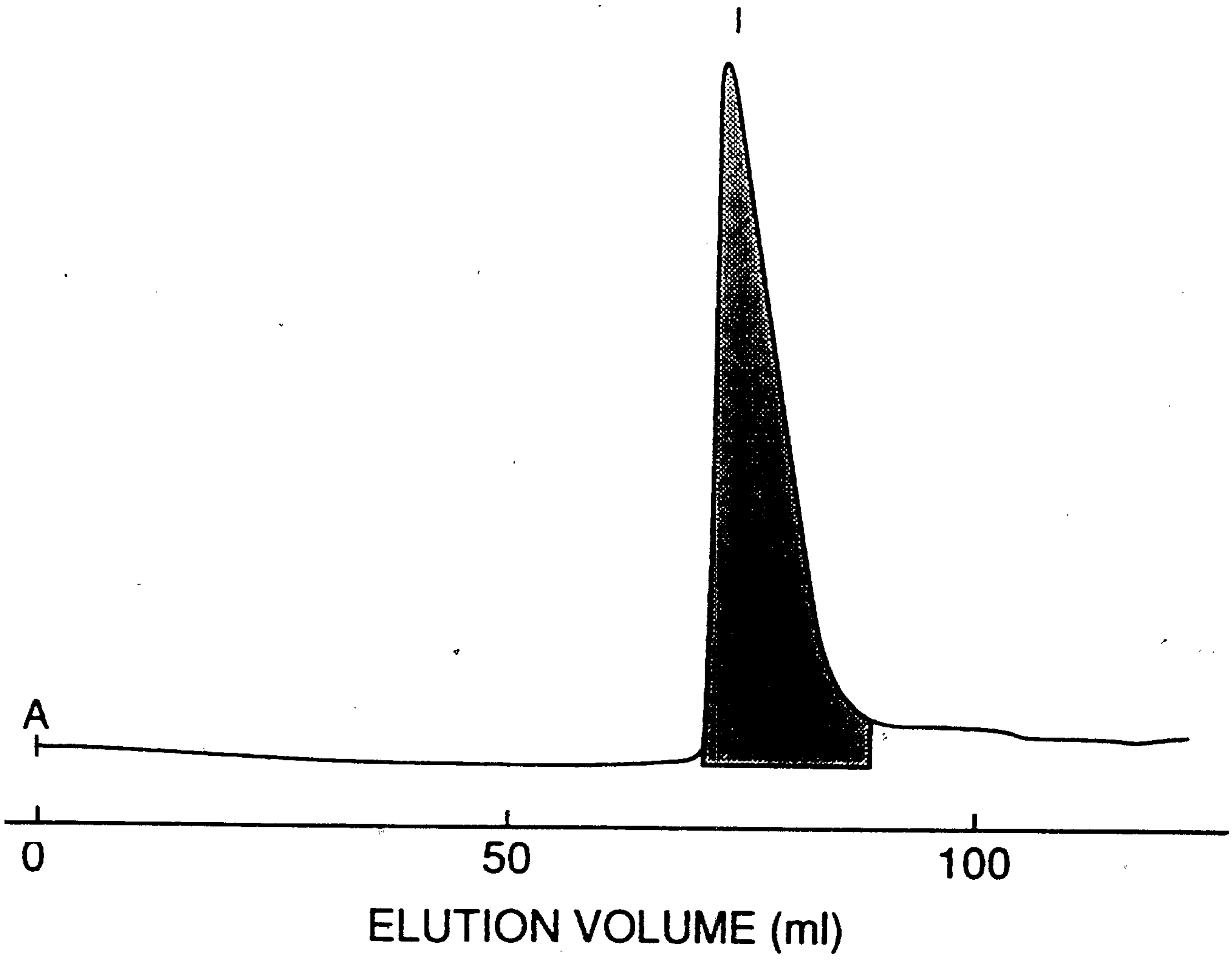
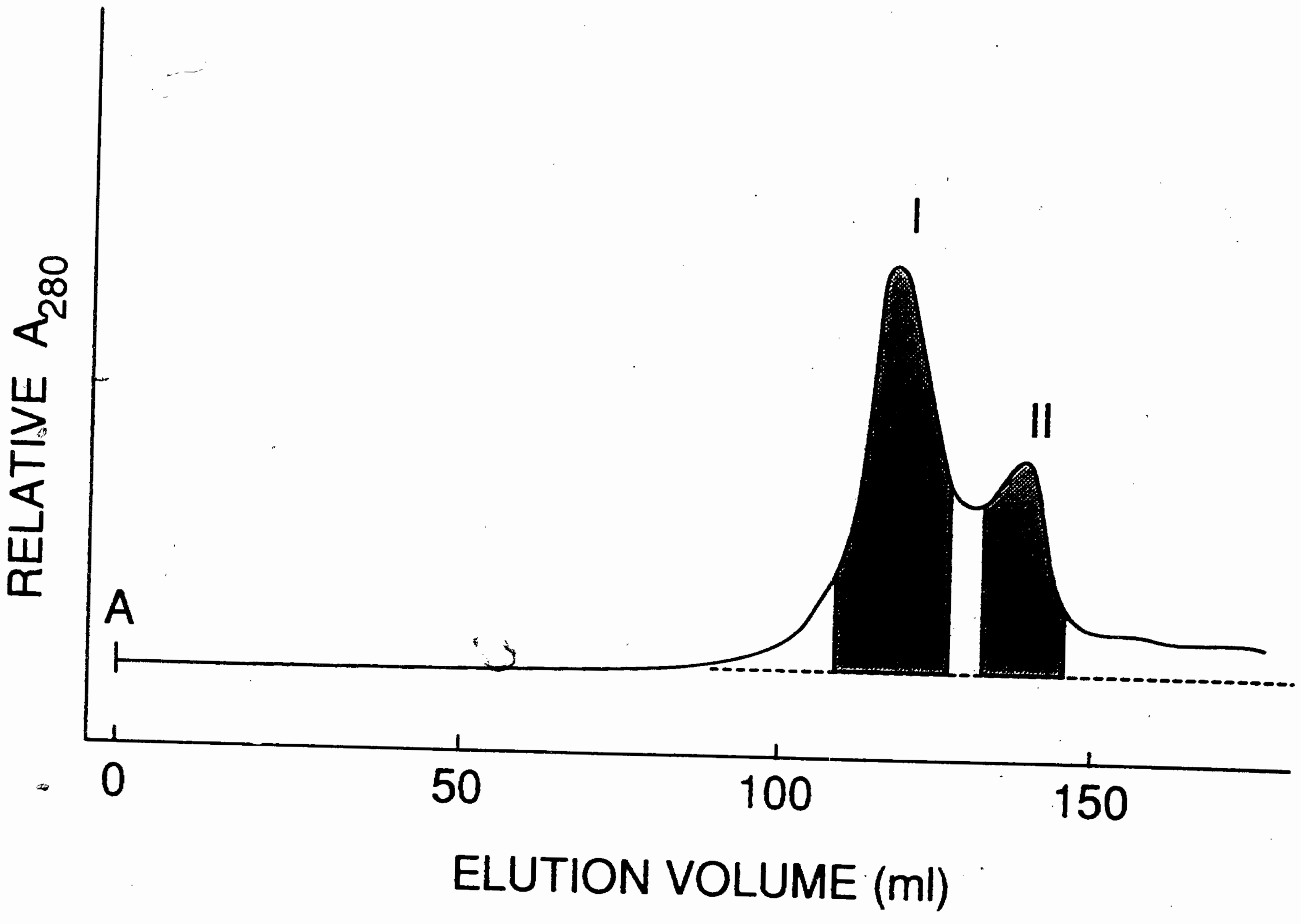

A
I
RELATIVE A_{280}
0
50
100
ELUTION VOLUME (ml)

Figure 11. Separation of B870α and B850α polypeptides by high-resolution gel filtration on Sephadex LH 60 (A) Elution with C/M/NH_4OAc containing 20% (v/v) acetic acid. Both peaks I (B870α polypeptide) and II (B850α polypeptide) were subjected to re-chromatography on the same column until sufficient purity was achieved.



RELATIVE A280
A
I
II
0
50
100
150
ELUTION VOLUME (ml)

The mixture of polypeptides in the "breakthrough" fraction (peak I) eluted from the DEAE-cellulose column (cf. Figure 9), was diluted 1:1 (v/v) with chloroform and rapidly applied to a CM-cellulose (CM 52) column (1.4 X 40 cm) which had been equilibrated in chloroform/methanol (3:1, v/v) containing 50 mM NH_4OAc. The column was washed with one column volume of the same solvent mixture. Subsequently, the polypeptides were eluted quantitatively and in high concentration with 20% (v/v) acetic acid in C/M/NH_4OAc at a flow rate of 15 ml/h (cf Figure 10). The volume of the sample was further reduced to about 2 ml by rotary evaporation prior to application to a high resolution Sephadex LH-60 column (2.1 X 80 cm). The column was eluted with C/M/NH_4OAc containing 20% (v/v) acetic acid with a flow rate of 8 ml/h. Fractions of 4 ml were collected, and pooled fractions (cf. Figure 11) were concentrated by rotary evaporation and rechromatographed on the same column until sufficient purity, as determined by the appearance of a single peak, was achieved. Finally, the polypeptides were dialyzed against distilled water and the precipitated polypeptides were freeze-dried.

2.2.16 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Mini-slab gels (100 X 84 X 1.5 mm) were prepared with 12-18% (w/v) gradients of polyacrylamide and were run verticaly using the discontinous SDS-buffer system of Laemmli

(60). Samples of membranes and light-harvesting polypeptides were solubilized prior to electrophoresis by heating at 100°C for 90 s in sample buffer containing 2% SDS (w/v), 62.5 mM Tris-HCl, pH 6.8, 5 mM β-mercaptoethanol, 10% glycerol (v/v), and 0.01% bromphenol blue (w/v). Samples were cooled and centrifuged prior to electrophoresis in a Johns Scientific Inc. microcentrifuge at 13,000 RPM for 10 min. Electrophoresis was carried out at a constant voltage of 75 mV for approximately 4-5 h at room temperature, or until the marker dye bromphenol blue had reached the bottom of the gel. Gels were stained in Coomassie blue R-250 in 25% isopropanol/10% acetic acid, destained in 10% methanol/10% acetic acid for one hour followed by further destaining in fresh solution until the gel was free from the back ground stain. Gels were photographed and then further destained to minimize color quenching during fluorography. The gels were impregnated with En^3Hance, dried using a Biorad gel drying apparatus, and exposed to Kodak XRP film for 8 weeks at -70°C.

2.2.17 High Performance Liquid Chromatography of Tryptic Fragments of B870α-polypeptides Purified from Radiolabeled Chromatophores and SDV

Lyophilized B870α protein purified from both types of membrane preprations (200 μg each) was dispersed in 0.2 M potassium phosphate buffer (200 μl), pH 8.1, using a Brownwill

Biosonic III ultrasonicator equipped with a microprobe, at a setting of 30, by a method identical to Cohn and Kaplan (21). The suspension was incubated at 37°C with trypsin at an enzyme-to-protein ratio of 1:40 (w/w) for 6 h. The reaction was stopped by the addition of 25 μl of formic acid. Samples were freeze-dried and redisolved in 100 μl of formic acid prior to HPLC analysis.

A Waters Associates Model 510 liquid chromatograph, equipped with a Model 481 variable wavelength detector and a Waters 840 data system, was employed. Separations were performed on a C_{18} spheri-5 reverse phase column (10 μm, 4 mm x 30 cm), from Brownlee Labs. Trypsin hydrolyzed protein (25 μg) was applied on the column and the elution of the peptides was achieved by a modified method of Miller *et al.* (74). A linear gradient between 100% solvent A (10% aqueous formic acid) and 100% solvent B (tetrahydrofuran) at a constant flow rate of 0.6 ml/min with a column pressure varying from 1200-1600 p.s.i. (cf. Figure 21) was used. The elution of the peptides was monitored at 280 nm. Fractions representing peaks I-IV (cf. Figure 21B & 21C) were individualy pooled together from four different runs (100 μg hydrolyzed 870α), from chromatophores and from SDV. The pooled fractions were freeze dried and resuspended in 10 ml of Aquasol for scintillation counting. Attempts to use other solvent systems (e.g., 0.1% aqueous phosphoric acid and acetonitrile or 0.1% aqueous

trifluoroacetic acid and acetonitrile) were unsuccessfull. However, use of 10% formic acid is damaging to the bonded silica surface of the packing material; therefore, exposure to formic acid was kept minimal. Generally, 10 min were required for equilibration of the column with solvent A after initial conditions on the reverse gradient programme were reached. Column precision was maintained by pumping tetrahydrofuran at 0.1 ml/min through the column overnight. The column was equilibrated in 60:40 methanol-water (v/v) for long-time storage. Solvents were degassed for 20 min under vacuum, with stirring. All runs were performed at room temperature.

Recently, Parkes-Loach *et al.* (82) have used hexafluoroacetone trihydrate to dissolve B881α and B881β of *R. rubrum* and purified them on a reverse-phase HPLC system employing a gradient of 0.1% aqueous trifluoroacetic acid and an acetonitrile/2-propanol (2:1, v/v) mixture.

CHAPTER 3

RESULTS

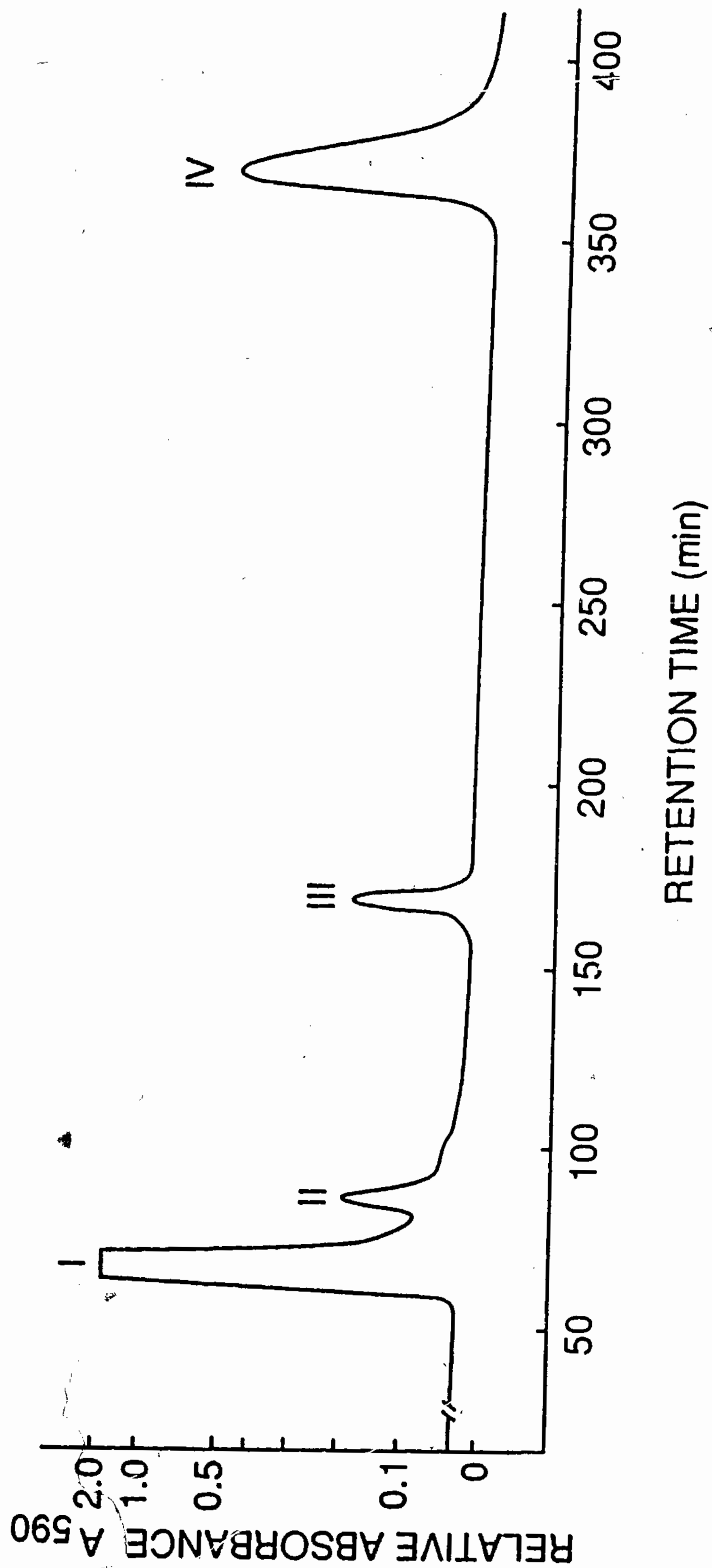
3.0.1 Reaction of the Guanidinating Reagent with Poly(L-lysine)

Following treatment of poly(L-lysine) with 2-S-thiuronium ethane sulfonate, the products were hydrolyzed with 6 M HCl. The acid hydrolysis products were separated on TLC system A, and visualized with ninhydrin. Two spots, corresponding to homoarginine (R_f 0.23) and lysine (R_f 0.47), were detected. When the acid hydrolysis products were also separated by an amino acid analyzer and compared with authentic samples of lysine (retention time 93 min) and homoarginine (retention time 368 min)] (cf. Figure 12), the same result was observed, confirming the coversion of lysine to homoarginine. Integration of the areas of peak II (lysine) and peak IV (homoarginine) from the acid hydrolysis products (cf. Figure 12) revealed that the conversion of lysine to homoarginine was 87% efficient.

