Isolation and Characterization of New Pterins From Nonmethanogenic Archaebacteria

by

Xinli Lin

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APPROVED:

R. White, Chairman

B. Anderson

G. Ferry

J. Hess

W. Niehaus

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Blacksburg, Virginia

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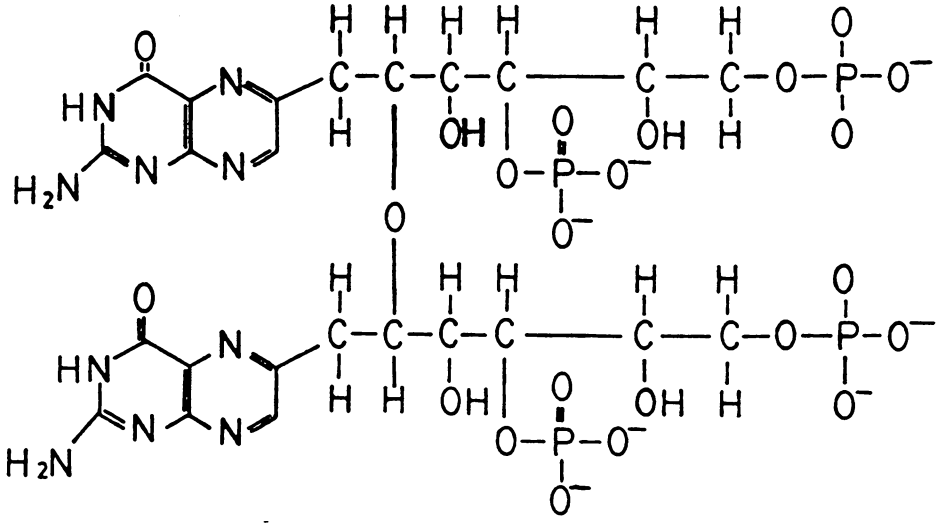
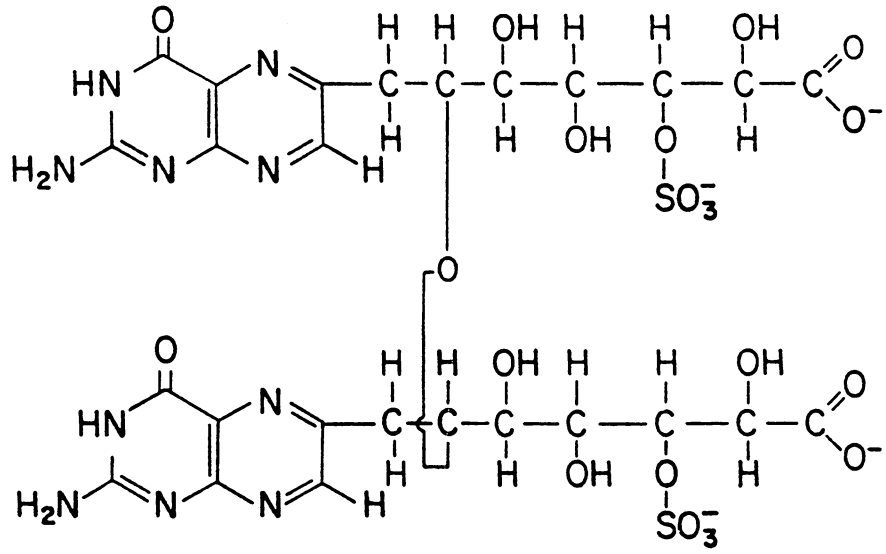
(ABSTRACT)

Several new pterins have been discovered in halophilic and thermoacidophilic archaebacteria. Two of these were identified in the extreme halophiles and were thus called halopterins. One of these halopterins is produced by *Halobacterium salinarium*, *Halobacterium halobium*, and *Halococcus morrhuae* and is called phosphohalopterin-1. It was given this name because it was the first halopterin discovered and it has four monophosphate esters per dimeric pterin. The proposed structure of phosphohalopterin-1 is as follows.



The other halopterin, which is produced by *Halobacterium marismortui*, *Halobacterium volcanii*, and Halobacterial strain GN-1, is called sulfohalopterin-2 because it has two sulfate esters per dimeric pterin and it was isolated and recognized after the isolation of phosphohalopterin-1. The proposed structure of sulfohalopterin-2 is as follows.



As shown above, both pterins are dimers with an ether linkage connecting the polyol side chains. Both of the halopterins are negatively charged because of the phosphate and sulfate esters on the side chains. In addition to the halopterins, a positively charged pterin has been isolated from *Sulfolobus solfataricus*. This pterin is very special since no positively charged unconjugated pterin had ever been found in nature before. This pterin is named solfapterin after the species name of the bacteria from which it was obtained. The structure of this pterin is still unknown but the preliminary data indicate that it is an unconjugated pterin with a polyol containing an amine on the side chain. Another positively charged pterin which is different from solfapterin was found in *Thermoplasma*. All of the above pterins are different from any previously described pterins and thus represent new pterins in the archaebacterial kingdom.

The discovery of these new pterins is important both to pterin biochemistry and to archaebacterial taxonomy. These discoveries also open up a new field, that is, the exploration of the function of these new pterins in nonmethanogenic archaebacteria.

Dedications

This thesis is dedicated to the two women I love most.

To my mother, Shuyun Lui, who passed away more than 10 years ago, but whose love is always with me.

To my wife, Jihua Wang, whose love and support is my life, just as my love and support is her life.

Acknowledgements

I thank Dr. R. H. White for his continuous support and intelligent advise during this work. He guided me through the oldest kingdom, the archaebacterial kingdom, and showed me a part of that kingdom that not many people have seen before. The knowledge I gained from him and from this work has been, and will continue to be, beneficial to my scientific carrier.

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I thank the Cunningham Fellowship Committee for honoring me with the Cunningham Fellowship for the 1984-1985 academic year, it was of great help to my research.

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I thank my wife for her support during this work and for her help in preparing this thesis.

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Chapter I

Introduction

A concept of molecular phylogeny was published in 1965 by Zuckerkandl and Pauling. In this classic paper, Zuckerkandl and Pauling defined semantides, which include primary semantides (DNA), secondary semantides (RNA) and tertiary semantides (polypeptides). They also defined episemantides which are molecules (usually "small molecules") that are synthesized under the control of tertiary semantides. The best molecules for molecular phylogeny are the semantides. However, due to the technical problems encountered with the identification and sequencing of specific genes in the genome, it was soon realized that the primary structures of the translation apparatus, mainly the rRNAs, offered the best hope for the establishment of molecular phylogeny (Sogin et al., 1972). The reasons are that the sequences of rRNAs are very conserved (Woese et al., 1975) and they are easy to isolate and identify. The primary structures of the rRNAs can be readily characterized in terms of the oligomers produced by digestion with specific nucleases. The 5S rRNA was used first in phylogenetic measurements (Sogin et al., 1972) and later the 16S rRNA was more widely used (Woese et al., 1975; Woese and Olsen, 1986). A major breakthrough using a comparative study of 16S rRNA as a criterion for molecular phylogeny was published in 1977 by Woese and Fox. They were the first to divide the present-day living organisms into three kingdoms (as

opposed to the traditional two kingdoms) based on a comparison of the partially sequenced 16S rRNA data from a wide range of organisms. The new kingdom, which they named archaebacteria, is not composed of "true bacteria" in the traditional sense since archaebacteria have properties that make them distinct from both eubacteria and eucaryotes. More studies on 16S rRNA have been published since the 1977 paper by Woese and Fox (Balch et al., 1977; Woese et al., 1978) and the complete sequence of 16S rRNAs are available now for comparative studies (Woese and Olsen, 1986; McGill et al., 1986). The results of this work have confirmed, refined and extended the concept of archaebacterial phylogeny. After analyzing the complete 16S rRNA data, Woese and Olsen (1986) found that in addition to the deep hiatus between the three major kingdoms, the archaebacteria themself also fall into two major divisions, the sulfur-dependent thermophilic archaebacterial branch and the methanogenic archaebacterial branch which includes the methanogens, the extreme halophiles, and two types of thermoacidophiles, *Thermoplasma acidophilum* and *Thermococcus celer*. All of the other phylogenetic studies support the present classification of organisms into three kingdoms, but not all are consistent with the above subdivisions. Some features, other than 16S rRNAs, that distinguish archaebacteria from eubacteria and eucaryotes follow.