3.0.2 Reaction of the Guanidinating Reagent with amino acid Functional Groups.

The reactivity of other amino acid side chains at basic pH (9.0) was investigated by reacting amino acids (with their α-amino groups blocked by a t-BOC group) with 2-S-thiuronium ethane sulfonate. The N^{α}-t-BOC derivatives of L-lysine,

Figure 12 Products of acid hydrolysis separated using Beckman Model-116 amino acid analyzer with a PA 28 ion exchange column. Peaks I-IV represent, ammonia, lysine, α-amino-β-guanidinopropionic acid (internal standard) and homoarginine respectively.



RELATIVE ABSORBANCE A 590
2.0
1.0
0.5
0.1
0
I
II
III
IV
50
100
150
200
250
300
350
400
RETENTION TIME (min)

L-tyrosine, L-histidine and L-serine were studied. The t-BOC moiety was removed by treating the respective products with trifluoroacetic acid. The unblocked amino acid products were analyzed by TLC system B. As observed earlier (in the case of the polylysine experiment), two spots were visible with ninhydrin for the treated lysine sample. One corresponded to authentic lysine (R_f 0.08) and the second one to homoarginine (R_f 0.13). With tyrosine, histidine, and serine, only single spots, corresponding to the authentic parent amino acids, were visible, indicating that 2-S-thiuronium ethane sulfonate did not react at pH 9.0 with the imidazole of histidine (R_f 0.06), the hydroxyl group of serine (R_f 0.22), nor the phenol of tyrosine (R_f 0.44).

To confirm the guanidination of lysine, 2-S-[^{14}C]thiuronium ethane sulfonate was also reacted with N^{α}-t-BOC-lysine. The product was analyzed on TLC with system B, and was found to co-migrate with starting material at R_f 0.75 (cf. Table 1). Upon removal of the t-BOC groups by treating the product with trifuoroacetic acid, lysine and homoarginine could now be separated by TLC in system B.

These spots were scraped from the TLC plate and counted for radioactivity (cf. Table 1). The homoarginine spot had a ca. 10-fold higher radioactivity than both the background and the lysine spot, thus confirming the incorporation of the ^{14}C-labeled guanidino group into homoarginine. When viewed

under ultraviolet light, the unreacted guanidinating reagent appeared as a dark spot at R_f 0.35-0.38. This spot was 19 fold higher in radioactivity than the homoarginine spot due to the use of a 20-fold molar excess of the guanidinating reagent to the amino acid derivatives in the labeling reaction.

N-acetyl-L-cysteine was also labeled with 2-S-thiuronium ethane sulfonate, as N-t-BOC-L-cysteine was not commercially available. The resulting products were analyzed by TLC with system B and visualized by two different methods:

1) KI/starch spray and
2) alkaline ferrocyanide/nitroprusside reagent.

The former reagent detects peptides, amides, ureides and thioureides while the latter detects guanidinium and thiuronium ions by a specific color reaction. At pH 9.0, the thiol of N-acetyl-L-cysteine reacted with 2-S-thiuronium ethane sulfonate; the results of TLC analysis in system B are shown in Table 2. The product of this reaction exhibited a major unidentified red spot, detected with the nitroprusside reagent, at R_f 0.60. The unreacted N-acetyl-L-cysteine appeared as a blue spot at R_f 0.21. Although thiourea has a similar R_f (0.59) to the major unidentified spot (R_f 0.60), thiourea gives a purple color with the nitroprusside reagent. The product was shown not to be N,N'-diacetyl-L-cystine (R_f 0.38), eliminating the possibility of air oxidation of

Table 1: Thin-Layer Chromatography of the Products of the Guanidination of N -t-BOC-L-lysine with 2-S-Thiuronium ethane sulfonate.

sample	R_f values[a]				
Nα-t-BOC-L-lysine					0.75
Nα-t-BOC-L-homoarginine					0.75
L-lysine	0.08				
L-homoarginine		0.13			
2-S-thiuronium ethane sulfonate				0.35	
N -t-BOC-L-lysine +	0.08	0.13		0.35	
2-S [^{14}C]thiuronium ethane-sulfonate + CF_3COOH	[64]	[629]	[64]	[10994]	[51]

[a] On silica gel $60F_{254}$ sheets in 2-propanol/acetic acid/water (4:1:1 v/v); total dpm recovered from spots in square brackets.

Table 2: Thin-Layer Chromatography of the Products of the Guanidination of N^{α}-Acetyl-L-cysteine with 2-S-Thiuronium ethane sulfonate.

sample	R_f values[a]				
2-S-thiuronium ethane sulfonate		0.35			
2-S-thiuronium ethane sulfonate + CF_3COOH		0.42			(0.59)
urea				0.52	
thiourea					0.59
thiourea + CF_3COOH					0.59
N-acetyl-L-cysteine				0.51	
N,N'-diacetyl-L-cystine		0.38			
N-acetyl-S-(2-sulfoethyl)-L-cysteine		0.35			
N-acetyl-L-cysteine + 2-S-thiuronium ethane sulfonate	(0.21)	0.36		0.50	0.60
N-acetyl-L-cysteine + 2-S-[^{14}C]thiuronium ethane sulfonate + CF_3COOH	(0.21) [64]	0.38 [3792]	0.45 [371]	(0.50) [0.60]	

[a] On silica gel $60F_{254}$ sheets in 2-propanol/acetic acid/water (4:1:1 v/v); total dpm recovered from spots in square brackets.

N-acetyl-L-cystine to the disulfide during TLC. The product was also found not to be N-acetyl-S-(2-sulfoethyl)-L-cysteine (cf. Equation 4, Discussion). We could not detect, however, whether some of this product had in fact formed, as it exhibited the same R_f value as the 2-S-thiuronium ethane sulfonate in the solvent system used (cf. Table 2).

The major product (R_f 0.60) was shown to be another reaction product, N-acetyl-S-amidino-L-cysteine (cf. Equation 5, Discussion), by the following results:

1) it gave a red color with the alkaline ferricyanide/nitroprusside reagent charateristic of an amidino group (eg. homoarginine and 2-S-thiuronium ethane sulfonate both give red colors with this reagent, whereas N-acetyl-L-cysteine gives a blue color and thiourea a purple-blue color).

2) the structural assignment of N-acetyl-S-amidino-L-cysteine to the major product purified from a large-scale reaction of the guanidinating reagent with N-acetyl-L-cysteine (cf. Materials and Methods) was consistent with the assigned peaks in both the ^{13}C and ^{1}H NMR spectra (Figures 5 and 7, respectively).

3) when 2-S-[^{14}C]thiouronium ethane sulfonate was used as the guanidinating reagent, the product was shown to contain radioactivity by the following procedure:

Analysis of the radioactivity contained in the product spot at R_f 0.60 was not possible because it co-chromatographed with traces of [^{14}C]thiourea (present as an impurity in the guanidinating reagent). Treatment with trifluoroacetic acid, however, was found to convert the R_f 0.60 spot to another spot at R_f 0.45, which was now well-separated from both the guanidinating reagent and thiourea. Treatment with 2.4 M HCl also yielded the same spot at R_f 0.45 (results not shown). Neither the guanidinating reagent nor thiourea was altered significantly by treatment with trifluoroacetic acid, although the former compound yielded some thiourea (cf. Table 2) due, perhaps, to an elimination reaction. When the TLC plates were analyzed for radioactivity (cf. Table 2), the level in the R_f 0.45 product was 6-fold above the background spot, confirming the fact that it still contained the ^{14}C atom derived from the thiouronium group of the guanidinating reagent. It is very likely, although it has not been shown definitively, that the labeled compound at R_f 0.45 is 2-iminothiazolidine-4-carboxylic acid (cf. Equation 6, Discussion).

3.0.3 Reaction of the Guanidinating Reagent with an α-amino Group.

Glycylglycine was treated with 2-S-thiuronium ethane sulfonate, and the product was applied to a Sephadex G-10 column (cf. Figure 13). N-Amidinoglycylglycine (peak I) was eluted before the labeling reagent (peak II). N-Amidinoglycylglycine and the labeling reagent have nearly identical molecular weights. However, Sephadex G-10 is known to behave unpredictably at lower molecular weights (84) because separation may depend on other factors such as the shapes of the molecules. When analyzed on TLC in system A, N-amidinoglycylglycine had an R_f of 0.48 (cf. Table 3). The purified N-amidinoglycylglycine was treated with 6 M HCl; two spots corresponding to synthesized N-amidinoglycine (R_f 0.27) and authentic glycine (R_f 0.38) were observed (cf. Table 3), thus confirming the reaction of a peptide α-amino group with 2-S-thiuronium ethane sulfonate.

3.0.4 Homoarginine as a Potential Cleavage Site for Trypsin

TLC analysis of the 30 min trypsin hydrolysis products of the pentapeptide, **Arg-Lys-Asp-Val-Tyr**, when analyzed on cellulose TLC sheets in butanol/pyridine/acetic acid/water solvent system, gave three major spots (cf. Table 4). The spot at R_f 0.11 was most likely due to the dipeptide, Arg-Lys, since it did not correspond to either authentic lysine (R_f

Figure 13 Elution profile of N-amidinoglycylglycine using a Sephadex G-10 column. Peaks I and II represent N-amidinoglycylglycine and the guanidinating reagent respectively.

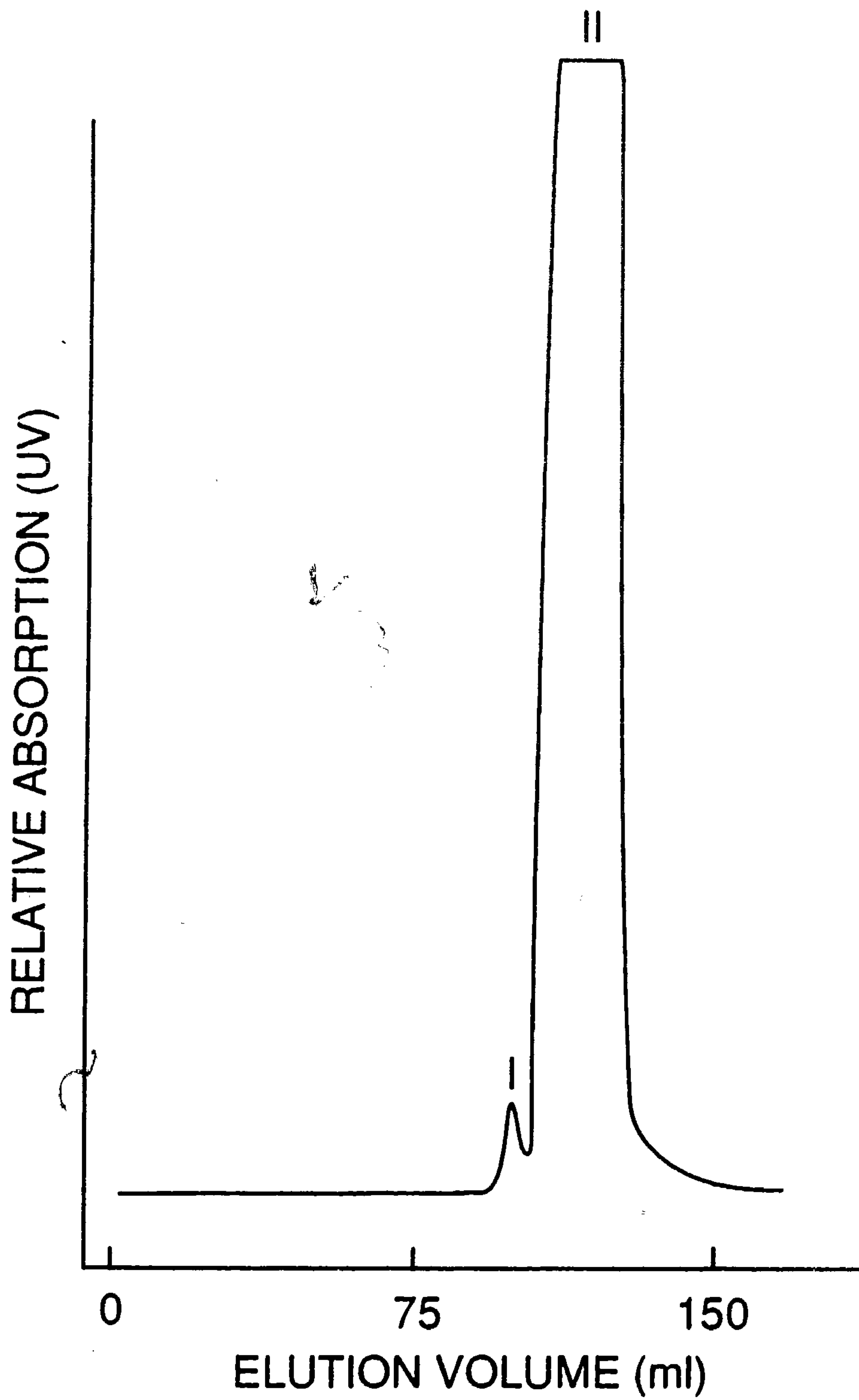

II
I
RELATIVE ABSORPTION (UV)
0
75
150
ELUTION VOLUME (ml)

Table 3: Thin-Layer Chromatography of the Products of the Guanidination of Glycylglycine with 2-S-Thiuronium ethane sulfonate.

sample	R_f values[a]			
glycine		0.38		
glycine + 2-S-thiuronium-ethane sulfonate	0.27	0.38		0.53
glycylglycine				0.52
glycylglycine + 2-S-thiuronium-ethane sulfonate			0.48	0.53
2-S-thiuronium ethane sulfonate				0.50
N-amidinoglycylglycine[b]			0.48	
N-amidinoglycylglycine[b] + 6 M HCL	0.27	0.38		

[a] On silica gel 60F254 sheets in chloroform/methanol/9 M ammonia (2:2:1 v/v); Spots were visualised with ninhydrin and alkaline ferricyanide/nitroprusside reagent.

[b] isolated using sephadex-G10 column.

0.16) or arginine (R_f 0.21). Spots at R_f 0.30 and 0.50 were due to undigested pentapeptide and the tripeptide, Asp-Val-Tyr, respectively.

The pentapeptide was reacted with 2-S-thiuronium ethane sulfonate. The guanidinated pentapeptide was isolated by gel filtration on a Sephadex G-10 column (cf. Figure 14) and subjected to trypsin hydrolysis for 3 h. TLC analysis (cf. Table 4) revealed a minor spot corresponding to either L-arginine or L-homoarginine, or both; since they appeared at the same spot (R_f 0.22) they were undistinguishable. A major spot corresponding to guanidinated undigested pentapeptide (R_f 0.36) was also observed. A spot at R_f 0.44 may have been due to a resulting tetrapeptide Har-Asp-Val-Tyr, and a minor spot at R_f 0.51 due to the tripeptide, Asp-Val-Tyr (cf. Discussion). Trypsin hydrolysis for 30 min did not show any spots other than that at R_f 0.36, demonstrating that the hydrolysis reaction was rather slow in the case of guanidinated pentapeptide.

3.0.5 Reaction of the Guanidinating Reagent with Membranes of R. sphaeroides

Figure 15 shows the results of varying the incubation conditions during the labeling of purified chromatophores with 2-S-[^{14}C]thiuronium ethane sulfonate. With increasing time the rate of radiolabeling was very fast for the initial 30 min,

Figure 14. Elution profile of guanidinated pentapeptide **Arg-Lys-Asp-Val-Tyr** on a Sephadex G-10 column. Peaks I and II represent guanidinated pentapeptide and guanidinating reagent respectively.

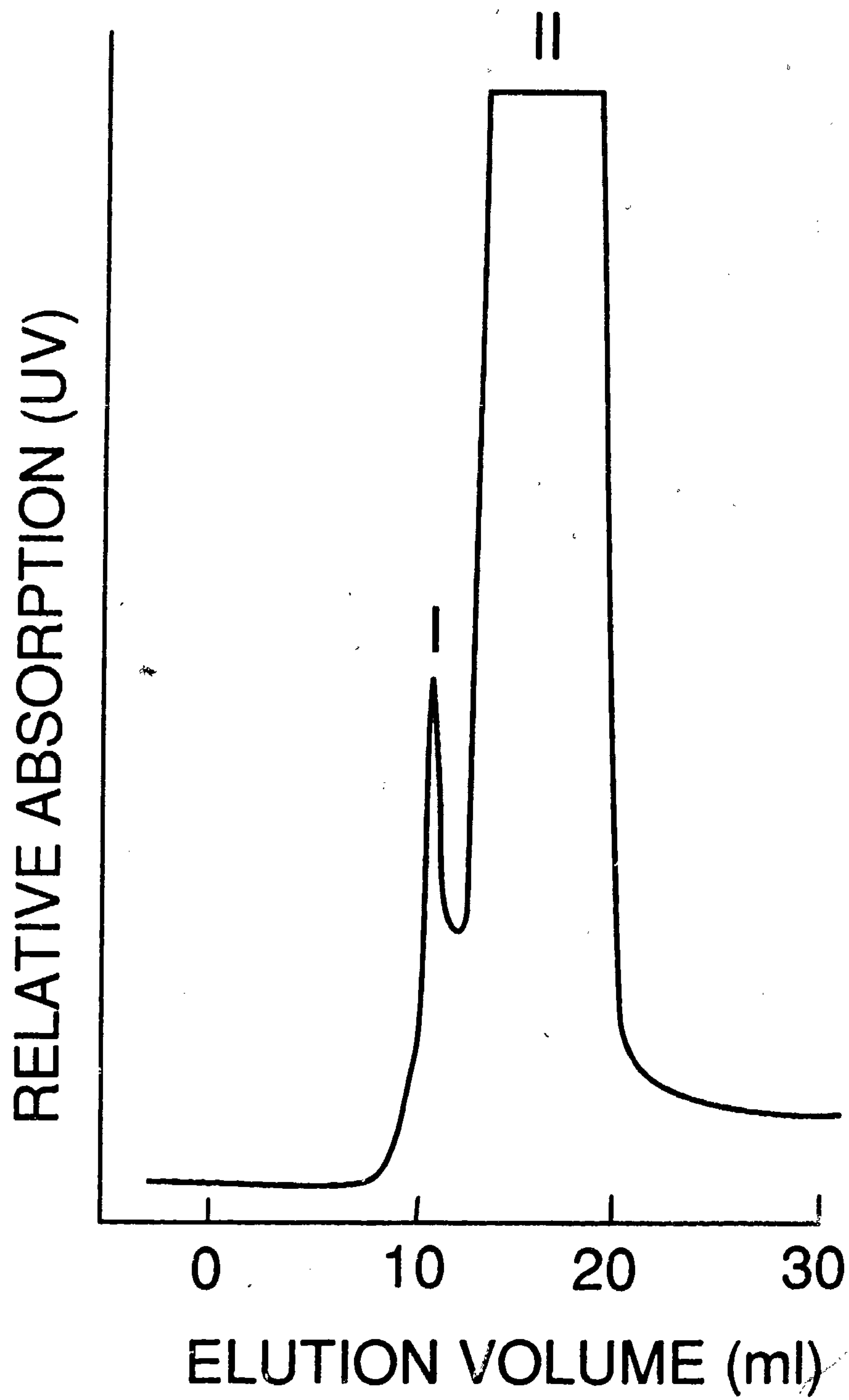

II
I
RELATIVE ABSORPTION (UV)
0
10
20
30
ELUTION VOLUME (ml)

Table 4: Thin-Layer Chromatography of the Trypsin hydrolysis products of 2-S-Thiuronium ethane sulfonate Guanidinated Pentapeptide.

sample	R_f values[a]						
L-lysine		0.16					
L-arginine			0.21				
L-homoarginine			0.22				
pentapeptide				0.31			
pentapeptide[b]					0.36		
pentapeptide + trypsin[c]	0.11			0.30			0.50
pentapeptide[b] + trypsin[d]			(0.22)		0.36	0.44	(0.51)

a On cellulose TLC sheets in butanol/pyridine/acetic acid/water (50:33:1:40 v/v); minor bands in parenthesis;

b Pentapeptide labeled with 2-S-thiuronium ethane sulfonate and isolated on sephadex G-10 column.

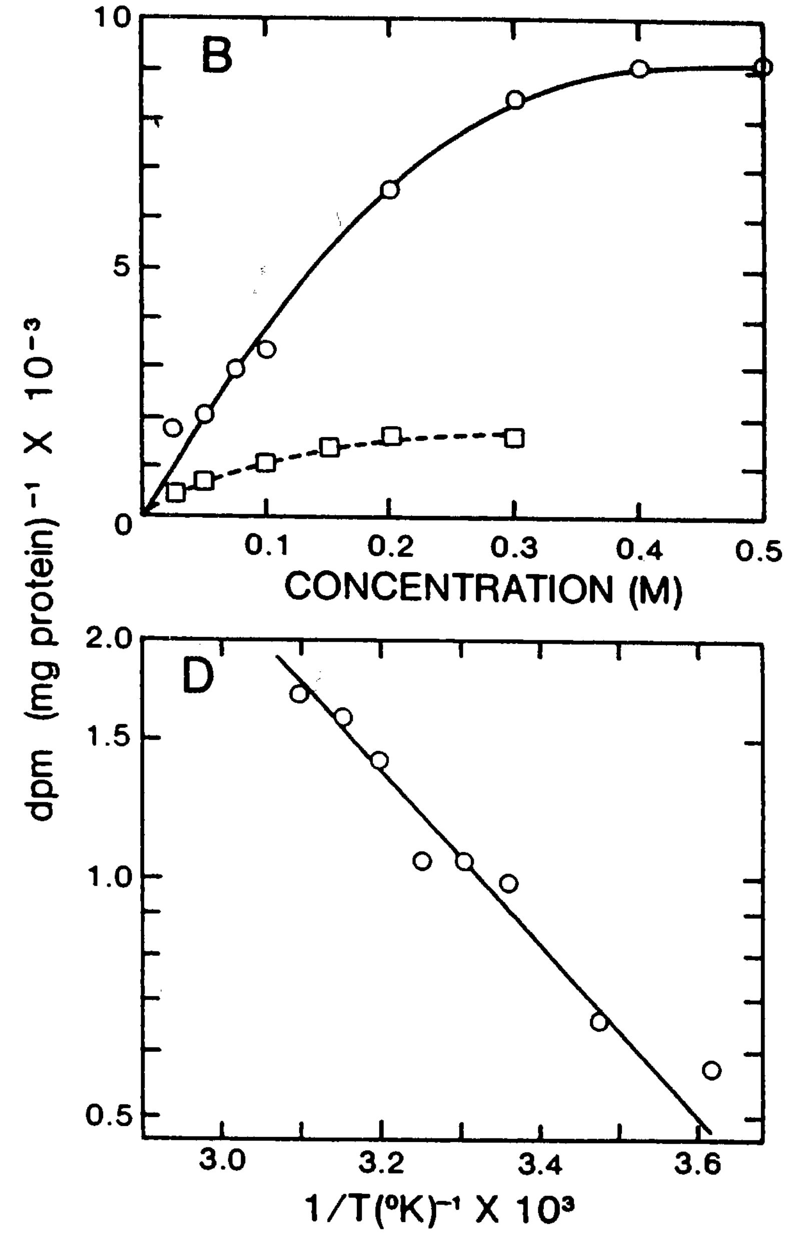
c Trypsin hydrolysis for 30 min.

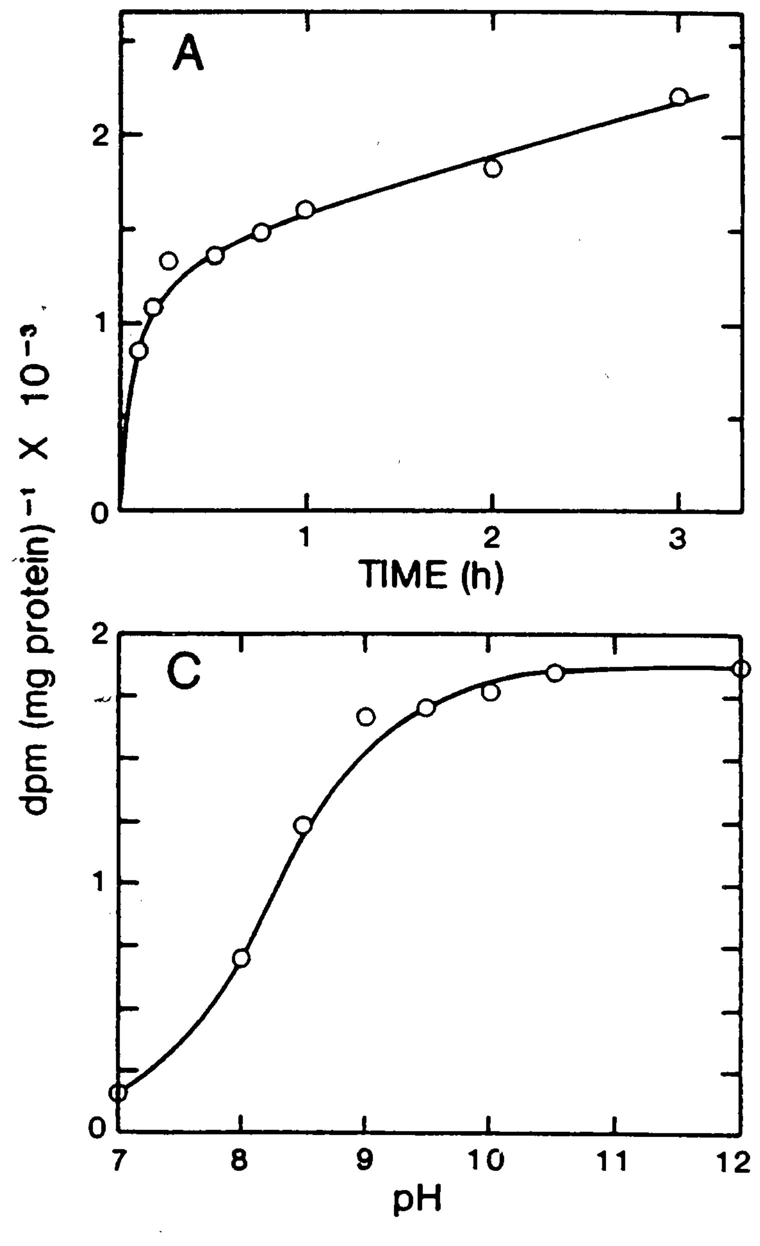
d Trypsin hydrolysis for 3 h.

followed by a slow increase without reaching saturation even after 3 h (cf. Figure 15A). When the concentration of the reagent was increased (cf. Figure 15B) under standard conditions (0.2 M 2-S-[^{14}C]thiuronium ethane sulfonate incubated for 2 h at 30°C at pH 9.0), a saturation curve was observed. The extent of labeling was ca. 90% of its maximum value at the saturation point of the reagent in water (0.3 M). A sigmoidal curve was, however, observed with increasing pH (cf. Figure 15C) with 90% of maximum label attained at pH 9.5. When the temperature was varied (cf. Figure 15D), the extent of labeling did not reach saturation even at 50°C.

When the labeling of whole cells (followed by isolation and purification of the chromatophore fraction) was compared to the labeling of purified chromatophores (cf. Figure 15B), the level of activity in the former was found to be only 20% of the level of activity in purified chromatophores when equivalent concentrations of guanidinating reagent were used. In an experiment labeling whole cells with undiluted 2-S-[^{14}C]thiuronium ethane sulfonate (at an estimated concentration of 0.1 M) a purified chromatophore fraction with a specific activity of 5100 dpm/mg protein resulted. This was considered too low a value to study the labeling of membrane proteins from the periplasmic side of the membrane; hence SDV were prepared from *R. sphaeroides* in a manner designed to ensure that the majority were of the "periplasmic-side-out"

Figure 15. Specific activities (per mg. of protein) of washed chromatophores after labeling purified chromatophores (O) or whole cells (□) of *R. sphaeroides* with 2-S-[^{14}C]thiuronium ethane sulfonate as a function of (A) time of incubation, (B) concentration of guanidinating reagent, (C) pH of the incubation buffer, and (D) temperature of the incubation.


A
B
TIME (h)
CONCENTRATION (M)
dpm (mg protein)⁻¹ X 10⁻³
0
1
2
3
0
5
10
0.1
0.2
0.3
0.4
0.5


C
D
pH
1/T(°K)⁻¹ X 10³
0
1
2
7
8
9
10
11
12
0.5
1.0
1.5
2.0
3.0
3.2
3.4
3.6

orientation (cf. Discussion).