DNA: It is known that all the covalently closed circular DNA duplexes which have been isolated from eucaryotes and eubacteria and their viruses are negatively supercoiled. Recently, a positively supercoiled DNA was found in *Sulfolobus* isolate B12 (Nadal, 1986). This could be a clue for establishing an unique architecture of the archaebacterial genome. The DNA sequences which are homologous to the nif-H gene (a gene that codes for the subunit of the iron protein in nitrogenase of eubacteria) were also studied in the archaebacteria (Possot et al., 1986) and it was found that the gene is present in the methanogens and in the extreme halophiles but not in the thermoacidophilic archaebacteria. These data differentiate the thermoacidophiles from the rest of the archaebacteria and are in agreement with the phylogenetic tree of Woese and Olsen (1986).

tRNAs: By studying the base modification patterns of tRNAs, it was found that archaebacterial base modification patterns, although different from those of both eubacteria and eucaryotes, are more like the latter than the former (Best, 1978; Gupta and Woese, 1980). The first complete sequence of archaebacterial tRNA published was that of $tRNA_M^{Met}$ from *Thermoplasma acidophilum* (Kilpatrick and Walker, 1981). While some modifications of the tRNA resemble eubacteria, i.e., substitution of 4-thiouridine at position 8 (S^4U-8) and others resemble eucaryotes with the substitution of N^2,N^2-dimethylguanosine at position 26 (m_2^2G-26). The sequence of $G\psi\psi C_m$ (53-56) (ψ: pseudouridine; C_m : 2′-O-methylcytidine) is different from the $GT\psi C$ sequence normally found in both eubacteria and eucaryotes. Similar results were produced from the sequence data of 41 tRNAs from *Halobacterium volcanii* (Gupta, 1984). In some respects, the archaebacterial tRNAs resemble the tRNAs from eubacteria in having an unmodified A_{37}, a U_{54} and a U_{60} in their initiators (eucaryotic initiators have a modified base *N*-[*N*-β-*D*-Ribofuranosylpurin-6-yl) carbamayl]threonine (^6A) at position 37, A_{54} and A_{60}). In other respects, they resemble eucaryotic tRNAs in having a terminal A_1-U_{72} pair (eubacteria have a noncanonical C_1-A_{72} pair) and a base A_{20} (modified base dihydrouridine (D) at position 20 in eubacteria). The archaebacteria tRNAs also have some unique patterns such as G_{11}-C_{24} (C_{11}-G_{24} in eucaryotes and A_{11}-U_{24} in eubacteria).

The similarities that exist between archaebacteria and eucaryotes are also found in tRNA genes. The most interesting of these is the presence of introns in the archaebacteria tRNA genes. A 105-bp intron was found in the gene for $tRNA^{Trp}$ from *Halobacterium volcanii* (Daniels et al., 1986). It was also found that there are two putative intron-containing tRNA genes in *Sulfolobus solfataricus* (Keine et al., 1983). Another similarity between the tRNA genes of archaebacteria and eubacteria is the lack of 3′-terminal CCA residues in the gene (Daniels et al., 1986; Hui and Dannis, 1985; Keine et al., 1983; Jarsch and Bock, 1983).

DNA-dependent RNA polymerases: It was found that the subunit structure of the DNA-dependent RNA polymerase of archaebacteria is more like that of eucaryotes than of eubacteria (Huet et al., 1983). Antibodies raised against the individual subunit of RNA polymerase A and B from

Saccharomyces cerevisiae cross-react with the subunit of archaebacteria RNA polymerase to a much greater extent than they cross-react with the polypeptide components of eubacterial RNA polymerase (Huet et al., 1983). There are functional similarities as well between archaebacterial and eucaryotic RNA polymerases. The elongation reaction catalyzed by eucaryotic RNA polymerase A and by the archaebacterial RNA polymerase of *Thermoplasma acidophilum* are both stimulated by silybin, which suggests a similar reaction mechanism for both enzymes (Schnabel et al., 1982).

The archaebacterial RNA polymerases have some unique properties. All archaebacterial RNA polymerases are completely resistant to rifampin and streptolydigin, antibiotics which inhibit eubacterial RNA polymerases. They are also insensitive to α-amanitin, an inhibitor of eucaryotic RNA polymerases B and C (Zilig et al., 1985). Recently, Gropp et al. (1986) have shown that the large subunits of the three different eucaryotic nuclear RNA polymerases are more related to the corresponding components of the enzymes from archaebacteria than to one another. It is possible that these three eucaryotic RNA polymerases evolved from an ancestral enzyme that was very similar to the RNA polymerases as they are found today in archaebacteria, especially the sulfur-dependent branch.

Elongation factors: With hybrid polyphenylalanine synthesis systems, it was shown that translocating elongation factors can only function with ribosomes from their own kingdom (Klink et al.,1983; Klink, 1985). Within the same kingdom there are no severe restrictions, with the exception of mammalian mitochondrial ribosomes (Eberly et al., 1985). Therefore, elongation factors are very useful in marking the boundaries of urkingdoms. Analysis of the elongation factors from archaebacteria using antibiotic sensitivity methods showed a remarkable intralineage heterogeneity (Klink, 1985; Londei et al., 1986). These same properties were not present in eubacteria and eucaryotes. While eucaryotic elongation factor 2 (EF2) is sensitive to diphtheria toxin and eubacterial elongation factor-G (EF-G) is not, all strains of archaebacteria EF-G (EF2)-equivalent proteins tested are sensitive to the toxin (Klink, 1985). In this case, the archaebacteria are more like eucaryotes than eubacteria. By testing with three factor-targeted inhibitors (other than diphtheria toxin) of protein synthesis, Londei et al. (1986) distinguished the sulfur-dependent

thermoacidophiles from the rest of the archaebacteria. It was known that eubacterial EF-Tu is sensitive to both pulvomycin and kirromycin but eucaryotes are not sensitive to either of them. Londei et al. (1986) found that methanobacterial and halobacterial EF-Tu(EF1)-equivalent proteins are sensitive to pulvomycin but not kirromycin. They also found that methanobacterial and halobacterial EF-G(EF2)-equivalent proteins are sensitive to fusidic acid as is the case with eubacteria EF-G and eucaryotic EF2. A very interesting finding of their work is that almost all sulfur-dependent thermophiles are not sensitive to all three of the antibiotics mentioned above. These results clearly separate sulfur-dependent archaebacteria from methanogenic-halophilic archaebacteria. The same kind of results were produced by comparing the sequence of ADP-ribosylated tryptic peptide from archaebacterial EF-G(EF2)-equivalent protein with the sequence of eucaryotic EF2 (Gehrmann et al., 1986; Gehrmann et al., 1985). All of the results mentioned above indicate a remarkable diversity among archaebacterial orders and a deep cleft between the sulfur-metabolizing order and the others. It was also shown that there is an unusual hiatus between Sulfolobus and Thermoproteals which indicates a closer relationship between thermoplasma and the methanogenic/halophilic branch which is consistent with Woese's 16S rRNA sequence analysis results (Woese and Olsen, 1986).

Ribosomal 'A' protein: When the primary structure of the ribosomal 'A' protein was studied, it was found that the archaebacteria form a group of microorganisms which is different from that of both eubacteria and eucaryotes (Matheson et al., 1986). It was also found that the extreme halophiles, which are closer to eubacteria, are quite different from methanogen and sulfur-dependent bacteria, which are closer to eucaryotes. These data are in conflict with the evolutionary tree proposed by Woese and Olsen (1986).

Lipids: The unique archaebacterial isoprenyl glycerol ether lipids were found long before the concept of archaebacteria was articulated (Kates et al., 1966; Kates, 1978; Langworthy, 1977a, b). The hydrocarbon chains of the glycerol ethers are isoprenoids and are generally fixed at a chain length of either 20- or 40-carbon atoms. An exception, however, are the C_{25} diether lipids which are found

in certain species of archaebacteria (Rosa et al., 1983). There were also intensive studies on the chemical structural changes of the lipids induced by change in temperature (Gliozzi et al., 1983) and on the morphism of the lipids (Luzzati and Gulik, 1986). Not only are the phytanyl diethers or dibiphytanyl diglycerol tetraethers unique and found exclusively in archaebacteria, these lipids also contain glycerol with the opposite stereochemistry of the glycerol found in eubacterial and eucaryotic lipids (Langworthy and Pond, 1986; Langworthy, 1985). The above properties form an excellent molecular marker for the presence of archaebacteria.

Coenzymes: Several of the coenzymes or cofactors which are found in methanogenic archaebacteria have never been found in eubacteria or eucaryotes. Among these are coenzyme M (Balch and Wolfe, 1979), methanofuran (CRF) (Romesser and Wolfe, 1982), and methanopterin (Van Beelen et al., 1984b). All these coenzymes are involved in the process of metabolizing CO_2 and H_2 to methane and other metabolites. F_{420} has been found in a wide range of the archaebacteria (Lin and White, 1986) as well as in several different *Streptomyces* species (Daniels et al., 1985). The possibility that unknown coenzymes exist in the nonmethanogenic archaebacteria is the basis for the research in this thesis.

All of the above properties which have been used to define the archaebacteria are semantides (with the exception of the lipids and coenzymes) which are best for molecular phylogeny as mentioned earlier in this Chapter. Most of the episemantides like ATP and FAD are not suitable for molecular taxonomy since they are present in all cells. For this reason, the discovery that cyclic AMP (Leichtling et al., 1986) and polyamines (Kneifl et al., 1986) are present in archaebacteria is not very informative in terms of archaebacterial taxonomy. However, there are exceptions like the archaebacterial lipids and the new coenzymes mentioned above. According to Zuckerkandl and Pauling (1965), episemantides are of use in defining parts of the phylogenetic tree. Ideally, a certain kingdom will contain specific chemical markers such as the ether lipids in archaebacteria. Groups of organisms within this kingdom will also have a particular chemical marker such as the new coenzymes in methanogenic bacteria which will define these groups. The advantage of using this kind of marker is that the "small molecules" are usually much easier to identify. For example, it is

much easier to identify the ether lipids than it is to sequence DNA, RNA, and proteins. The question is, can we find such markers in such nonmethanogenic archaebacteria as the extreme halophiles and sulfur-dependent thermoacidophiles. The archaebacteria, as viewed from the physical condition of our planet surface, all live in extreme environments. Actually, they live in such bizarre conditions as high salt concentration, high temperature, low pH, and absolute anaerobic conditions that we suspect that they must have some special properties in order to adapt to these extreme environments. Furthermore, the archaebacteria are believed to have evolved very early, and separately from the eubacteria and the eucaryotes (Woese and Olsen, 1986), so that it is possible that they would have evolved coenzymes that have never evolved or existed in either eubacteria or eucaryotes. No successful studies have been done on these kind of molecules, with the exception of the ether lipids in archaebacteria and the coenzymes in methanogens. The work in this thesis is the first attempt to explore such molecules in the nonmethanogenic archaebacteria.