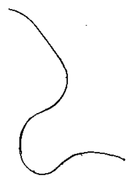
3.0.6 Purification of Spheroplast Derived Vesicles using Affinity Chromatography

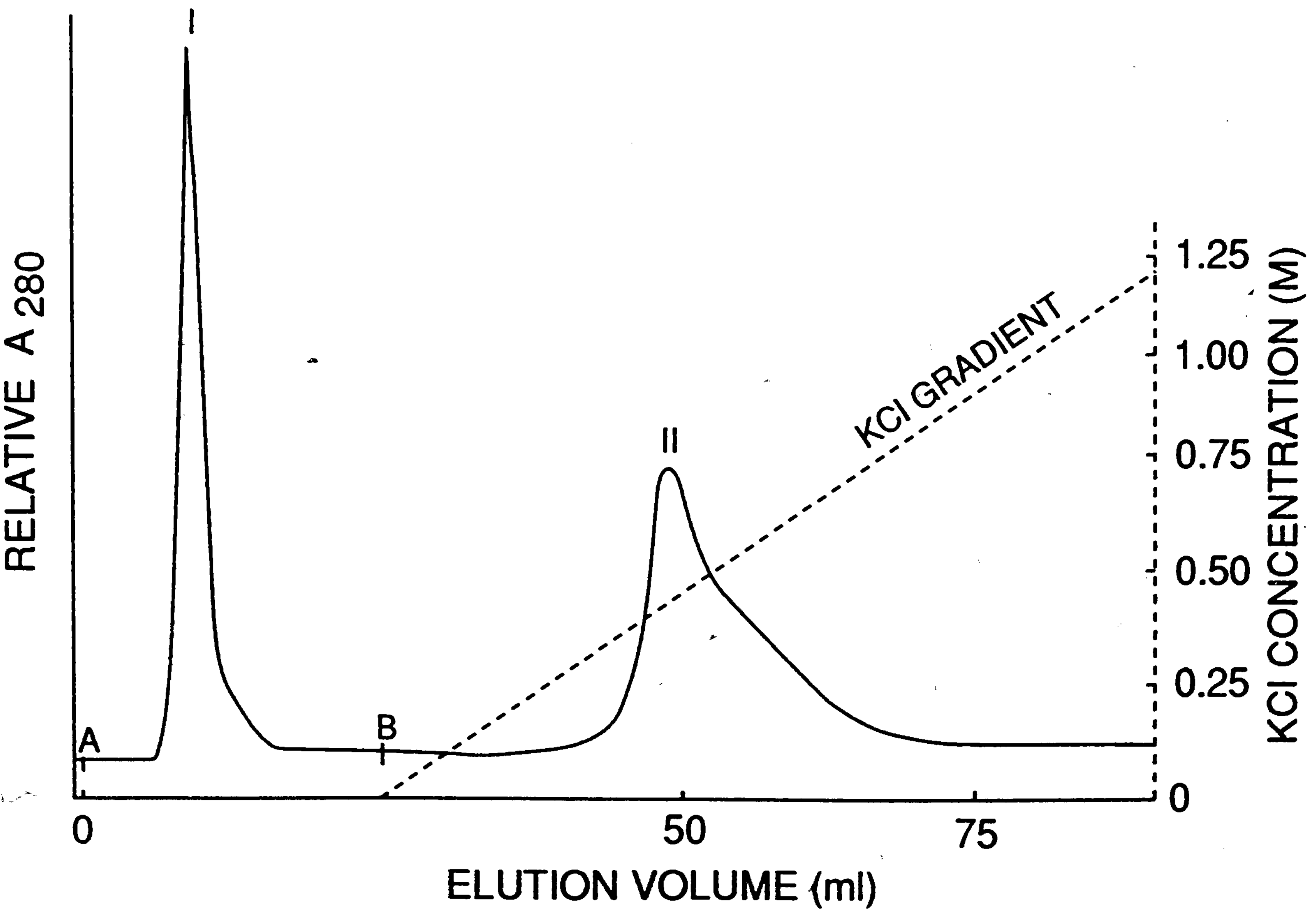
The SDV were purified using a cytochrome c-linked Sepharose 4B column (cf. Figure 16). Fraction I was eluted with the equilibration buffer at low ionic strength (0.15 M KCl) and represented unbound vesicles which lacked a binding site for cytochrome c and, thus, were of "cytoplasmic-side-out" orientation. Fraction II was eluted with a gradient of increasing KCl. Vesicles eluted at ca. 0.3-0.6 M KCl in the gradient represented the bound fraction of "periplasmic-side-out" orientation. No more peaks were observed even at 1.5 M KCl concentration. When the column was washed with the equilibrium buffer containing 0.1% Triton X-100, another peak (not shown in Figure 16) was observed. This other peak could have been due to the disaggregation of aggregated SDV which had failed to enter the gel, or to the dissolution of their membranes by the detergent.

3.0.7 The Near-Infrared Spectrum of Membranes

The near-infrared spectrum of purified chromatophores (cf. Figure 17) demonstrated two maxima, one at 800 nm and a second one at 850 nm. The absorption at 870 nm was almost equal to that at 850 nm. The spectrum obtained with SDV from bacteria grown under very high light intensity showed a reduction in

Figure 16. Separation of "cytoplasmic-side-out" and "periplasmic-side-out" vesicles from a preparation of SDV using a cytochrome *c*-linked sepharose 4B column. Peaks I and II represent "cytoplasmic-side-out" "periplasmic-side-out" vesicles, respectively.




I
II
A
B
KCl GRADIENT
RELATIVE A 280
KCl CONCENTRATION (M)
0
0.25
0.50
0.75
1.00
1.25
ELUTION VOLUME (ml)
0
50
75

absorption at the 850 nm band by 50% with respect to the 870 nm band when compared to the chromatophore spectrum. This is a classic example of where the B800-850 complex can be regulated by various environmental conditions such as changes in light intensity (97). This enhanced predominance of the B870 complex was desirable for SDV preprations since B870α (which is associated with the B870 complex) was required in large amounts for trypsin digestion and subsequent high performance liquid chromatographic analysis.

3.0.8 SDS-PAGE of Membranes Demonstrating Impermiability to the Guanidinating Reagent

The results of the separation of membrane proteins by SDS-PAGE and analysis of the gels for radioactive proteins by flourography are shown in Figure 18 for the purified chromatophore and SDV fractions. The major proteins labeled in the chromatophore fraction included polypeptides with apparent M_r-values similar to the α- and β-subunits of the coupling factor (M_r 55,000 and 51,000 Da), the RC_H (M_r 28,000 Da), RC_L (M_r 21,000 Da), and light-harvesting polypeptides. In the case of the SDV (cf. Figure 18), more polypeptides were visible in the gel due to the presence of cytoplasmic membrane and apparent outer membrane components. Although the B870α polypeptide (which appears in the upper light-harvesting band) and a polypeptide with an apparent M_r-value similar to the RC_M (M_r 24,000 Da) polypeptide were observed to be heavily

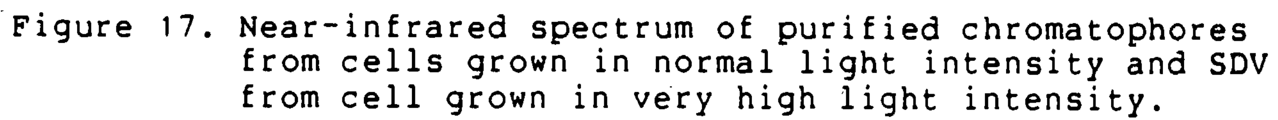
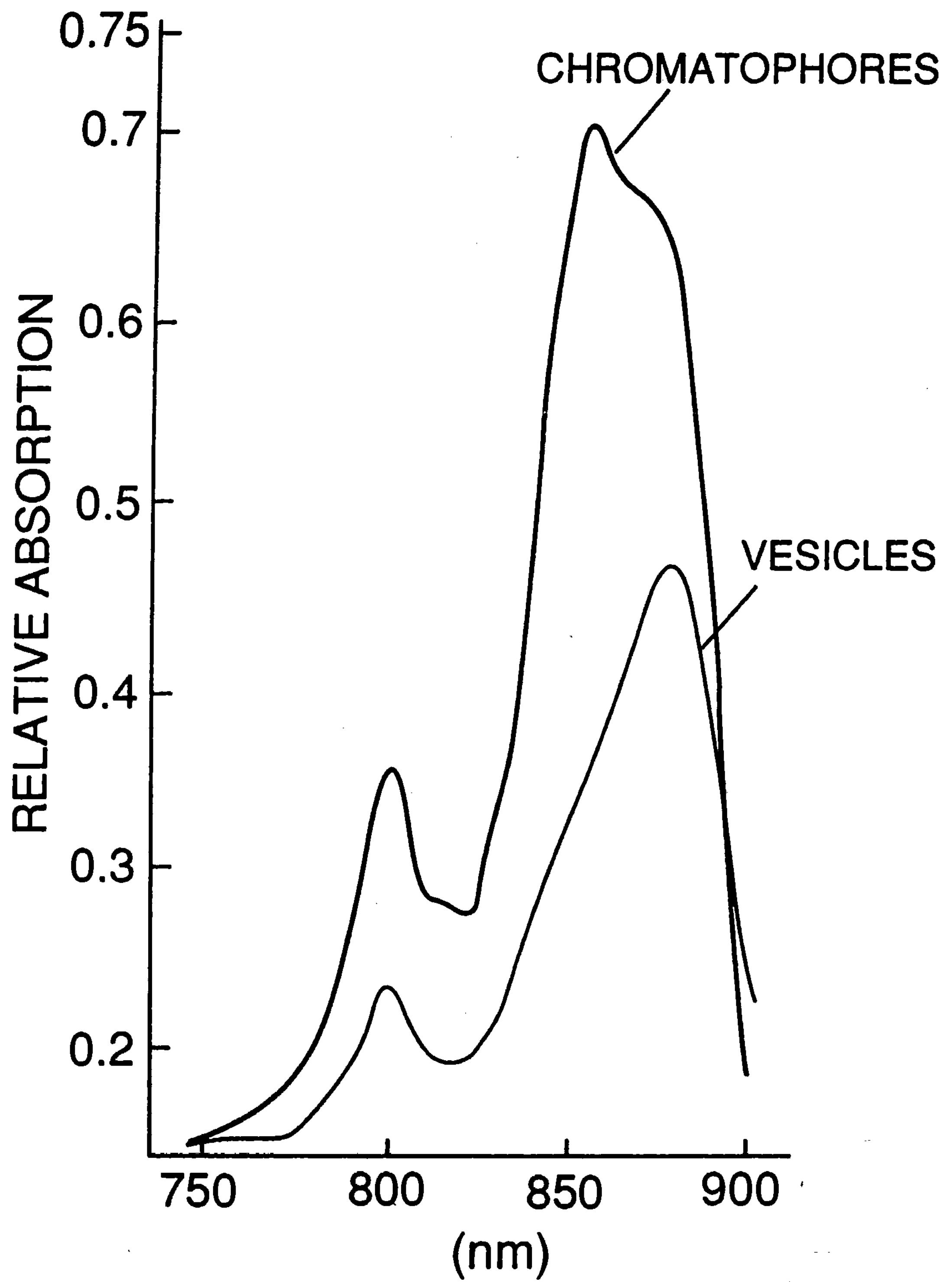


Figure 17. Near-infrared spectrum of purified chromatophores from cells grown in normal light intensity and SDV from cell grown in very high light intensity.


0.75
0.7
0.6
0.5
0.4
0.3
0.2
RELATIVE ABSORPTION
CHROMATOPHORES
VESICLES
750
800
850
900
(nm)

labeled, no activity was detected in the α- and β-subunits of the coupling factor after 8 weeks of fluorography, indicating that the guanidinating reagent had not penetrated through the membrane.

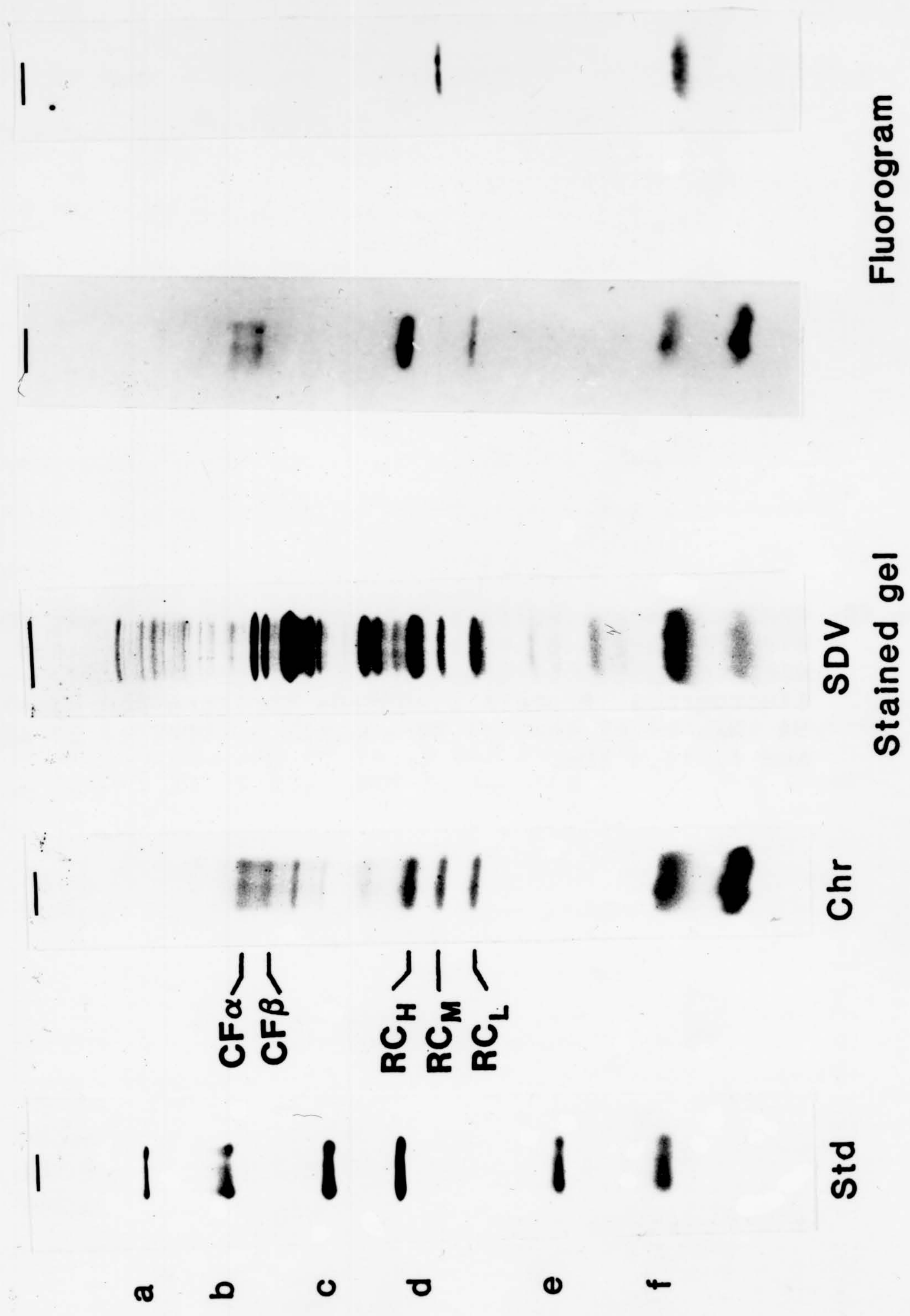
3.0.9 SDS-PAGE of Light-Harvesting Proteins purified from Labeled Chromatophores

The four light-harvesting proteins were purified as described in Materials and Methods from radiolabeled chromatophores and run on SDS-PAGE (cf. Figure 19). The light-harvesting proteins did not show any significant contamination due to any other proteins. Fluorography after 8 weeks demonstrated that all four proteins, i.e., the B870α, B850α, B870β and B850β polypeptides, were radiolabeled. The specific activities of the purified polypeptides is given in Table 5.

Table 5: Specific activities of Purified Light-Harvesting Proteins from Radiolabeled Chromatophores and SDV.

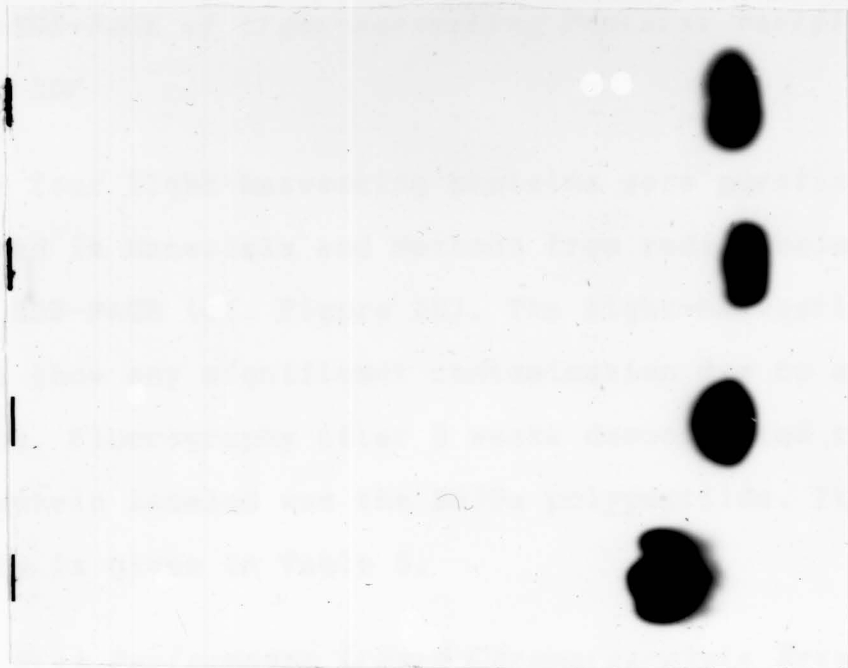
	DPM/mg protein	
protein	chromatophore	SDV
B870α	25,700	24,300
B850α	23,900	-
B870β	24,600	-
B850β	18,900	-

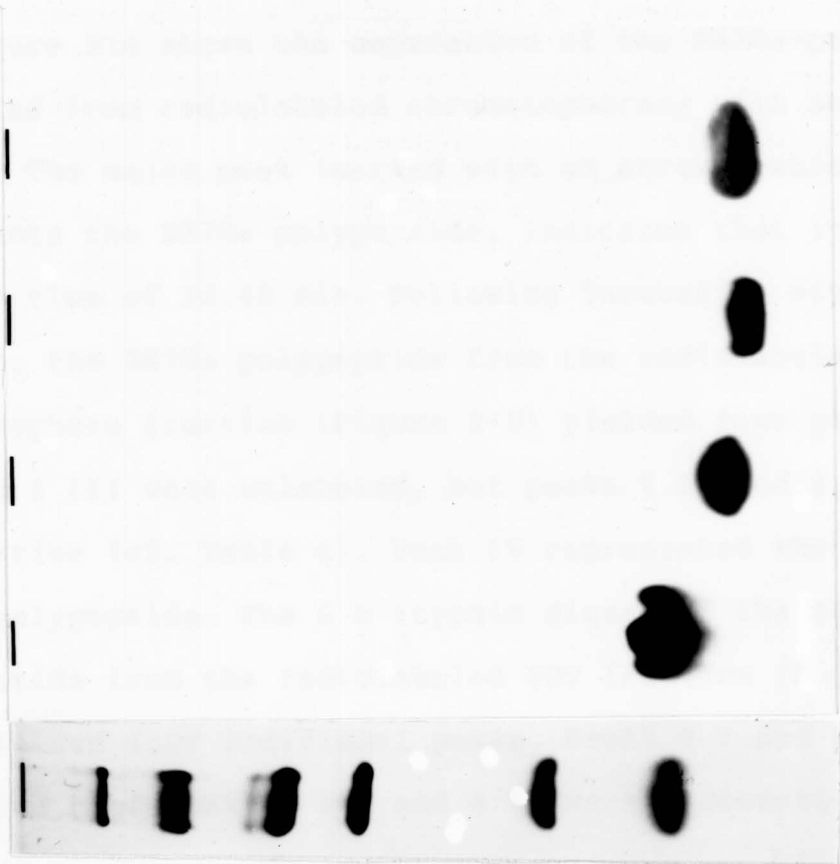
Figure 18. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoreis of chromatophores and SDV stained with Coomassie blue and the corresponding fluorograms. Protein standards employed had M_r a) 94 kDa, b) 67 kDa, c) 43 kDa, d) 30 kDa, e) 20 kDa, and f) 14.4 kDa.