Of particular interest is the isolation and identification of pterins, as pterins are widespread in living organisms and their structure, and maybe function differ from organism to organism (see Table 1 on page 9 for the naturally occurring pterins and their different origins). Furthermore, pterins have never been studied in nonmethanogenic archaebacteria. Bagnara and Obika (1985), who found several pterins (including biopterin, pterin, xanthopterin, 6-carboxyl-pterin, and drosopterin) in amphibians, found that the distribution of the pterins is different from species to species. They suggested that the pterin distribution could be used as a taxonomic tool in understanding some of the dilemmas of amphibian phylogeny. On the other hand, phylogeny may assist in tracing the evolution of the metabolic pathways of pterins. This same idea has been employed in this study. By comparing the structures and distribution of pterins in different archaebacteria, yet another criterion is added to defining the phylogenetic tree in archaebacteria. Conversely, the taxonomy of the archaebacteria, as well as other kingdoms, may be used to follow the evolution of pterin structure and function.

Pterins have very important functions in living organisms and different kinds of pterins function differently. Pterins were first isolated from butterflies by Hopkins who first isolated a yellow pigment (Hopkins, 1889) and then a white pigment (Hopkins, 1895). These two pigments were later

named xanthopterin and leucopterin from the Greek terms *xanthos* (yellow) and *leucos* (white) and *pteron* (wing). With the discovery of folic acid by Mitchell et al. in 1941, it became apparent that pterins were more than just pigments in the wings of butterflies and in the eyes of insects. Folic acid, which is called a "conjugated" pterin because it contains p-aminobenzoic acid and polyglutamate in its side chains, is a well-known single carbon carrier which is present in all the living organisms studied so far with the possible exception of methanogens (Leigh, 1983). Whether folic acid is absent in all archaebacteria is still not very clear and remains an interesting question. Biopterin, which is called an "unconjugated" pterin because it lacks p-aminobenzoic acid and polyglutamate side chains, was initially found as a growth factor required by *Crithidia fasciculata* (Patterson et al., 1956a, b) and later it was found to function as a coenzyme in phenylalanine and tyrosine metabolism (Shiman, 1985; Kaufman and Kaufman, 1985; Kuhn and Lovenberg, 1985). Biopterin is important clinically (Curtius, 1985) as well as biochemically. There are pterins present in many organisms (see Table 1 on page 9), however, their function in most of them is far from clear.

Several pterins have been discovered only recently. Molybdenum pterin was found as a cofactor in xanthine dehydrogenase and other molybdenum containing oxido-reductases (Johnson and Rajagopalan, 1982; Johnson et al., 1984). Methanopterin, first discovered as the formalaldehyde activation factor, is present in all methanogenic bacteria and is very similar to folic acid in its chemical structure and properties (Daniels and Zeikus, 1978; Beelen et al., 1984; Escalante-Semerena et al., 1984). 7-Methylpterin, found only in methanogenic bacteria (Keltjens et al., 1983), has been shown to be an oxidative degradation product of methanopterin (White, 1985). These 7-methyl-substituted pterins have only been found in methanogens so far. Recently, another pterin was found from carbon monoxide dehydrogenase of *Pseudomonas carboxydoflava*. This pterin was named bactopterin and, at present, its structure is unknown (Krüger and Meyer, 1986).

The research from this thesis has added several new pterins to the list of pterins found in living organisms. The finding of these pterins is important both to archaebacterial phylogeny and pterin biochemistry. The structural features of these new pterins will be discussed in detail in the following Chapters.

Table 1 (Part 1 of 8). Naturally Occurring Pterins

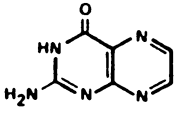
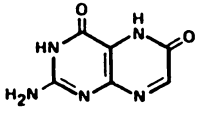
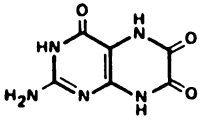
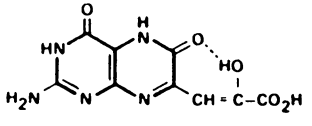
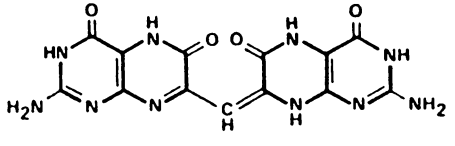
NAME	STRUCTURE	SOURCE	REFERENCE
PTERIN.		Methane-Oxd.Bact. Humane Urine Amphibians Amphibians Reptile Reptile	Urushibara, 1970 Fukushima, 1972 Bagnara, 1965 Hama, 1960 Blair, 1957 Ortiz et al., 1963a
XANTHOPTERIN		Butterfly Pseudomonas Ascidians Amphibians Reptile	Hopkins, 1889 Hopkins, 1891 Guroff, 1966 Gaill, 1975 Bagnara, 1965 Ortiz, 1963a
LEUKOPTERIN		Butterfly.	Hopkins, 1895
ERYTHROPTERIN		Tubecle Bacillus Mycobacteria	Crowe, 1949 Tschesshe, 1953
RHODOPTERIN		Butterfly Butterfly	Russell, 1949 Tschesche, 1952

Table 1 (Part 2 of 8). Naturally Occurring Pterins

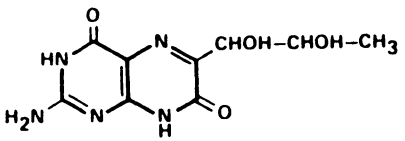
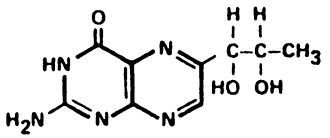
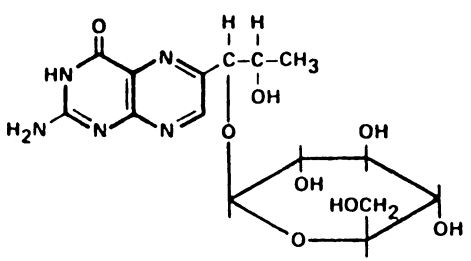
NAME	STRUCTURE	SOURCE	REFERENCE
ICHTHYOPTERIN		Fish Fish Fish	Matsuura, 1955 Tschesche, 1958 Huttel, 1943
BIOPTERIN		Humane urine Humane urine Fish Humane urine Rat Parasite Amphibians Bullfrog Reptile Amphibians	Patterson, 1956a Patterson, 1956b Mori, 1960 Fukushima, 1972 Haberle, 1978 Fukushima, 1970a Bagnara, 1965 Fukushima, 1970b Ortiz, 1963 Bagnara et al., 1965
BIOPTERIN GLUCOSIDE		Blue-green alga Blue-green alga	Forrest et al., 1958 Maclean et al., 1966

Table 1 (Part 3 of 8). Naturally Occurring Pterins

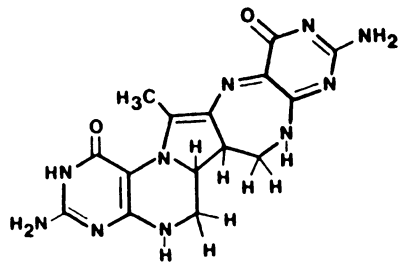
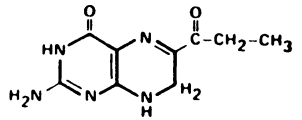
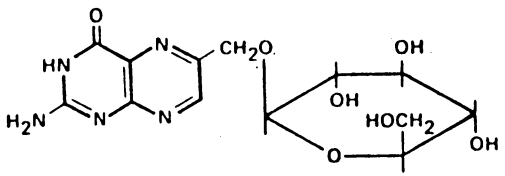
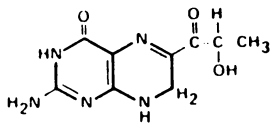
NAME	STRUCTURE	SOURCE	REFERENCE
DROSOPTERIN		Anoles (reptile) Reptile Eye of flies Eye of flies Eye of flies Amphibians Reptile	Ortiz et al., 1963b Ortiz, 1962 Baglioni, 1959 Theobald et al., 1978 Wiederrecht, 1981 Bagnara et al., 1965 Ortiz et al., 1963a
2'-DEOXYSEPIAPTERIN		Blue-green alga Eye of flies	Forrest et al., 1959a Forrest et al., 1959b
6-HYDROXY-METHYLPTERIN-GLUCOSIDE		Blue-green-algal	Hatfield, 1961
SEPIAPTERIN		Eye of flies Eye of flies Humane urine Amphibians Amphibians Bullfrog tadpole	Forrest et al., 1962 Pfleiderer, 1979 Fukushima et al., 1972 Bagnara et al, 1965 Hama et al., 1960 Fukushima, 1970b

Table 1 (Part 4 of 8). Naturally Occurring Pterins

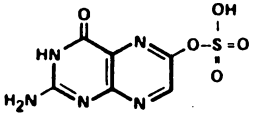
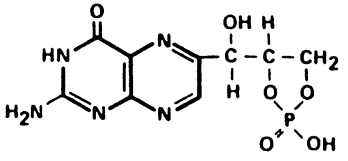
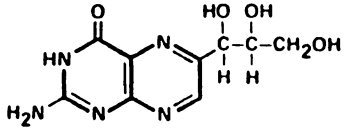
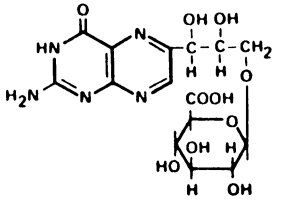
NAME	STRUCTURE	SOURCE	REFERENCE
XANTHOPTERINYL SULFATE		Azotomonas insolita	Goto and Forrest, 1965
NEOPTERIN-2', 3'-CYCLIC MONOPHOSPHATE		Pseudomonas Comanonas sp Methylococcus capsulatus	Guroff et al., 1966 Cone and Guroff, 1971 Urushibara et al., 1971
NEOPTERIN		Higher plant Humane urine Amphibians	Yoshida et al., 1968 Fukushima et al., 1972 Bagnara et al., 1965
NEOPTERIN-3'-B-D-GLUCURONIDE		Azotobacter agilis	Suzuki et al., 1968

Table 1 (Part 5 of 8). Naturally Occurring Pterins

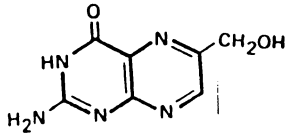
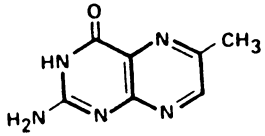
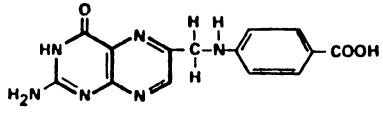
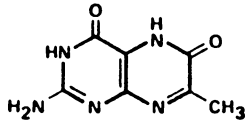
NAME	STRUCTURE	SOURCE	REFERENCE
6-HYDROXYMETHYL-PTERIN		Pseudomonas sp Spinach chloroplasts Spinach chloroplasts Higher plant Amphibians	Viscontini, 1968 Iwai et al., 1980 Iwai et al., 1976 Yoshida et al., 1980 Bagnara et al., 1965
6-METHYL-PTERIN		Methane-oxidizing bacteria	Urushibara and Forrest, 1970
PTEROIC ACID		Spinach leaves Spinach leaves	Mitchell et al., 1941 Angier et al., 1946
CHRYSOPTERIN		Butterflies	Tschesche & Korte, 1951

Table 1 (Part 6 of 8). Naturally Occurring Pterins

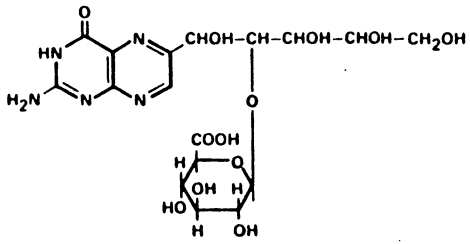
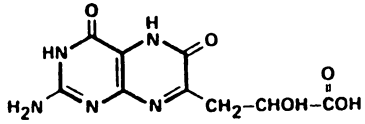
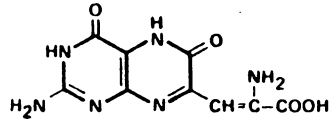
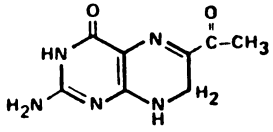
NAME	STRUCTURE	SOURCE	REFERENCE
		Mycobacteria	Goto and Kobayashi, 1965
EKAPTERIN		Head of flies	Nixon, 1985
LEPIDOPTERIN		Head of flies	Nixon, 1985
SEPIAPTERIN C		Flies	Sugiura et al., 1973

Table 1 (Part 7 of 8). Naturally Occurring Pterins

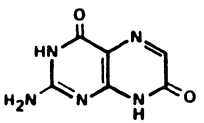
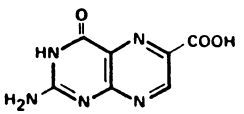
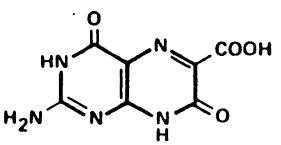
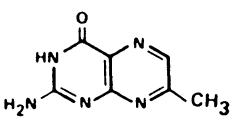
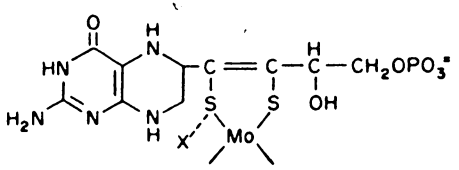
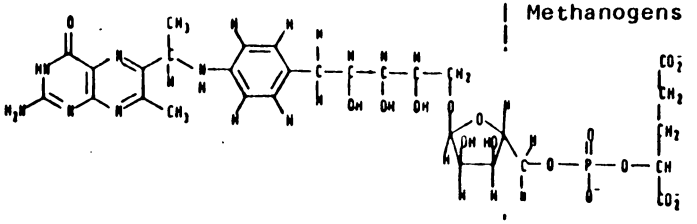
NAME	STRUCTURE	SOURCE	REFERENCE
ISOXANTHOPTERIN		Reptile	Ortiz et al., 1963b
		Reptile	Blair, 1957
		Higher plant	Yoshida et al., 1980
		Fish	Matsuura et al., 1955
		Humane urine	Fukushima et al., 1972
		Ascidians	Gaill et al., 1975
		Amphibians	Hama et al., 1960
6-CARBOXYPTERIN		Amphibians	Bagnara et al., 1965
		Amphibians	Hama et al., 1960
		Reptile	Blair, 1957
6-CARBOXY-ISOXANTHOPTERIN		Fish	Matsuura et al., 1955
7-METHYL PTERIN		Methanogens	Keltjens et al., 1983

Table 1 (Part 8 of 8). Naturally Occurring Pterins

NAME	STRUCTURE	SOURCE	REFERENCE
MO-COFACTOR (proposed structure)		Sulfate oxidase Xinthine dehydrogenase	Johnson et al., 1982 Johnson et al., 1984
METHANOPTERIN		Methanogens	Daniels et al., 1978 Van Beelen et al., 1984 Escalante- Semerena et al., 1984

Chapter II

The Distribution of Pterins in Nonmethanogenic Archaebacteria

2.1 Abstract

Pterin content was surveyed in nine different species of nonmethanogenic archaebacteria comprised of six species of halobacteria, two species of *Sulfolobus* and one specie of *Thermoplasma*. The results indicated the presence of a phosphate-containing pterin, phosphohalopterin-1, in three species of halobacteria, *Halobacterium salinarium*, *Halobacterium halobium*, and *Halococcus morrhuae*. The sulfate-containing pterin, sulfohalopterin-2, was present in three other species of halobacteria, *Halobacterium marismortui*, halobacterial strain GN-1, and *Halobacterium volcanii*.

A positively charged pterin, solfapterin, was present in *Sulfolobus solfataricus* and, more interestingly, a negatively charged pterin was induced when *S. solfataricus* was grown in a medium containing homogentisic acid (2,5-dihydroxyphenylacetic acid). This negatively charged pterin was

not present when the cells were grown in the normal medium. Another positively charged pterin found in *Thermoplasma* was determined to be different from solfapterin according to its paper electrophoresis properties. Uncharged pterins were also identified in all of these bacteria, however, no attempt was made to elucidate the structures of these uncharged pterins. Pterin content was found to be different in the different bacteria and to depend on the growth conditions.

All of the charged pterins described above differ from any previously described naturally occurring pterins and therefore represent new pterins. From a phylogenetic point of view, halopterins are good chemical markers for the extreme halophilic archaebacteria and the occurrence of the two different types of halopterins can also be used to separate the halobacteria into two sub-branches. The positively charged pterins are unique to thermoacidophilic archaebacteria and, therefore, could be used as chemical markers for those species that contain these pterins. Clearly all of the above pterins can be readily utilized in the development of archaebacterial taxonomy.