Std
Chr
SDV
Stained gel
Fluorogram
a
b
c
d
e
f
CFα
CFβ
RC_H
RC_M
RC_L

Figure 19. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoreis of light-harvesting proteins purified from radiolabeled chromatophores, showing the gel stained with Coomassie blue (left) and the corresponding fluorograms (right). Protein standards employed had M_r a) 94 kDa, b) 67 kDa, c) 43 kDa, d) 30 kDa, e) 20 kDa, and f) 14.4 kDa.


Fluorogram


Std
B870α
B850α
B870β
B850β
Stained gel
a
b
c
d
e
f

3.0.10 SDS-PAGE of Light-Harvesting Proteins Purified from Labeled SDV

The four light-harvesting proteins were purified as described in Materials and Methods from radiolabeled SDV and run on SDS-PAGE (cf. Figure 20). The light-harvesting proteins did not show any significant contamination due to any other proteins. Fluorography after 8 weeks demonstrated that the only protein labeled was the B870α polypeptiide. Its specific activity is given in Table 5.

3.0.11 High Performance Liquid Chromatograhpic Results of the Trypsin Digest of B870α from Labeled Chromatophores and SDV

Figure 21A shows the separation of the B870α-polypeptide (purified from radiolabeled chromatophores) with an HPLC column. The major peak (marked with an arrow), which represents the B870α polypeptide, indicates that it has an elution time of 26.45 min. Following incubation with trypsin for 6 h, the B870α polypeptide from the radiolabeled chromatophore fraction (Figure 21B) yielded four peaks. Peaks # 1 and # III were unlabeled, but peaks # II and # IV were radioactive (cf. Table 6). Peak IV represented the undigested B870α polypeptide. The 6 h trypsin digest of the B870α polypeptide from the radiolabeled SDV fraction (Figure 21C) also yielded four individual peaks. Peaks # I and # IV were unlabeled, but peaks # III and # IV were radioactive (cf.

Figure 20. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoreis of light-harvesting proteins purified from radiolabeled SDV, showing the gel stained with Coomassie blue (left) and the corresponding fluorograms (right). Protein standards employed had M_r a) 94 kDa, b) 67 kDa, c) 43 kDa, d) 30 kDa, e) 20 kDa, and f) 14.4 kDa.

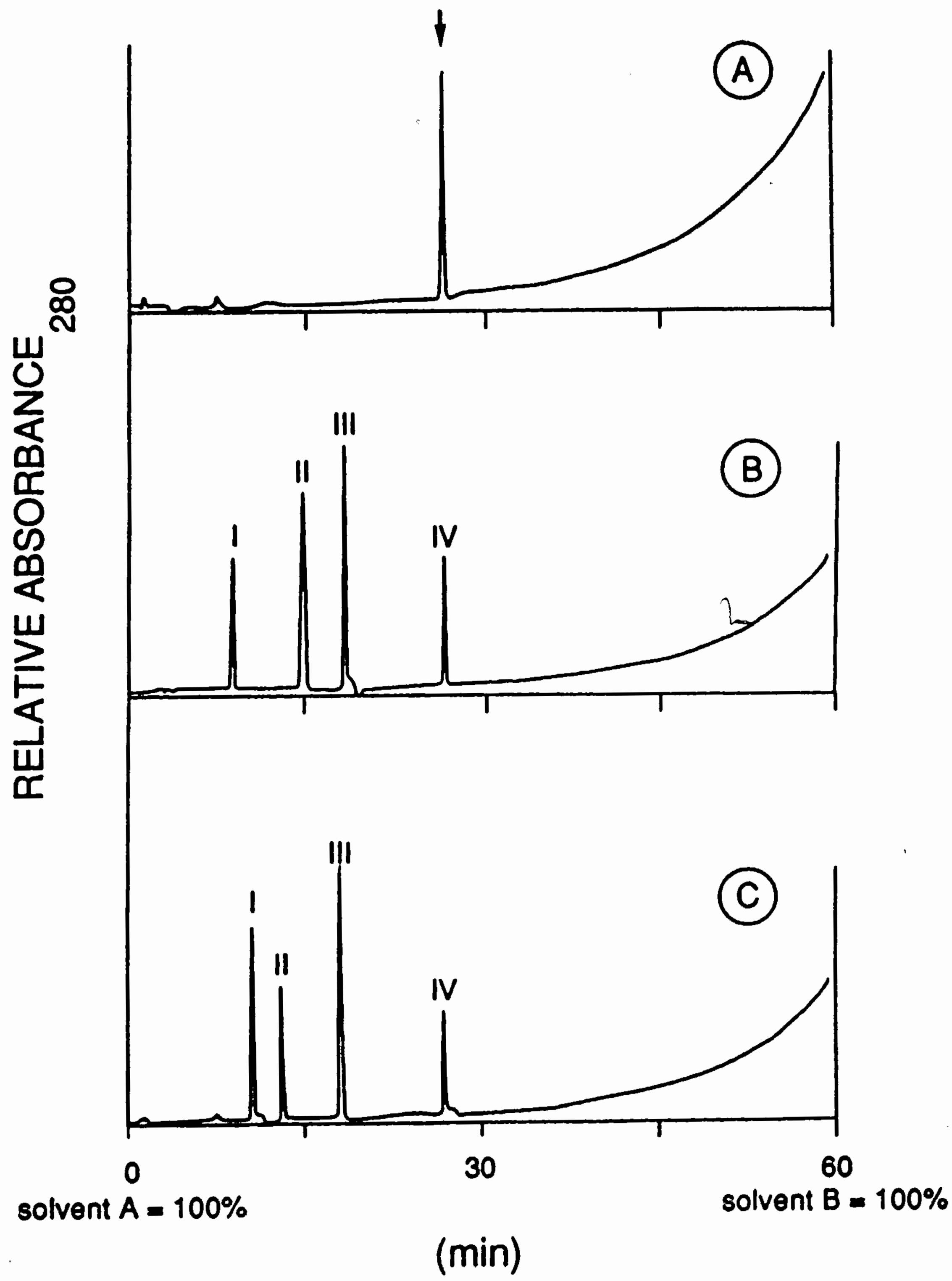
Fluorogram
Stained gel
Std
870α
850α
870β
850β
a
b
c
d
e
f

Table 6). Again, peak # IV represented the undigested B870α polypeptide.

Table 6: Radioactivity of Trypsin Fragments of B870α Purified by HPLC.

	DPM for trypsin peptide fragments from	
Peak #	chromatophores	SDV
I	31	31
II	1361	28
III	29	1239
IV	477	320

Figure 21. High performance liquid chromatograms of (A) Purified B870α, and trypsin digests of B870α purified from (B) chromatophores (C) SDV.


A
B
C
I
II
III
IV
I
II
III
IV
RELATIVE ABSORBANCE 280
0
30
60
solvent A = 100%
solvent B = 100%
(min)

CHAPTER 4

DISCUSSION

Both the chemical analysis of 2-S-thiuronium ethane sulfonate and its ^1H and ^{13}C NMR spectra are consistent with its structure (cf. Materials & Methods). Since the compound is a salt, its solubility is limited to water only. A mass spectrum of the compound was not obtained since it decomposed at high temperature before being vaporized. The guanidinating reagent reacted with the ϵ-amino group of lysyl residues in the case of both poly(L-lysine) and N^{α}-t-BOC-lysine, to give homoarginyl residues. It also reacted with the thiol group of N-acetyl-L-cysteine to yield N-acetyl-S-amidino-L-cysteine. The reagent did not react with the functional groups of tyrosine, histidine, or serine at pH 9.0. Although O-methylisourea has been shown to modify histidyl residues of β-lipoprotein, the expected product, γ-guanidino histidine, was not identified upon labeling of histidine or histidylhistidine by Margolis *et al.* (66). In various other proteins, eg. chymotrypsinogen (17) and lysozyme (34), however, histidine has not been found to react with O-methylisourea. 2-S-Thiuronum ethane sulfonate, however, did not modify histidine as determined by TLC.

Since proteins containing reduced cysteinyl residues are rare, as opposed to cysteins in (oxidized) cystine disulfides,

and some proteins have no cysteins at all [eg: light harvesting proteins (cf. Figure 1)], the most common consequence of the use of the guanidinating reagent would be to convert lysyl residues to homoarginyl residues (cf. Equation 2) in about 80-90% yield. In addition, the N-terminal α amino group should also be guanidinated. Shield *et al.* (96) observed that seven lysine residues out of eight of mercuripapain can be converted to homoarginine by the guanidination procedure using O-methylisourea. Similarly, Hettinger and Hartbury observed the conversion of all the lysine residues in horse and tuna cytochrome c to homoarginine residues (40).

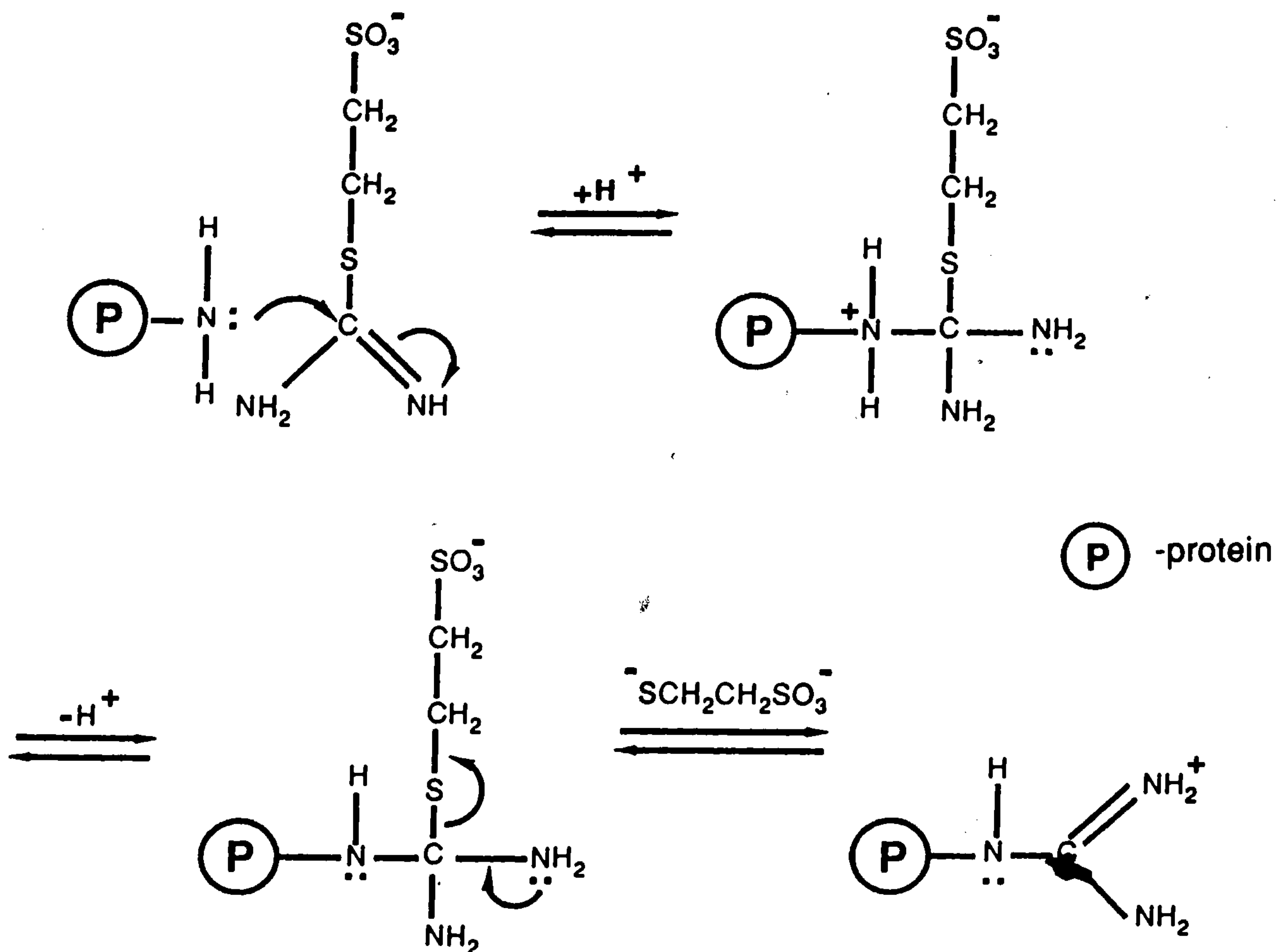
In the case of 2-S-thiuronium ethane sulfonate, the pH dependence of the guanidination reaction was demonstrated by the almost 90% of maximum labeling attained at pH 9.5 (cf. Figure 15C). This indicates that most of the labeling reaction takes place below the pK_a of lysine (10.5); therefore, lysine must be primarily in the protonated form; neverthless, enough lysine remains unprotonated for the reaction to occur. However, pK_a of the labeling reagent, which was determined to be 9.2[1], indicated that more than 50% of the reagent was in the unprotonated form, and the reaction can still occur when the reagent is completely unprotonated since the reaction is maximal out to pH 12 (cf. Figure 15C). Hence, the most likely

----

[1]determined by titration of aqueous solution of 2-S-TES with NaOH.

mechanism may be that described by the Equation 3.