2.2 *Materials and Methods*

Halobacterium marismortui, *Halobacterium volcanii*, *Halobacterium halobium* R_1, and halobacterial strain GN-1 were supplied by Barbara J. Javor (Scripps Institution of Oceanography, LaJolla, CA). *Halobacterium salinarium*, *S. solfataricus*, and *Thermoplasma* strain 122-1B3 were supplied by Tom Langworthy (University of South Dakota, Vermillion). *Halococcus morrhuae* (ATCC 17082) and *Sulfolobus acidocaldarius* (ATCC 33909) were obtained from the American Type Culture Collection, Rockville, Md. The halobacteria were grown at 39°C in 100-ml Erlenmeyer flasks fitted with cotton plugs for the times indicated in the individual experiments. The flasks were shaken while under constant illumination from a 15-W, soft-white fluorescent light positioned ~30 cm above the cultures. The medium consisted of 50 mL of the complex medium described by Javor (Javor, 1984). *S. solfataricus* and *S. acidocaldarius* were grown at 75°C at pH 3.0 for 3 days with light aeration on a medium consisting of 1.3 g of $(NH_4)_2SO_4$, 280 mg of

KH_2PO_4, 250 mg of $MgSO_4 \bullet 7H_2O$, 75 mg of $CaCl_2 \bullet 2H_2O$, and 1.0 g of yeast extract per L of water. In an experiment designed for tracing the biosynthesis of caldariellaquinone which is a special quinone found only in *Sulfolobus* (Thurl, 1986), homogentisic acid (50 mg), a suspected intermediate in the biosynthesis of the quinone, was added to the *S. solfataricus* growth culture (100 mL). An unexpected result of this experiment is that a negatively charged pterin was induced by the homogentisic acid (shown in Results and Discussion). The *Thermoplasma* isolate was grown at 56°C at pH 2 for 3 days on a medium consisting of 0.2 g of $(NH_4)_2SO_4$, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \bullet 7H_2O$, 0.25 g of $CaCl_2 \bullet 2H_2O$, and 1.0 g of yeast extract per L of water. Halobacterial cells from 40 mL of growth medium and *Sulfolobus* and *Thermoplasma* cells from 400 mL of medium were isolated by centrifugation at 15000g for 20 min, and the resulting cell pellet was suspended in 35 mL of 70% ethanol. This suspension was heated for 30 min at 70°C, cooled, and centrifuged. The resulting clear extract was removed and the pellet was reextracted. The 70% ethanol-insoluble pellet was then dried at 110°C to a constant weight in order to obtain the dry weight of the extracted cells. This will be referred to as the dry weight residue (dwr). The combined 70% ethanol extract was rotary evaporated to dryness at 60 °C and the resulting residue was dissolved in the smallest possible volume of water. An equal volume of chloroform was added to the water and the resulting two-phase system was well mixed and centrifuged. The resulting clear, yellow water layer was removed, concentrated *in vacuo*, and purified by paper chromatography or paper electrophoresis. For analysis, the areas which contained the desired pterin bands were cut from the paper and the pterins were then eluted with water. Quantitation was accomplished by fluorescence spectroscopy of the samples disolved in water with excitation at 360 nm and emission at 445 nm. Known amounts of neopterin disolved in water were used as standard. The indicated molarities of the unknown pterins in this and the following Chapters represent the molarities of the pterin chromophore in the molecules. This point should be stressed because that the halopterins are dimeric pterins.

2.3 *Results and Discussion*

As shown in Figure 1 on page 21, two different negatively charged pterins are present in the six species of halobacteria studied (Figure 1, lanes 1-6). The identity of each of these two different pterins in the different species of halobacteria is further confirmed by their properties in acid hydrolysis and periodate cleavage (Figure 1, lanes 1′-6′ and 1″-6″, respectively). A detailed discussion about the above properties will be given in the following chapters.

The paper electrophoresis and thin-layer chromatographic properties of pterins from *Sulfolobus* and *Thermoplasma* are shown in Figure 2 on page 23. As shown in the figure (Figure 2A, lanes 4 and 5), different positively charged pterins are present in *S. solfataricus* and *Thermoplasma.* The preliminary work on the structure of solfapterin will be shown in Chapter VI. No further studies have been done with the positively charged pterin from *Thermoplasma* because of its extremely low concentration in the cells (see Table 2 on page 25). Figure 2A, lane 1 shows that the solfapterin is not present in another species of *Sulfolobus*, *S. acidocaldarius.* This property clearly separates the two species of *Sulfolobus* and is, therefore, a good chemical marker. As shown in Figure 2A, lane 3, when *S. solfataricus* was grown in the presence of homogentisic acid, a negatively charged pterin was induced which was not detected when the cells were grown in normal medium (Figure 2A, lane4). The properties of this negatively charged pterin are similar to those of phosphohalopterin-1 in paper electrophoresis, however, it was differentiated from the latter by thin-layer chromatography (Figure 2B, lanes 2 and 3). Based on the fluorescent intensity of the homogentisic acid-induced pterin, it appears that this pterin occurs in the cells at about the same concentration as solfapterin. No further work has been done on elucidating the structure and function of this pterin, but the discovery has raised an interesting and challenging question as to the function of this pterin and the other charged pterins. Possibly the homogentisic acid-induced negatively charged pterin is involved in the metabolism of homogentisic acid. The question remains as to how it is involved and how the biosynthesis of this pterin is induced by homogentisic acid.

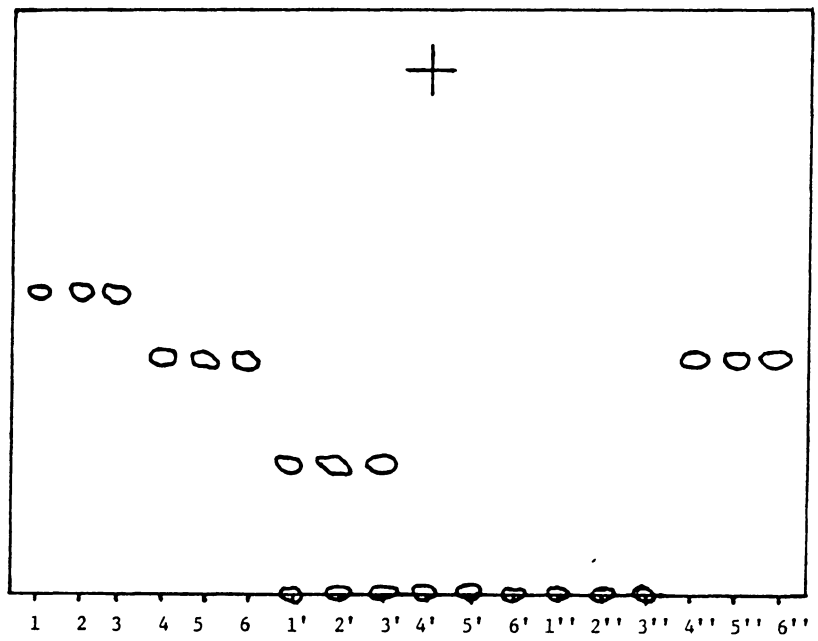


Figure 1. Paper electrophoresis of halopterins: Schematic representation of paper electrophoresis of halopterins from six species of halobacteria. Lanes 1-6 represent samples from *H. marismortui*, strain GN-1, *H. volcanii*, *H. salinarium*, *H. halobium*, and *Halococcus morrhuae*, respectively. Lanes 1'-6' and 1''-6'' represent the same sequence of samples except that the former were hydrolyzed with 1 M HCl at 100°C for 3 h, and the latter were reacted with 0.1 M $NaIO_4$ before being applied to the paper for electrophoresis. The electrophoresis was done on Whatman no. 3 paper (16 x 35 cm) with puridine:AcOH:water (1.0:0.4:98.6, v/v/v) buffer at pH 5.3 and at a constant power of 30 W for 40 min. The crude halobacterial extracts were prepurified with paper chromatography developed with n-butanol:water:AcOH (12:5:3, v/v/v) (referred to as S1) on Whatman no. 3 paper (23 x 55 cm). The lowest R_f, most intensive blue fluorescent bands were cut off and the halopterins were eluted off with water. The resulting solutions were concentrated in vacuo and readied for the electrophoresis.

The answers may provide clues to the functions of other negatively charged pterins such as the halopterins found in halobacteria.

Table 2 on page 25 shows the levels of these new pterins found in the cells. As shown in the table, the maximum yield of phosphohalopterin-1 was produced by *Halococcus*, however, *H. salinarium* was selected for the structural studies of phosphohalopterin-1 because of the low growth yield of the *Halococcus*. *H. marismortui* was used in the structural studies of sulfohalopterin-2 because it produced the maximum amount of halopterins (the halopterin in *H. marismortui* is approximately ten times that of *H. salinarium*).

A comparison of the calculated in vivo concentrations of the new pterins found in nonmethanogenic archaebacteria with those of coenzymes and pterins found in other bacteria is reported in Table 3 on page 26. The table shows that the concentration of halopterins is at least 100 times that of the folic acid found in *P. fluorescens* but that the concentration of the positively charged pterin from *Thermoplasma* is approximately the same as that of folic acid. This could suggest that the positively charged pterin found in the thermoacidophiles might function as a one-carbon carrier like folic acid. An amine on the side chain of solfapterin has been identified (see Chapter V) and, chemically, this amine could function in the same way as the benzyl amine in folic acid. table 3 on page 26 also shows that the concentration of halopterins in halobacteria and the concentration of pterins in photosynthetic bacteria (Maclean et al., 1966) are about the same. This could suggest that halopterins may function in halobacteria in the same way as the pterins function in photosynthetic bacteria (Maclean et al., 1966), i.e., in the photoreaction center.

Figure 3 on page 24 contains the growth curves and halopterin production for *H. halobium* and *H. marismortui.* It is clear that phosphohalopterin-1 continued to be synthesized after the cells stopped growing whereas sulfohalopterin-2 synthesis stopped. For all cases, however, the pterins reached their maximum level after about 5 days of growth.