Equation #3



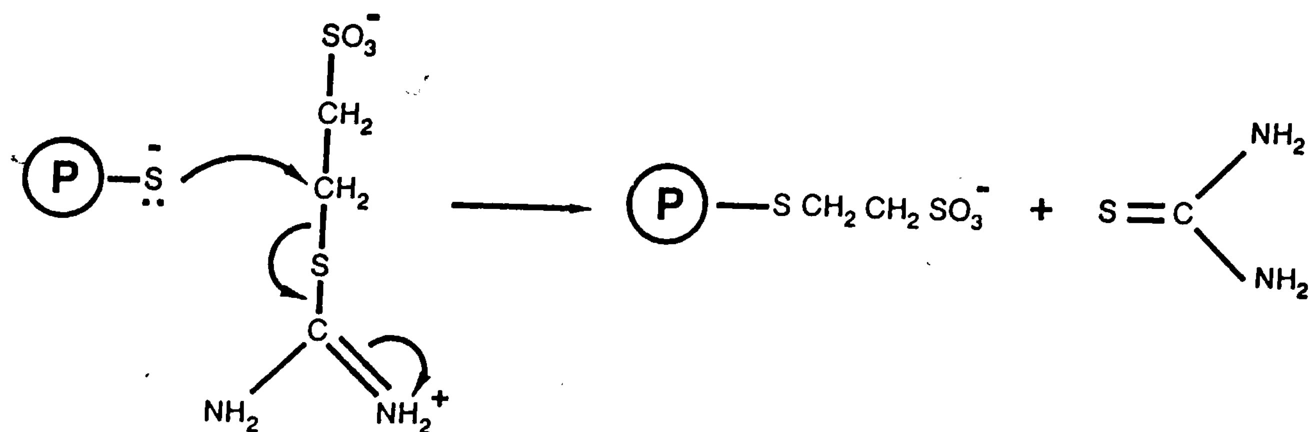
The conversion of lysyl to homoarginyl residues should not lead to any drastic changes in the properties of parent polypeptides, although homoarginine (pk_a = 12.5) is distinctly more basic than lysine (pk_a= 10.5). The properties of polypeptides with altered residues would, however, not be expected to be significantly different from the parent polypeptides; hence, solubilization, isolation, and analysis procedures (including chromatography, gel filtration,

SDS-PAGE, etc.) could be carried out and retain altered and unaltered polypeptides in the same fraction. However, Hughes *et al.* (44) observed a decrease in the aqueous solubility of serum albumin with increased conversion of amino groups to guanidino groups. A similar effect was observed on chymotrypsinogen by Chervenka and Wilcox (17), who also observed alterations in electrophoretic and sedimentation behavior. However, Klee and Richards (56) did not observe any major changes in the electrophoretic or sedimentation behavior of ribonuclease upon guanidination, and we did not see any significant difference in chromatographic or electrophoretic behavior of the guanidinated light-harvesting proteins in our study.

In order to determine the product of the reaction of the guanidinating reagent with thiol groups, N-acetyl-L-cysteine was used as a model compound. At the pH of the guanidination reaction (>9.0), the attacking species of N-acetyl-L-cysteine is very likely to be the thiolate anion; two reactions are possible.

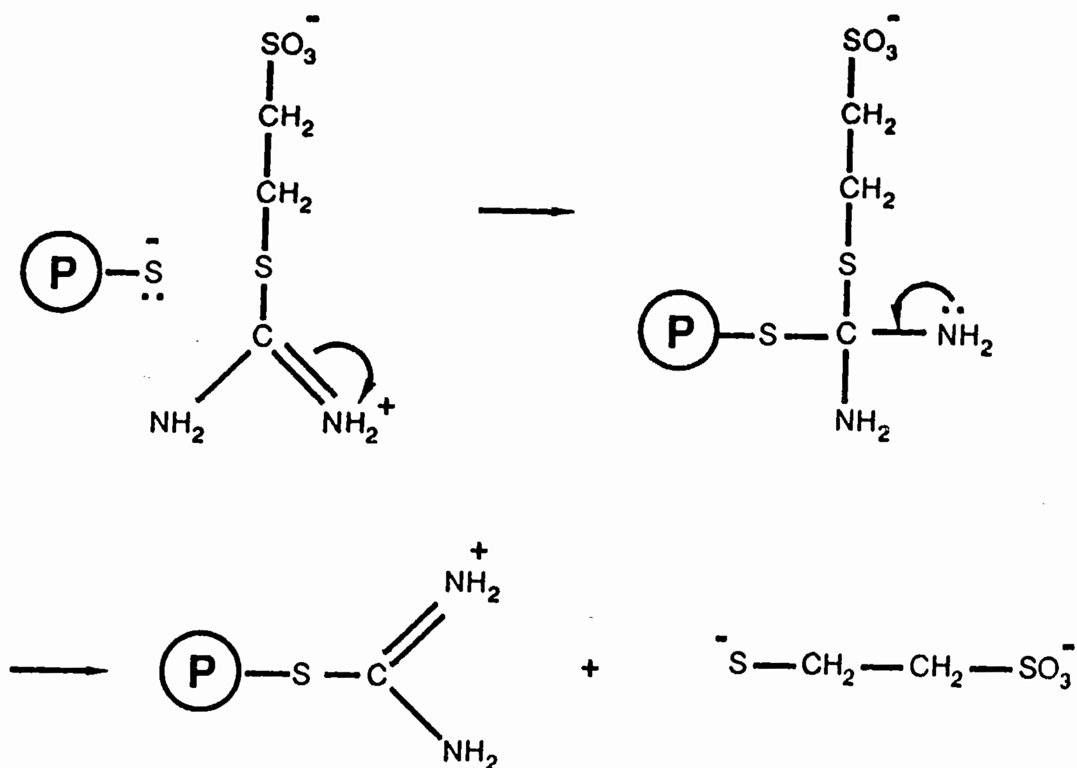
In the first possibility (cf. Equation 4) attack of the thiolate on a saturated carbon (C2 of ethylene) leads to alkylation of the thiol and elimination of thiourea.

Equation #4



Banks and Shafer (5) observed a high reactivity of O-methylisourea at pH 9.8 towards the thiol groups of papain by a similar mechanism, resulting in S-methylation of the cysteinyl residues. In the second possibility (cf. Equation 5), attack of the thiolate anion on the unsaturated thiouronium carbon leads to elimination of 2-mercaptoethane sulfonate

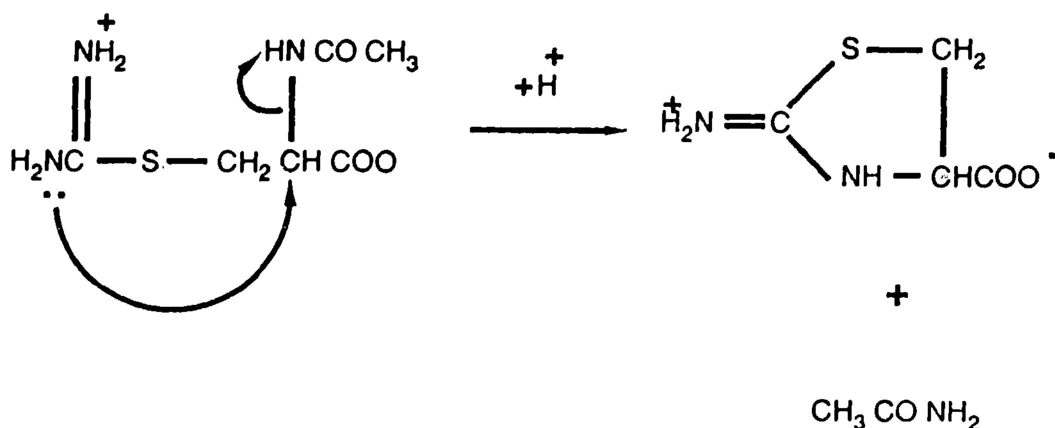
Equation #5



(which would be much more facile than in the case of an O-containing leaving group) and converts N-acetyl-L-cysteine to a thiuronium derivative (N-acetyl-S-amidino-L-cysteine). The major product at R_f 0.60 (cf. Table 2) was shown to have this structure by its NMR spectrum (cf. Materials and Methods). This product was synthesized by Rambacher (87) using N-acetyl-L-cysteine and disodium cyanamide, and the product (N-acetyl-S-amidino-L-cysteine) was converted by 2.4 M HCl to 2-iminothiazolidine-4-carboxylic acid (cf. Equation 6). Hence

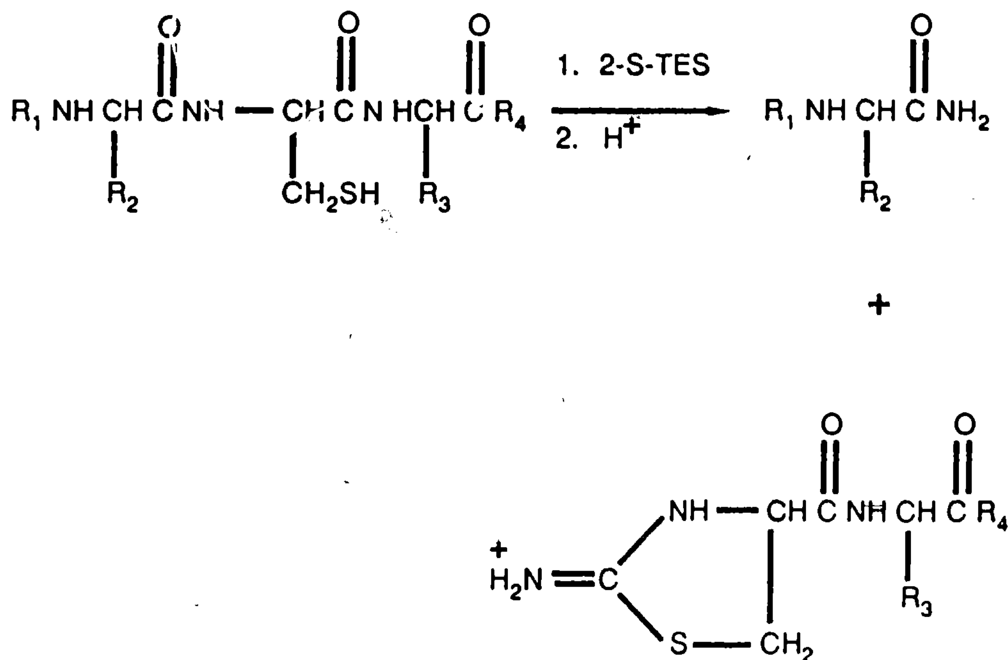
it is very likely that the labeled compound at R_f 0.45 (cf. Table 2, Results), derived from N-acetyl-L-cysteine by treatment with 2-S-thiuronium ethane sulfonate and trifluoroacetic acid, is the above cyclization product.

Equation #6



It is likely that a protein containing reduced disulfide bonds, when labeled with 2-S-thiuronium ethane sulfonate followed by treatment with anhydrous trifluoroacetic acid, would result in cleavage of the peptide on the amino side of the cysteinyl residues. The resulting peptides will have 2-iminothiazolidine-4-carboxamide derivatives of the residue on the carboxyl side of each cysteinyl residue (cf. Equation 7).

Equation #7



Jacobson *et al.* (48) developed a similar peptide cleavage procedure. They used 2-nitro-5-thiocyanobenzoic acid to generate S-cyanocysteinyl residues (from either cysteine or cystine residues), which were cyclized to 2-iminothiazolidine-4-carboxamide derivatives (plus the free carboxylate of the residue on the amino side of each cysteinyl residue). The 2-iminothiazolidine-4-carboxamide residues may be catalytically reduced with $NiCl_2$ plus $NaBH_4$ to alanyl residues (93); hence, 2-S-thiuronium ethane sulfonate represents a new cysteine-specific protein cleavage reagent for peptide analysis that can be employed under relatively

mild conditions.

The results of varying the duration of the labeling reaction of the guanidinating reagent with chromatophores (cf. Figure 15A) at pH 9.0 demonstrated a biphasic curve, i.e. an initial rapid rate of labeling for 15 minutes followed by a much slower labeling rate. This slower labeling rate did not reach saturation even after 3 h of incubation. An incubation time of two hours was taken as optimal.

When the pH of the incubation buffer was increased from 7 to 12 (cf. Figure 15C) only about 50% of the groups reacting in 2 h at pH >10.5 had reacted at pH 8.3. This would indicate that perhaps the initial rapid labeling rate in Figure 15A (which was carried out at pH 9.0) was due to the labeling of groups with pK_a values less than 9.0. As shown in the Results (cf. Figure 13, Table 3), the labeling reagent reacted with the α-amino group of glycylglycine. Banks and Shafer (5) have also suggested that the S-methylation of cysteine residues with O-methylisourea is predominant at pH below 9.0, while guanidination was predominant at pH above 9.0. So the N-terminal amino groups, together with the thiols of cysteinyl residues, may, therefore, have been labeled in the rapid labeling phase. Peak III (representing lipids and pigments) in Figure 8 also demonstrated radioactivity (results not shown) indicating that lipids as well as proteins are labeled by the guanidinating reagent. Hence, the amino groups of

phosphatidylethanolamine and lysyl residues may have been labeled in the slower labeling phase, as they both have pK_a values greater than 10.0 and would be largely protonated at pH 9.0. The sigmoidal pH curve can also be explained, as suggested by Whitley and Berg (124), by the possibility that amino groups of proteins in membrane may be more accessible at higher pH.

The results of varying the temperature of the incubation of the guanidinating reagent with chromatophores (cf. Figure 15D) have demonstrated a linear relationship between the log of the degree of labeling and 1/T in the temperature range 4-50°C, with no sign of reaching a saturation level. We did not test whether the highest temperature had led to the unmasking of labeling sites due to partial denaturation of membrane proteins, but this seemed unlikely due the continuous nature of the temperature variation plot. It is possible that higher temperature might cause slow penetration of the label into membranes due to increased fluidity of the bilayer. The optimum temperature used for labeling studies was 35-37°C.

Glycylglycine was treated with the guanidinating reagent to determine the labeling of an N-terminal amino group of a peptide. The product, which was purified on a Sephadex G-10 column (R_f 0.48) (cf. Figure 13) was indeed N-amidinoglycylglycine (cf. Table 3). Thus, the guanidinating reagent does label the α-amino groups of N-terminal amino

acids. The related S-ethylisothiourea has been shown to convert glycine to guanidinoacetic acid (11). O-methylisourea is also known to react with the α-amino group of the terminal glycine residue of insulin (29), as well as with the α-amino groups of bovine serum albumin (44), casein, & ovalbumin (89). Shields *et al*. observed only partial conversion of N-terminal isoleucine to N-amidinoisoleucine in papain (96). This indicates that the labeling of the α-amino group of the amino acid at the N-terminal end of a protein might depend on the type or availability of that amino acid or perhaps the nature of the guanidinating reagent. In general, α-amino groups in most proteins can be guanidinated.

Weil & Tilka (122), using α-lactoalbumin, and Shields *et al*. (96), using mercuripapain, observed that homoarginyl residues are not substrates for trypsin. However, Kenelly *et al*. observed cleavage of guanidinated cytochrome c (coupled to a solid innert matrix) with trypsin at the carboxyl side of homoarginine residues (53) (cf. Figure 22). In a control experiment, guanidinated cytochrome c with no coupling to the gel matrix was also treated with trypsin and its cleavage at homoarginine residues was observed. The reaction was carried out for 12 h. Weil & Tilka did not observe any cleavage even up to 24 h. This discrepency in results could be due to the influence exerted by neighbouring amino acids in the primary structure of the polypeptide chain or by the tertiary

structure of the protein (52).

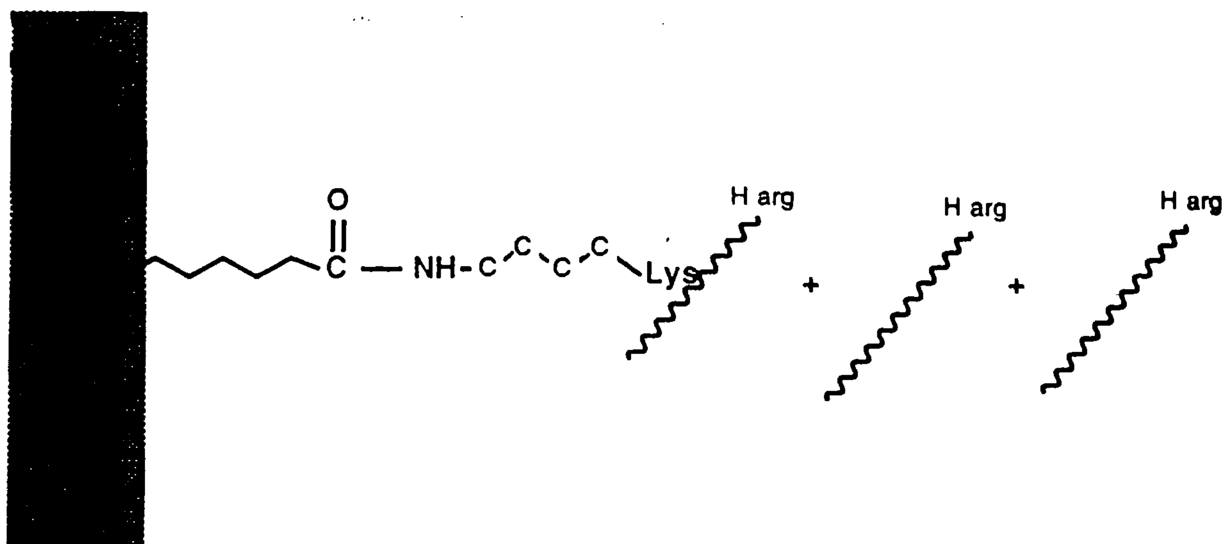


Figure 22. Schematic diagram showing cleavage of guanidinated cytochrome c coupled to an inert solid support (shaded area). Coupling was done via the ε-amino groups of remaining (unguanidinated) lysyl residues of cytochrome c to matrix carboxylic acids. Trypsin is shown to cleave at homoarginine residues.