No halopterins were detected in *E. coli*, *Nostoc muscorum*, Chlorobium cells, yeast cells or *M. formicicum* by paper chromatography of cell extracts. Compounds detected in *M. formicicum* with about the same R_f as halopterins were shown not to be these compounds by acid hydrolysis of the extract followed by paper chromatography.

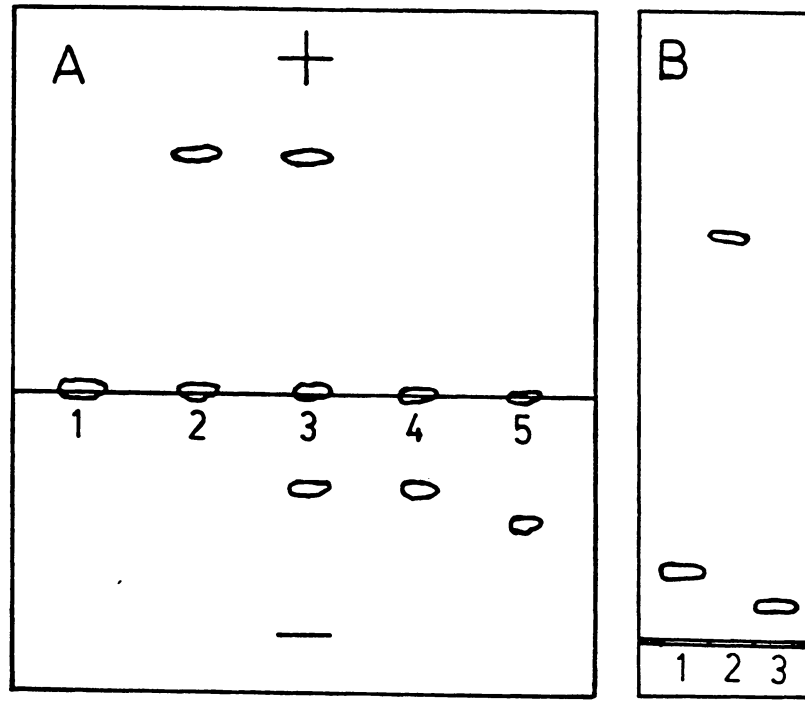


Figure 2. Paper electrophoresis (A) and TLC (B) of pterins from thermoacidophilic archaebacteria: A: The paper electrophoresis procedures and the phosphohalopterin-1 sample (lane 2) are the same as in Figure 1. For the thermoacidophiles, the crude extracts as described in Materials and Methods were directly applied to the paper for the electrophoresis. Lane 1, *S. acidocaldarius*; lane 2, phosphohalopterin-1; lane 3, *S. solfataricus* grown with homogentisic acid; lane 4, *S. solfataricus*; lane 5, *Thermoplasma*. B: The TLC was done on silica gel 60 F-254 (5 x 18.5 cm, E. Merk AG, Darmstadt, Federal Republic of Germany) which was developed by S1. Lane 1, solfapterin; lane 2, homogentisic acid induced negatively charged pterin; lane 3, phosphohalopterin-1.

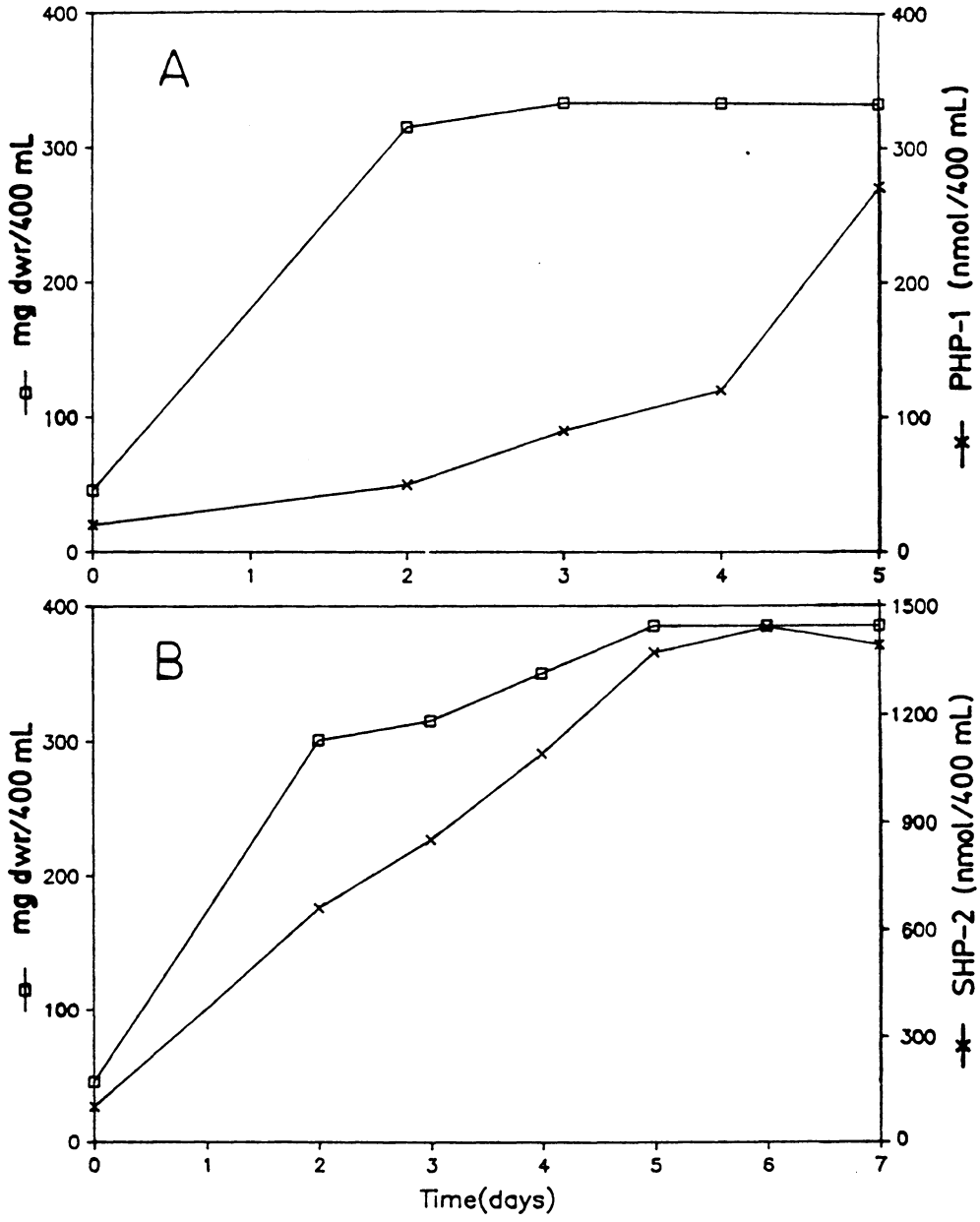


Figure 3. Growth and halopterin synthesis: A: Growth and phosphohalopterin-1 synthesis in *H. halobium*, and B: Growth and sulfohalopterin-2 synthesis in *H. marismortui*. The experimental conditions have been given in "Materials and Methods" except that 400 mL of growth cultures was used for each point. Note the scale differences between A and B.

Table 2. Amounts of Charged Pterins in Nonmethanogenic Archaebacteria

Cell Type	Pterin	nmol/mg dwr [1]
H. salinarium	Phosphohalopterin-1	0.38
H. halobium	Phosphohalopterin-1	0.35
Halococcus	Phosphohalopterin-1	0.80
H. volcanii	Sulfohalopterin-2	0.35
GN-1	Sulfohalopterin-2	1.9
H. marismortui	Sulfohalopterin-2	3.8
S. solfataricus	Solfapterin	0.21
Thermoplasma	Positively charged pterin	0.016 [2]

[1] dwr: dry weight residue or insoluble material after the cells are extracted with 70% ethanol.

[2] Estimated value according to the comparison of fluorescent intensity with solfapterin.

Table 3 (Part 1 of 2). Comparison of Pterin and Vitamin Levels

Cell Type	Pterin or Vitamin	Conc. (mM)[1]
H. salinarium	Phosphohalopterin-1	0.12[2]
H. halobium	Phosphohalopterin-1	0.11[2]
Halococcus	Phosphohalopterin-1	0.25[2]
H. volcanii	Sulfohalopterin-2	0.11[2]
GN-1	Sulfohalopterin-2	0.61[2]
H. marismortui	Sulfohalopterin-2	1.2[2]
S. solfataricus	Solfapterin	0.067[2]
Thermoplasma	Positively charged pterin	0.005[5]
P. fluorescens	Folic acid	0.0024[3]
E. coli	Riboflavin	0.26[3]
E. coli	Thiamine	0.0069[3]
B.thetaiotaomicron	Pantothenic acid	0.27[3]
B. subtilis	Nicotinic acid	3.5[3]
B.thetaiotaomicron	Pyridoxine	13.8[3]
R.vannielli	Pterin (total)	0.76[4]

Table 3 (Part 2 of 2). Comparison of Pterin and Vitamin Levels

R.palustris	Pterin (total)	0.81[4]
R.rubrum	Pterin (total)	0.18[4]

[1] Results are reported as the in vivo concentration, assuming that 22% of the wet weight of cells is equal to the dry weight and that the wet weight of cells contains 70% water (Watson J. D., 1965. in Molecular Biology of the Gene. Ed. C. Levinthal. pp. 85).

[2] The dry-weight value in this calculation was that of the 70% ethanol-insoluble material and thus is less than the actual dry weight of cells.

[3] Calculated from the data of Leigh (1983).

[4] Calculated from the data of Maclean et al.. (1966).

[5] Estimated value according to the comparison of fluorescent intensity with solfapterin.

The fact that the above pterins are present in nonmethanogenic archaebacteria and absent in eubacteria and eucaryotes is further evidence of the uniqueness of archaebacteria. While several new coenzymes have been found in methanogenic archaebacteria as mentioned in Chapter I, the discovery of these new pterins could represent new coenzymes from the nonmethanogenic archaebacteria. It is reasonable to believe that more new coenzymes will be found with further work in this field. Sulfohalopterin-2 is in the reduced form (tetrahydrosulfohalopterin-2) in vivo in *H. marismortui* and it is an unconjugated pterin (see Chapter III) so that it could serve as a cofactor in an enzymatic hydroxylation as does biopterin in the hydroxylation of phenylalanine (Ayling and Baily, 1982; Benkovic, 1980; Kaufman, 1971).

As mentioned above, positively charged unconjugated pterins have never been found in nature before. Chemically, the reduced form of these kinds of pterins could have the same properties as tetrahydrofolate or tetrahydromethanopterin and could serve as a coenzyme in one-carbon metabolism. For this reason, the positively charged pterins from thermoacidophilic archaebacteria could function as a substitute for folic acid in these cells.

The structure of these new pterins will be discussed in the following chapters, where it will be shown that there are several unusual structural features of the halopterins. These special structural features provide another example of the uniqueness of archaebacteria. First, the dimeric pterins are not common in naturally occurring pterins (see Table 1). The only dimeric pterin is rhodopterin, in which the two pterins are connected by a carbon-carbon bond, not an ether linkage as in halopterin. Second, the ether linkage of sugars is very unusual in nature. Though it is not difficult for the cell to make the ether bond (like the ether lipids of archaebacteria), it is very difficult to synthesize this kind of sugar ether chemically. That is why, until now, no good synthetic strategies have been proposed to chemically synthesize this compound.

These findings are also evidence for the diversity of archaebacteria. It was surprising to learn that there are two different forms of halopterin in the different strains of halobacteria. It was also a surprise to find that solfapterin is not present in *S. acidocaldarius*. The halopterins clearly separate the halobacteria into two sub-groups and if more extensive studies are performed, the solfapterin may also be found to separate *Sulfolobus* into two sub-groups, with or without this pterin. It is

hoped that this work will bring about extensive studies of the structures of all forms of pterins (charged or uncharged) in archaebacteria and thereby contribute to archaebacterial taxonomy. On the other hand, the study of the evolution of the living organisms and their pterin structures and content will provide a better understanding of the evolution of pterins and coenzymes.

Chapter III

Isolation and Characterization of Sulfohalopterin-2 from Halobacterium marismortui

3.1 Abstract

Sulfohalopterin-2 (SHP-2) (39 μmol) was isolated from 700 g wet weight of cells of *H. marismortui* from 40 L of fully grown culture. The structure of this compound was elucidated by various methods. UV-visible and fluorescence spectra revealed that it is a typical unconjugated pterin. Paper electrophoresis proved that both SHP-2 and its acid hydrolysis product halopterin-2 (HP-2) were negatively charged. The origin of the negative charge was shown to result from sulfate esters and carboxylate on the side chain of the pterin by sulfate assay, ^{35}S-labeling, and specific infrared absorbance bands. The PMR spectra of SHP-2 and HP-2 and the ^{13}C-NMR (CMR) of SHP-2 proved that there is a methylene and a five-carbon polyol connected together on the side chain and that SHP-2 is a 6-substituted unconjugated pterin. The mass spectrum of the TMS-derivative of the $NaBH_4$-reduced pterin aldehyde produced by periodate cleavage of SHP-2

proved that sulfohalopterin-2 is a novel pterin dimer which has an ether linkage on the polyol side chain. The dimer is a symmetrical molecule generated by an ether bond at the 2′-carbons of the pterin side chains. This was proven by the mass spectra of the TMS derivatives of the periodate cleavage and borohydride reduction products derived from SHP-2. The proposed structure of SHP-2 follows.

3.2 Materials and Methods

3.2.1 Purification of SHP-2

H. marismortui cells were grown as described in "Materials and Methods" in Chapter 2 except that 1.1-L cultures were grown in 3-L Erlenmeyer flasks at 39°C for 5 days with shaking and constant illumination. The cells were isolated by centrifugation at 17000g for 30 min and 700 g of wet cells were collected from 40 L of the growth medium (most of the wet weight of the cell pellet is salt). The resulting cell pellet was mixed with 3 L of 70% ethanol and placed in the dark at room temperature for 12 h to complete the oxidation of the reduced pterin. The homogenous mixture was centrifuged, the clear 70% ethanol fraction was separated, and the insoluble material was re-extracted with 3 L of 70% ethanol 10 more times. The combined 70% ethanol fractions were

evaporated, dissolved in 500 mL of distilled water, and mixed with 500 mL of chloroform. The well-shaken water and chloroform mixture was centrifuged at 25000g for 30 min and the resulting clear, yellow water layer, which contained a high concentration of salt, was removed and diluted to 20 L with distilled water to reach a final concentration of sodium chloride of 0.15 M as measured by conductivity. The water solution was then passed through a 3.5 x 23 cm column of QAE-Sephadex (Q-25-120) from Sigma Chemical Co. which was preequilibrated with 0.1 M NH_4HCO_3. The column was further washed with 2 L of 0.2 M NH_4HCO_3 and the anionic compounds were then eluted with a linear gradient of 0.2 to 2 M NH_4HCO_3. Fractions (7 mL) were collected from the eluent (800 mL) and SHP-2 was detected by its absorbance at 350 nm. Those fractions containing the major SHP-2 peak (high A_{350}, ~ 1.5-2.0 M NH_4HCO_3 fractions) were combined, lyophilized, dissolved in approximately 3 L of distilled water, and applied to another QAE-Sephadex column (1.5 x 27 cm) which was preequilibrated with 0.1 M NH_4HCO_3. A 0.2 - 2 M NH_4HCO_3 linear gradient was applied and the fractions containing SHP-2 were collected and lyophilized. The resulting lyophilized material was dissolved in the smallest possible volume of distilled water (5 - 10 mL) and applied to a 3.5 x 37 cm column of Bio-Gel P-2 (200-400 mesh) from Bio-Rad Laboratories which was preequilibrated with distilled water. Distilled water was used as the eluting solvent and SHP-2 fractions were quantitated by absorption at 342 nm ($E = 6.3 \times 10^{+3}$ $M^{-1}. cm^{-1}$). The combined SHP-2 fractions were evaporated, dissolved in a minimal amount of water, and purified by paper chromatography developed with n-Butanol:AcOH:water (12:3:5 v/v/v) on Whatman no. 3 paper (23 x 55 cm). This solvent system will be referred to as S1. The major blue fluorescent band with the lowest Rf was cut off, eluted from the paper with water, dried, dissolved in a minimal amount of water, and reapplied to the above Bio-Gel P-2 column which was eluted with distilled water. The fractions containing SHP-2 (a total of 39 μmol of SHP-2) were combined, evaporated, and dissolved in D_2O for NMR and subsequent analyses.

3.2.2 Sulfate and Phosphate Assay

Three samples of SHP-2 (24 nmol per sample) were hydrolyzed with 1 M HCl at 100°C for 2 h to release the bound sulfate for assay. For a control, 3 samples of SHP-2, which were not subjected to the hydrolysis procedure, were analyzed at the same time as the samples. In addition, three blanks with the same amount of 1 M HCl as the samples were subjected to the same "hydrolysis" procedures and assayed the same way as the samples. A water solution of Na_2SO_4 (4 mM) was used to do the standard curve. The sulfate assay method used was basically the same as that described by Terho and Hartiala (1971) which is based on the reaction of barium rhodizonate with sulfate.

The stock solutions were a $BaCl_2$ solution composed of 10 mL of 2 M acetic acid, 2 mL of 0.005 M $BaCl_2$, and 8 mL of 0.02 M $NaHCO_3$ which was brought to 100 mL with ethanol and a rhodizonate solution which was made by dissolving 5 mg of sodium rhodizonate (from Sigma) in 20 mL of water followed by the addition of 100 mg of L-ascorbic acid (from Fisher). The rhodizonate solution was shaken until all solids were dissolved and the volume was brought to 100 mL with ethanol. The solutions can be used after 30 min and remain stable for about 2 days.

All the glassware was cleaned with nitric acid and rinsed repeatedly with distilled water. The standard and the samples were dissolved in 0.5 mL of water after which 1 mL of $BaCl_2$ solution and 1.5 mL of rhodizonate solution were added. The mixtures were shaken well and kept in the dark for 15 min before the absorbance was read at 520 nm.

The phosphate assay used was that of Martine et al. (1971). The stock solution is a mixture of 1 part 10% ascorbic acid and 6 parts molybdate mix composed of 0.42% ammonium molybdate in 1 N H_2SO_4. Both standard phosphate solutions and the samples (prepared by the acid hydrolysis of SHP-2 in 1 M HCl at 100 °C for 12 h and then dried under nitrogen) were dissolved in 0.3 mL of 0.5 M HCl. Stock solution (0.7 mL) was added to the standard and the samples and these were mixed well and heated at 45 °C for 20 min before the absorbance was read at 820 nm.