However, we observed that, whereas, there was no cleavage at the homoarginyl residue after 30 min of trypsin hydrolysis, after 3 h of reaction, the tripeptide was detected, although in very small amounts (cf. Table 4). Thus, homoarginyl residues are slow hydrolysis sites for trypsin. Another observation was made that in tryptic digests of unguanidinated pentapeptide, no arginine or lysine was detected and the presence of the dipeptide, Arg-Lys was inferred, indicating

that the terminal arginine was not cleaved by trypsin. When the arginine was guanidinated at the α-amino group, the N-terminal residue may now be cleaved because the α-guanidino group may be recognized by the enzyme as an additional peptide bond. No spot was observed to run in the region of the dipeptide Arg-Lys, although we do not know that the expected dipeptide, N^{α}-amidinoarginylhomoarginine, would have a similar R_f value. It is possible that this dipeptide may have migrated with the same R_f (0.22) as homoarginine (cf. Table 4) and thus have escaped detection. We also could not detect the amino acid derivative, N^{α}-amidinoarginine, which may also have migrated with the same R_f-value (0.22) as free homoarginine and arginine. However, since a spot which had an R_f value (0.44) intermediate between that of the guanidinated pentapeptide (R_f 0.36) and the tripeptide (R_f 0.51) may have been due to a tetrapeptide (cf. Table 4), it is possible that cleavage by trypsin at the N^{α}-amidinoarginine site was even more prevalent than cleavage at the homoarginine site.

The chromatophore vesicles of purple photosynthetic bacteria (derived from the ICM during cellular disruption by ultrasonication or pressure release in the French pressure cell) are orientated predominantly "inside-out" (or cytoplasmic-side-out), while SDV are orientated predominantly "right-side-out" (or periplasmic-side-out) (62,67,73,110). Numerous studies, using one or both of these membrane vesicle

prepations, have been carried out recently to identify the components that have accessible reactive residues on either side of the ICM, including both lipids (1) and proteins (131). The yield of SDV and the fidelity of their orientation is rather low compared to that of chromatophores. For radiolabeling studies, the homogeneity of vesicle orientation is a very important factor. One can achieve complete homogeneity of periplasmic labeling by incubating whole cells with the guanidinating reagent and assuming that the reagent will freely pass through the outer (but not the inner) membrane. Francis and Richards (32), using pyridoxal phosphate and KB^3H_4, labeled whole cells as well as prepared chromatophores and SDV. In both that study and the present work, the specific activity of the chromatophores derived from labeled whole cells was too low to study by fluourography (cf. Reference 32 & Figure 15B). The yield of SDV with the "right-side-out" orientation can be greatly increased (in *R. sphaeroides*) by growth of the bacteria in very high light intensity, during which time the cells produce very little ICM, thus yielding a higher proportion of "right-side-out" SDV during the osmotic shock step in the prepration procedure. In order to purify these SDV further, cytochrome c-linked affinity chromatography was employed.

Mitochondrial cytochrome c (horse), which is a very efficient electron donor to bacterial photosynthetic reaction

centers *in vitro*, has been shown to bind to the reaction center of *R. rubrum* (10). This binding site on the reaction center is close (approx 10 Å) to both the L and M subunits on the periplasmic side (90). Brudvig *et al.* (12), using horse heart cytochrome c linked to Sepharose 4B, purified reaction centers from *R. sphaeroides* R-26. They also observed the binding of wild-type *R. sphaeroides* and *R. capsulatus* reaction centers to cytochrome c. These studies led us to use CNBr-Sepharose 4B-linked cytochrome c to purify SDV with a homogenious "right-side-out" orientation. The vesicles with no cytochrome c binding site, were not retained on the column, while vesicles which had their cytochrome c binding site exposed (i.e., SDV with a correct orientation) were eluted with KCl. This is a very useful technique since one can achieve significant purification of vesicles with homogenous single-sided orientation. Similar procedures have been used previously by other workers to purify mitochondrial mitoplasts from inverted vesicles (33,63).

The impermeability of the labeling reagent was demonstrated by the different fluorographic pattern obtained from chromatophores and SDV (cf. Figure 18). The α- and β-subunits of the coupling factor were only labeled in the case of chromatophores, since they are only exposed on the cytoplasmic side of the membrane (28,110). All three reaction center subunits (H, M, & L) span the ICM in *R. sphaeroides* as

they can be labeled from either side of the membrane by radioiodination (3). Neverthless, only the H subunit (and to a lesser extent the L subunit) was shown previously to have been labeled from the cytoplasmic side of the ICM by Francis and Richards (32) by a lysine-specific radiochemical labeling reagent. This result has been confirmed in this study with the guanidinating reagent.

Cogdell *et al.* have demonstrated that the B800-850 light-harvesting complex of *R. sphaeroides* has antigenic sites on both sides of the membrane (20). In the present study, both B850α and B850β as well as B870β were labeled from the cytoplasmic side only (cf. Figure 19). From the amino acid sequence of all four light-harvesting polypeptides (cf. Figure 1), it is clear that while each of the four peptides has at least one lysine present on its cytoplasmic surface, only one (B870α) has a lysine on its periplasmic surface. Our results confirm the findings of Francis and Richards (32) that the B870α polypepide is exposed on the periplasmic surface of the ICM (cf. Figure 20). Our result is also in agreement with results of Takemoto *et al.* (109) that the C-terminal end of B870α is exposed on the periplasmic surface. Bachmann *et al.* (3) also observed radiolabeling of B870α at the periplasmic face. Takemoto *et al.* (109), however, did not observe any cleavage of B870α with either proteinase K or trypsin from the chromatophore surface. Our results (cf. Figures 19 and 21)

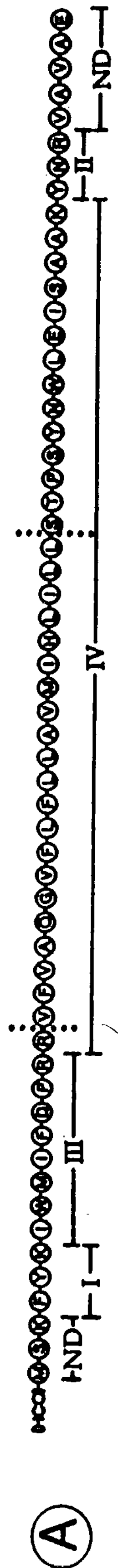
demonstrate that at least part of this polypetide is exposed to the much smaller molecular weight guanidinating reagent on the cytoplasmic surface. Brunisholz *et al.* (16) in *R. rubrum* G-9⁺ (a mutant lacking carotenoids) and *Tadros et al.* (104) in *R. capsulatus* Ala⁺ (a mutant lacking the B800-850 light-harvesting complex), used similar proteolytic digestion and sequencing techniques to determine cleaved sequences and demonstrated that the B870α polypeptide is exposed on the cytoplasmic surface in both organisms. However, when carotenoids were present in *R. rubrum* S1, or the B800-850 complex was present in *R. capsulatus*, the N-terminal was insensitive to digestion by proteinase K (13,16). This indicates that the presence of the B800-850 complex in wild type *R. capsulatus* and *R. sphaeroides* limits the exposure of the B870α polypeptide to proteolytic digestion on the cytoplasmic surface. The B870α polypeptide might be partially buried in the membrane at the water-lipid interface. Evidence for such an arrangement comes from the work of Meister *et al.* (69), who used photogenerative hydrophobic labels in *R. rubrum* G-9⁺ to demonstrate that not only do these reagents bind specifically to the central hydrophobic domain but, surprisingly, along the N-terminal end of the B870α polypeptide as well. This suggests that in the presence of the B800-850 complex, the mode of assembly might be such that the N-terminal of the B870α polypeptide is less exposed to the large molecular weight proteolytic enzymes. In wild type *R.*

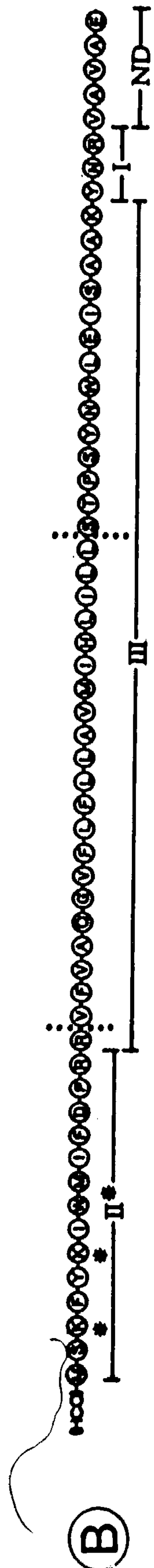
capsulatus, the B800-850 complex is also protected from the cytoplasmic side (108). However, in the Y5 mutant, which lacks the reaction center and the B875 complex, both the B850α and B850β polypeptides are exposed on the chromatophore surface (107). In the present results, exposure of all four light-harvesting polypeptides was clearly demonstrated on the cytoplasmic surface (cf. Figure 19).

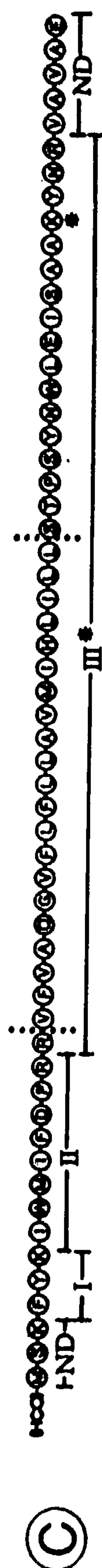
The transmembrane and asymmetric nature of the B870α polypeptide was also confirmed in the present study. From a tryspin digestion of the B870α polypeptide, six tryptic fragments are to be expected due to cleavage at lysine and arginine residues (cf. Figure 23A). Assuming that there is no cleavage at homoarginine residues (which seems likely), only four fragments are to be expected from the B870α protein isolated from chromatophores, if both the lysines on the cytoplasmic side have been modified to homoarginines (cf. Figure 23B).

The HPLC analysis of the trypsin digest of the B870α polypeptide purified from the chromatophore sample (cf. Figure 21B) showed only three peaks (I-III) due to tryptic fragments, and another peak (IV) due to the undigested B870α polypeptide. The absorbance of the HPLC eluate was monitored at 280 nm due to the high ultraviolet cutoff value for tetrahydrofuran. Hence, not all of the possible tryptic fragments can be

Figure 23 Sites of action of trypsin on B870α (A) the unmodified peptide, (B) from guanidinated chromatophores and (C) from guanidinated SDV. * indicates guanidinated lysines, and, hence, labeled homoarginyl fragments which would yield correspondingly labeled tryptic peptides.


A
ND
I
III
IV
II
ND


B
II
III
I
ND


C
ND
I
II
III
ND

detected at 280 nm due to a lack of aromatic amino acids. One such fragment (the C-terminal fragment) lacks both tryptophan and tyrosine (cf. Figure 23B). Hence, only three fragments would be detected, and only one of these (the N-terminal fragment) would be expected to be labeled. The results have confirmed these predictions. A tentative assignment of the three visible peaks is indicated in Figure 23B, based on their similarity to the undigested parent peak, and the labeling results of Table 6.

The HPLC analysis of the trypsin digest of the B870α polypeptide purified from the SDV sample (cf. Figure 21C) also showed only three peaks (I-III) due to tryptic fragments, plus another peak (IV) due to the undigested protein. Five tryptic fragments were to be expected if the lysine on the periplasmic side of the membrane had been modified to homoarginine (cf. Figure 23C). In this case, however, two residues (both the N- and C-terminal fragments) contain no tryptophan or tyrosine and would not, therefore, be detected. Hence, only three fragments would be detected and only one (the large fragment containing the hydrophobic domain) would be expected to be labeled. Again, the results have confirmed all of these predictions. Once more, tentative assignments of the three visible peaks in the SDV sample is indicated in Figure 23C; these should be compared with the assignments of the peaks in the chromatophore sample. It should be noted that the three

peaks in each sample represent different tryptic fragments and have, therefore, different retention times. Also, a different fragment is labeled in the SDV sample (peak III in Figure 21C) than in the chromatophore sample (peak II in Figure 21B).

In conclusion, the labeling of the B870α polypeptide from both sides of the membrane confirms its transmembrane orientation. The different pattern for the radiolabeling of the typtic fragments isolated from the B870α polypeptide labeled from both sides of the membrane (as well as the labeling of the B870β, B850α, and B850β polypeptides from only one side of the membrane) demonstrates the asymmetry of the four light-harvesting polypeptides in the photosynthetic membrane. These results have also demonstrated the effectiveness of the labeling reagent for future membrane-protein orientation studies.

REFERENCES

1. AL-BAYATTI, K. K. AND J. Y. TAKEMOTO (1981) *Biochemistry* **20**, 5489-5495

2. ALLEN, J. P., G. FEHER, T. O. YEATES, H. KOMIYA AND D. C. REES (1987) *Proc. Natl. Acad. Sci. USA.* **84**, 6162-6166

3. BACHMANN, R. C., K. GILLIES AND J. Y. TAKEMOTO (1981) *Biochemistry* **20**, 4590-4596

4. BACHOFEN, R. (1979) *FEBS Lett.* **107**, 409-412

5. BANKS, T. E. AND J. A. SHAFER (1972) *Biochemistry* **11**, 110-114

6. BERARD, J., G. BELANGER, P. CORRIVEAU AND G. GINGRAS (1986) *J. Biol. Chem.* **261**, 82-87

7. BELANGER, G., J. BERARD, P. CORRIVEAU AND G. GINGRAS (1988) *J. Biol. Chem.* **263**, 7632-7638

8. BISSIG, I., R. A. BRUNISHOLZ, F. SUTER, R. J. COGDELL AND H. ZUBER (1988) *Z. Naturforsch.* **43c**, 77-83

9. BORISOV, A. Y. (1978) In: **The Photosynthetic Bacteria** (eds. Clayton, R. K. and W. R. Sistrom) pp. 323-330, Plenum Press, New York and London.

10. BOSSHARD, H. R., M. SNOZZI AND R. BACHOFEN (1987) *J. of Bioenergetics and Biomembranes* **19**, 341-375

11. BRAND, E. AND F. C. BRAND (1955) **Organic syntheses, collect.** *vol. III*, pp. 440-442. Wiley, New York.

12. BRUDVIG, G. W., S. T. WORLAND AND K. SAUER (1983) *Proc. Natl. Acad. Sci. USA.* **80**, 683-686

13. BRUINSHOLZ, R. A., H. ZUBER, J. VALENTINE, J. G. LINDSAY, K. J. WOOLLEY AND R. J. COGDELL (1986) *Biochim. Biophys. Acta* **849**, 295-303

14. BRUNISHOLZ, R. A., F. JAY, F. SUTER AND H. ZUBER (1985) *Hoppe-Seyler Biol. Chem.* **366**, 87-98

15. BRUNISHOLZ, R. A., P. A. CUENDET, R. THEILER AND H. ZUBER (1981) *FEBS Lett.* **129**, 150-154

16. BRUNISHOLZ, R. A., V. WIEMKEN, F. SUTER, R. BACHOFEN AND H. ZUBER (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 689-701

17. CHERVENKA, C. H. AND P. E. WILCOX (1956) *J. Biol. Chem.* **222**, 635-647

18. CHORY, J., T. J. DONOHUE, A. R. VARGA, L. A. STAEHELIN AND S. KAPLAN (1984) *J. Bact.* **159**, 540-554

19. COGDELL, R. J., G. LINDSAY, J. VALENTINE AND I. DURANT (1982) *FEBS Lett.* **150**, 151-154

20. COGDELL, R. J., J. VALENTINE AND G. LINDSAY (1983) In: **Structure AND function of membrane proteins.** (eds. E. Quagliariello and F. Palmieri) pp. 125-128. Elsevier Science Publishers.

21. COHN, L. K. AND S. KAPLAN (1981) *J. Biol. Chem.* **256**, 5909-5915

22. COLLINS, M. L. P., D. E. MELLON AND R. A. NIEDERMAN (1980) *J. Bact.* **143**, 221-230

23. CUENDET, P. A., H. ZURRER, M. SNOZZI AND H. ZUBER (1978) *FEBS Lett.* **88**, 309-312

24. DEBUS, R. J., G. FEHER AND M. Y. OKAMURA (1985) *Biochemistry* **24**, 2488-2500

25. DEBUS, R. J., G. FEHER AND M. Y. OKAMURA (1986) *Biochemistry* **25**, 2276-2287

26. DEISENHOFER, J., O. EPP, K. MIKI, R. HUBER AND H. MICHEL (1984) *J. Mol. Biol.* **180**, 385-398

27. DREWS, G. (1985) *Microbiol. Rev.* **49**, 59-70

28. ELFERINK, M. G. L., K. J. HELLINGWERF, P. A. M. MICHELS, H. G. SEYEN AND W. N. KONINGS (1979) *FEBS Lett.* **107**, 300-307

29. EVANS, R. L. AND H. A. SAROFF (1957) *J. Biol. Chem.* **228**, 295-304

30. FEHER, G. AND M. Y. OKAMURA (1978) In: **The photosynthetic bacteria.**, (eds. Clayton, R. K. and W. R. Sistrom) pp. 349-386, Plenum press, New York, London.

31. FEHER, G. AND M. Y. OKAMURA (1976) *Brookhaven Symp. Biol.* **28**, 183-194

32. FRANCIS, G. A. AND W. R. RICHARDS (1980) *Biochemistry* **19**, 5104-5111

33. GAUTHERON, D. C., C. GODINOT, H. MAIROVCH, B. BLANCHY, F. PENIN AND Z. WOJTKOWIAK (1977) In: **Bioenergetics of Membranes.** (eds. Pecker, L. *et al.*) pp. 501-512. Elsevier/North-Holland Biomedical Press, Amesterdam, New York.

34. GESCHWIND, I. I. AND C. H. LI (1957) *Biochim. Biophys. Acta* **25**, 171-178

35. GOGEL, G. E., P. S. PARKES, P. A. LOACH, R. A. BRUNISHOLZ, AND H. ZUBER (1983) *Biochim. Biophys. Acta* **746**, 32-39

36. GOGEL, G. E., M. MICHALSKI, H. MARCH, S. COYLE AND L. GENTILE (1986) *Biochemistry* **25**, 7105-7109

37. GREENSTEIN, J. P. (1935) *J. Biol. Chem.* **109**, 541-544

38. HALL, R. L., P. F. DOORLEY AND R. A. NIEDERMAN (1978) *Photochem. Photobiol.* **28**, 273-276

39. HELLINGWERF, K. J., P. A. M. MICHELS, J. W. DORPEMA AND W. N. KONINGS (1975) *Eur. J. Biochem.* **55**, 397-406

40. HETTINGER, T. P. AND H. A. HARBURY (1965) *Biochemistry* **4**, 2585-2589

41. HOGER, J. H. AND S. KAPLAN (1985) *J. Biol. Chem.* **260**, 6932-6937

42. HOLMQVIST, O. (1979) *FEMS Microbiol. Lett.* **6**, 37-40

43. HUBBARD, A. L. AND Z. A. COHEN (1976) In: **Biochemical analysis of membranes.** (ed. A. H. Maddy) pp. 427-501, Wiley, New York

44. HUGHES, W. L. Jr., H. A. SAROFF AND A. L. CARNEY (1949) *J. Am. Chem. Soc.* 71, 2476-2480

45. HUNDLE, B. S. AND W. R. RICHARDS (1987) *Biochemistry* **26**, 4505-4511

46. ITOH, M., D. HAGIWARA AND T. KAMIYA (1975) *Tetrahedron Lett.* 4393-4394

47. JACOB, J. S. AND K. R. MILLER (1983) *Arch. Biochem. Biophys.* **223**, 282-290

48. JACOBSON, G. R., M. H. SCHAFFER, G. R. STARK AND T. C. VANAMAN (1973) *J. Biol. Chem.* **248**, 6583-6591

49. JACQUES, B. AND E. NABEDRYK (1984) *FEBS Lett.* **176**, 355-359

50. JAY, F. AND M. LAMBILLOTTE (1985) *Eur. J. Cell Biol.* **37**, 7-13

51. JAY, F., M. LAMBILLOTTE AND K. MUHLETHALER (1983) *Eur. J. Cell Biol.* **30**, 1-8

52. KEIL, B. (1971) In: **The Enzymes** (ed. P. D. BOYER) **Vol III** pp. 249-275

53. KENNELLY, P. J., R. TIMKOVICH AND M. A. CUSANOVICH (1981) *J. Mol. Biol.* **145**, 583-602

54. KILEY, P. J. AND S. KAPLAN (1987) *J. Bact.* **169**, 3268-3275

55. KIMMEL, J. R. (1967) *Methods Enzymol.* **11**, 584-589

56. KLEE, W. A. AND F. M. RICHARDS (1957) *J. Biol. Chem.* **229**, 489-504

57. KNOX, R. S. (1975) In: **Bioenergetics of photosynthesis** (ed. Govindjee) pp. 183-221. Academic Press, New York.

58. KOSAKOWASKY, M. M. AND S. KAPLAN (1974) *J. Bact.* **118**, 1114-1157

59. KOSHLAND, M. E., F. M. ENGLBERGER AND S. M. GADDONE (1963) *J. Biol. Chem.* **238**, 1349-1352

60. LAEMMLI, U. K. (1970) *Nature (London)* **227**, 680-685

61. LASCELLES, J. (1966) *Biochem. J.* **100**,175-183

62. LOMMEN, M. A. J. AND J. Y. TAKEMOTO (1978) *J. Bacteriol.* **136**, 730-741

63. LOTSCHER, H. R., K. SCHWERZMANN AND E. CARAFROLI (1979) *FEBS Lett.* **99**, 194-198

64. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL (1951) *J. Biol. Chem.* **193**, 265-275

65. LUNDBLAD, R. L. AND C. M. NOYES (1984) In: **Chemical reagents for protein modification, Vol. I**, pp. 127-170, CRC Press, Boca Raton,FL.

66. MARGOLIS, S. AND R. G. LANGDON (1966) *J. Biol. Chem.* **241**, 477-484

67. MATSUURA, K. AND M. NISHIMURA (1977) *Biochim. Biophys. Acta* **459**, 483-491

68. MEANS, G. E. AND R. E. FEENY (1971) In: **Chemical modification of proteins**, pp. 93-97, Holden-Day, San Francisco.

69. MEISTER, H. P. AND R. BACHOFEN (1984) *Biochim. Biophys. Acta* 771,103-106

70. MEISTER, H., R. BACHOFEN, G. SEMENZA AND J. BRUNNER (1985) *J. Biol. Chem.* **260**, 16326-16331

71. MICHEL, H., K. A. WEYER, H. GREENBERG AND F. LOTTSPEICH (1985) *EMBO. J.* **4**, 1667-1672

72. MICHEL, H., K. A. WEYER, H. GREENBERG, I. DENGER, D. OESTERHELT AND F. LOTTSPEICH (1985) *EMBO. J.* **5**, 1149-1158

73. MICHELS, P. A. M. AND W. N. KONINGS (1978) *Biochim. Biophys. Acta* **507**, 353-368

74. MILLER, J. F., S. B. HINCHIGERI, P. S. PARKES-LOACH, P. M. CALLAHAN, J. R. SPRINKLE, J. R. RICCOBONO AND P. A. LOACH (1987) *Biochemistry* **26**, 5055-5062

75. NIKETIC, V., J. THOMSEN AND K. KRISTIANSEN (1974) *Eur. J. Biochem.* **46**, 547-551

76. NORRIS, J. R. AND G. VAN BRAKEL (1986) In: **Photosynthesis Vol III**. (eds. L. A. Staehelin and C. J. Arntzen) pp. 353-370. Springer, New York.

77. ODERMATT, E., M. SNOZZI AND R. BACHOFEN (1980) *Biochim. Biophys. Acta* **591**, 372-380

78. OELZE, J. AND E. POST (1980) *Biochim. Biophys. Acta* **591**, 76-81

79. OELZE, J. AND G. DREWS (1972) *Biochim. Biophys. Acta* **265**, 209-239

80. OELZE, J. (1978) *Biochim. Biophys. Acta* **509**, 450-461

81. OKAMURA, M. Y., G. FEHER AND N. NELSON (1982) In **Photosynthesis Vol. I**, (ed. Govindjee) pp. 195-274. Academic Press, New York

82. PARKES-LOACH, P. S., J. R. SPRINKLE AND P. A. LOACH (1988) *Biochemistry* **27**, 2718-2727

83. PETERS, J. D. AND G. DREWS (1983) *FEBS Lett.* **162**, 57-60.

84. Pharmacia Fine Chemical handbook (1982) **Gel Filtration**, theory and practice pp. 34

85. PHILLIPS, D. R. AND M. MORRISON (1971) *Biochemistry* **10**, 1766-1771

86. PICOREL, R., G. BELANGER AND G. GINGRAS (1983) *Biochemistry* **22**, 2491-2497

87. RAMBACHER, P. (1968) *Chem. Ber.* **101**, 3433-3437

88. REED, D. W., D. RAVEED AND M. REPORTER (1975) *Biochim. Biophys. Acta* **387**, 368-378

89. ROCHE, J., MOURGNI, M. AND BARET, R. (1954) *Bull. Soc. Chim. Biol.* **36**, 85

90. ROSEN, D., M. Y. OKAMURA, E. C. ABRESCH, G. E. VALKIRS AND G. FEHER (1983) *Biochemistry* **22**, 335-341

91. RYDON, H. N. AND P. W. G. SMITH (1952) *Nature (London)* **169**, 922-923

92. SAUER, K. AND L. A. AUSTIN (1978) *Biochemistry* **17**, 2011-2019

93. SCHAFFER, M. H. AND G. R. STARK (1976) *Biochem. Biophys. Res. Commun.* 71, 1040-1047

94. SCHRAMM, C. H., H. LEMAIRE AND R. H. KARLSON (1955) *J. Am. Chem. Soc.* 77, 6231-6233

95. SCHUTTE, E. (1943) *Hoppe-Seyler's Z. Physiol. Chem.* **279**, 52-59

96. SHIELDS, G. S., R. L. HILL AND E. L. SMITH (1959) *J. Biol. Chem.* **234**, 1747-1753

97. SISTROM, W. R. (1978) In: **The Photosynthetic Bacteria** (eds. Clayton, R. K. and W. R. Sistrom) pp. 841-848, Plenum Press, New York and London

98. SNOZZI, M. (1977) *Ber. Dtsch. Bot. Ges.* **90**, 485-492

99. STEINER, L. A., A. D. LOPES, M. Y. OKAMURA, L. C. ACKERSON AND G. FEHER (1974) *Fed. Proc.* **33**, 1461

100. STELLWAGEN, E., F. SMITH, R. CASS, R. LEDGER AND H. WILGUS (1977) *Biochemistry* **16**, 3672-3679

101. STEPHENS, R. E. (1978) *Anal. Biochem.* **84**, 116-126

102. TADROS, M. H., F. SUTER, G. DREWS AND H. ZUBER (1983) *Eur. J. Biochem.* **129**, 533-536

103. TADROS, M. H., F. SUTER, H. H. SEYDEWITZ, I. WITT, H. ZUBER AND G. DREWS (1984) *Eur. J. Biochem.* **138**, 209-212

104. TADROS, M. H., R. FRANK AND G. DREWS (1985) *FEBS Lett.* **183**, 91-94

105. TADROS, M. H., G. FRANK, H. ZUBER AND G. DREWS (1985) *FEBS Lett.* **190**, 41-44

106. TADROS, M. H., R. FRANK, AND G. DREWS (1986) *FEBS Lett.* **196**, 233-236

107. TADROS, M. H., R. FRANK AND G. DREWS (1986) *J. Bact.* **167**, 96-100

108. TADROS, M. H., R. FRANK, B. DORGE, N. GAD'ON, J. Y. TAKEMOTO AND G. DREWS (1987) *Biochemistry* **26**, 7680-7687

109. TAKEMOTO, J. Y., R. L. PETERSON, M. H. TADROS AND G. DREWS (1987) *J. Bacteriol.* **169**, 4731-4736

110. TAKEMOTO, J. AND R. C. BACHMANN (1979) *Arch. Biochem. Biophys.* **195**, 526-534

111. THEILER, R., F. SUTER AND H. ZUBER (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 1765-1776

112. THEILER, R. AND H. ZUBER (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 721-729

113. THEILER, R., F. SUTER, H. ZUBER AND R. J. COGDELL (1984) *FEBS Lett.* **175**, 231-237

114. THEILER, R., F. SUTER, J. D. PENNOYER, H. ZUBER AND R. A. NEIDERMAN (1985) *FEBS Lett.* **184**, 231-236

115. THEILER, R., F. SUTER, V. WEIMKEN AND H. ZUBER (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 703-719

116. THORNBER, J. P., R. J. COGDELL, B. K. PIERSON AND R. E. B. SEFTOR (1983) *J. Cell. Biochem.* **23**, 159-169

117. VALKIRS, G. AND G. FEHER (1982) *J. Biol. Chem.* **249**, 6446-6453

118. VALKIRS, G. AND G. FEHER (1982) *J. Cell. Biol.* **95**, 179-188

119. VALKIRS, G.., D. ROSEN, K. T. TOKUYASU AND G. FEHER (1976) *Biophys. J.* **16**, 223a.

120. WALKER, J. B. (1955) *Arch. Biochem. Biophys.* **59**, 233-245

121. WEBSTER, G. D., R. J. COGDELL, J. G. LINDSAY AND M. A. REID (1980) *Biochem. Soc. Trans.* **8**, 329.

122. WEIL, L. AND TELKA, M. (1957) *Arch. Biochem. Biophys.* **71**, 473-474

123. WIEMKEN. V. AND R. BACHOFEN (1982) *Biochim. Biophys. Acta* **681**, 72-76

124. WHITELEY, N. M. AND H. C. BERG (1974) *J. Mol. Biol.* **87**, 541-561

125. WILLIAMS, J. C., L. A. STEINER, R. C. OGDEN, M. I. SIMON AND G. FEHER (1983) *Proc. Natl. Acad. Sci. USA.* **80**, 6505-6509

126. WILLIAMS, J. C., L. A. STEINER, G. FEHER AND M. I. SIMON (1984) *Proc. Natl. Acad. Sci. USA.* **81**, 7303-7307

127. WILLIAMS, J. C., L. A. STEINER AND G. FEHER (1986) *Proteins* 1, 312-325

128. WOFSEY, L. AND S. J. SINGER (1963) *Biochemistry* **2**, 104-116

129. YOUVAN, D. C., E. J. BYLINA, M. ALBERTI, H. BEGUSCH AND J. E. HEARST (1984) *Cell* **37**, 949-957

130. YOUVAN, D. C. AND S. ISMAIL (1985) *Proc. Natl. Acad. Sci. USA.* **82**, 58-62

131. ZUBER, H. (1985) *Photochem. Photobiol.* **42**, 821-844

132. ZURRER, H., M. SNOZZI AND R. BACHOFEN (1983) *FEBS Lett.* **153**, 151-155

133. ZURRER, H., M. SNOZZI, K. HANSELMANN AND R. BACHOFEN (1977) *Biochim. Biophys. Acta* **460**, 273-279