3.2.3 ^{35}S-Labeling Experiment

In order to get the highest specific activity of the radioactive sulfate with a given amount of $^{35}SO_4^=$, it was important to use a minimal amount of unlabeled sulfate in the growth medium so that the cells would grow normally and the yield of SHP-2 would not decrease. To determine this minimal sulfate concentration, the influence of the sulfate content in the growth medium on the growth of *H. marismortui* and the yield of SHP-2 was studied. The normal complex medium (CM) described by Javor (Javor, 1984) contains 81 mM $MgSO_4$. A sulfate-free medium was made which was essentially the same as the CM medium except that $MgCl_2$ was used instead of $MgSO_4$. Ten milliliters of *H. marismortui* cells grown for 2 days in the CM medium was transferred to 90 mL of sulfate-free medium to make the final sulfate concentration 8.1 mM. The cells were grown under normal conditions and 10 mL of the cells grown for 2 days on the 8.1 mM sulfate medium were transferred to the sulfate-free medium again to make the concentration of sulfate 0.81 mM. The growth curves of the cells which were grown in the 81 mM, 8.1 mM, and 0.81 mM sulfate CM media were compared as were the yields of SHP-2 in these growth media.

For the radioactive experiment, 10 mL of *H. marismortui* cells grown in the CM medium for 2 days were added to 90 mL of sulfate-free CM medium (0.81 mM sulfate) in a 250-mL Erlenmeyer flask. H_2SO_4 (0.14 mCi, carrier-free) from ICN Biomedicals, Inc. were added to the medium and the cells were grown for 5 days before being centrifuged and extracted as described earlier (Materials and Methods, Chapter II). The specific activity of the ^{35}S-labeled sulfate in the growth media is 6.8 x 10^3 dpm / nmol. This specific activity is the basis for the quantitative calculation of sulfate containt in the ^{35}S-labeled SHP-2, as will be shown in the following figures. The ^{35}S-labeled SHP-2 was isolated by paper chromatography, repurified two times with paper electrophoresis, and then purified by Bio-Gel P-2 column chromatography. The above procedures were basically the same as those described in Chapter II, "Materials and Methods" on page 18.

3.2.4 Oxidation of SHP-2 with Periodate and Reduction of the Resulting Aldehyde

SHP-2 (1.3 μmol) was dissolved in 0.1 mL of water and the resulting solution was mixed with 0.2 mL of 65 mM $NaIO_4$ (13 μmol) in water. The mixture was kept in the dark for about 2 h. The resulting pterin aldehyde precipitate was dissolved by adding 0.1 mL of 2 M NH_4OH. Approximately 0.1 mL of 1 mM $NaBH_4$ in 2 M NH_4OH was then added to the reaction mixture which was kept in the dark for about an h. The reaction mixture was then evaporated under a stream of nitrogen and the extra $NaBH_4$ was destroyed by adding 1 M HCl. A few drops of methanol were added to the acidic solution and the mixture was evaporated under nitrogen. The resulting material was dissolved in water and passed through a small Dowex 50W-8X H^+ (0.7 x3.0 cm) column. The column was washed with water and the pterin was eluted with 2 M NH_4OH into the top of a 0.7 x 4.5 cm column of Dowex-1 X8-200 OH^-. The Dowex-1 column was washed with water and 0.01 M HCl and the pterin was eluted off with 0.1 M HCl. This pterin, which will be called SHP-OR, was derivatized by the TMS reagent for mass spectral analysis.

3.2.5 Reduction and Reoxidation of SHP-2

The procedures used are basically those of Fukushima and Nixon (1980) Fukushima et al. (1978) and Kaufman (1967).

SHP-2 (0.15 μmol) was put into a small bottle (1 x 3 cm) fitted with a rubber septum. Platinum(IV) oxide (1 mg) was added to the bottle and the bottle was flushed with H_2. Forty microliters of 1 M HCl was then added and the bottle was pressurized with 24 psi H_2 and kept at room temperature with constant stirring for 2 h. The resulting pterin (in tetrahydro form, checked by UV-visible spectrum) in 1 M HCl was separated from the platinum by filtration and then equally distributed into two small bottles. The samples in both bottles were evaporated under nitrogen,

then 10 μL of 0.1% I_2 in 0.1 N NaOH was added to one bottle and 10 μL of 0.1% I_2 in 0.1 N HCl was added to the other bottle. The bottles were allowed to sit in the dark at room temperature for 30 min and the reaction product (oxidized pterin) was directly applied to the TLC plate for TLC analysis. For the control, standard neopterin was reduced and reoxidized in exactly the same manner.

3.2.6 Periodate Oxidation of HP-2 and Its Spectrum Change

HP-2 and neopterin (both 50 nmol) were dissolved in 2 mL of 0.01 M HCl separately and then 0.5 mL of 0.01 M HIO_4 was added to each. After standing at room temperature for 15 min, 0.5 mL of 0.1 M KOH was added to give a final pH of 10.5 and a UV-visible spectrum was taken immediately for each in order to determine the maximum absorbance. For the controls, HP-2 and neopterin were each dissolved in 2.5 mL of 0.01 M HCl and 0.5 mL of 0.1 M KOH was added before each spectrum was taken.

3.2.7 Sodium Borotritide Reduction Experiment

The experimental procedures were basically the same as in "Oxidation of SHP-2 with Periodate and Reduction of the Resulting Aldehyde" on page 35 except that $NaBT_4$ was used instead of $NaBH_4$ and some other minor modifications in the experiment.

SHP-2 (0.6 μmol) was reacted with 3.0 μmol of $NaIO_4$ in water and the reaction mixture was kept in the dark at room temperature for 3 h. NH_4OH (2 M) was added to dissolve the pterin precipitate and then about 3 μCi of $NaBT_4$ was added to the reaction mixture. The mixture was kept in a hood overnight and then about 6 μmol of $NaBH_4$ were added to make sure that the reduction was completed. The above reaction mixture was evaporated under nitrogen to remove excess ammonia, then 0.5 mL of 50% acetic acid was carefully added, and the solution evaporated

again. The residue was redissolved in ~ 0.5 mL of an equal mixture of 1 M HCl and methanol and then evaporated with heating under nitrogen. The evaporation of the acidic methanol was repeated one more time in order to insure the complete removal of boric acid. The total activity of the resulting labeled sample was 3.7 x 10^7 cpm.

Two μmol of SHP-2 was used to prepare a unlabeled sample which was subjected to the procedures as described in "Oxidation of SHP-2 with Periodate and Reduction of the Resulting Aldehyde" on page 35. The unlabeled sample was combined with the labeled sample and then directly applied to the paper for paper electrophoresis as described in Figure 1 on page 21.

3.2.8 Preparation of Threonic Acid and Erythronic Acid

The erythronic acid was prepared by oxidizing erythrose with iodine in base solution according to Schaffer and Isbell (1963). The threonic acid was prepared by two methods. The first was to epimerize erythronate to threonate in base by heating according to the method of Hamilton and Smith (1954). The second and preferred method was to oxidize ascorbic acid to threonic acid with H_2O_2 in base according to the method of Isbell and Frush (1979). The lactone of the resulting acid was prepared by passing the acid through a Dowex-50 column and drying the resulting acid solution with heat under a stream of nitrogen.

3.2.9 Instruments Used in These Studies

UV-visible spectra were recorded on a Varian Cary 219 double beam spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer 650-40 fluorescence spectrophotometer. NMR spectra were obtained on an IBM System WP-270 SY. Infrared spectra were run on a Perkin-Elmer 700 infrared spectrophotometer. Mass spectra and GC-MS were obtained using a VG 70-70HF mass spectrometer. A Varian 2400 gas chromatograph was used for the GC analyses.

CD spectra were recorded in the Department of Physiology at Duke University on an Instrument S.A. Auto-dichrograph Marlc.

3.3 Results and Discussions

3.3.1 UV-visible and Fluorescence Spectra of Sulfohalopterin-2

The UV-visible spectra of SHP-2 recorded under acidic, basic, and neutral conditions were the same as those of neopterin when the SHP-2 was purified by the above methods (Figure 4 on page 39).

The fluorescence excitation and emission spectra of SHP-2 were also the same as those of neopterin (Figure 5 on page 40). These spectra formed the basis for the identification of the chromophore of SHP-2 as a pterin.

3.3.2 Evidences that SHP-2 is in The Tetrahydro Form in vivo

A comparison of Bio-Gel P-2 column chromatography of aerobic and anaerobic extracts from *H. marismortui* is shown in Figure 6 on page 42. The figure shows that less than 10% of the total pterin in *H. marismortui* is in an oxidized form, or basically, that pterins are in reduced form in vivo. The fluorescence in this case can only distinguish the oxidized form from the reduced form as all forms of reduced pterins are non-fluorescent. Basically, there are two reduced forms of pterins, tetrahydropterin and dihydropterin. Neither of these forms is fluorescent. They can be distinguished by their absorbance since tetrahydropterins have λ_{max} at 287 nm in basic solution and

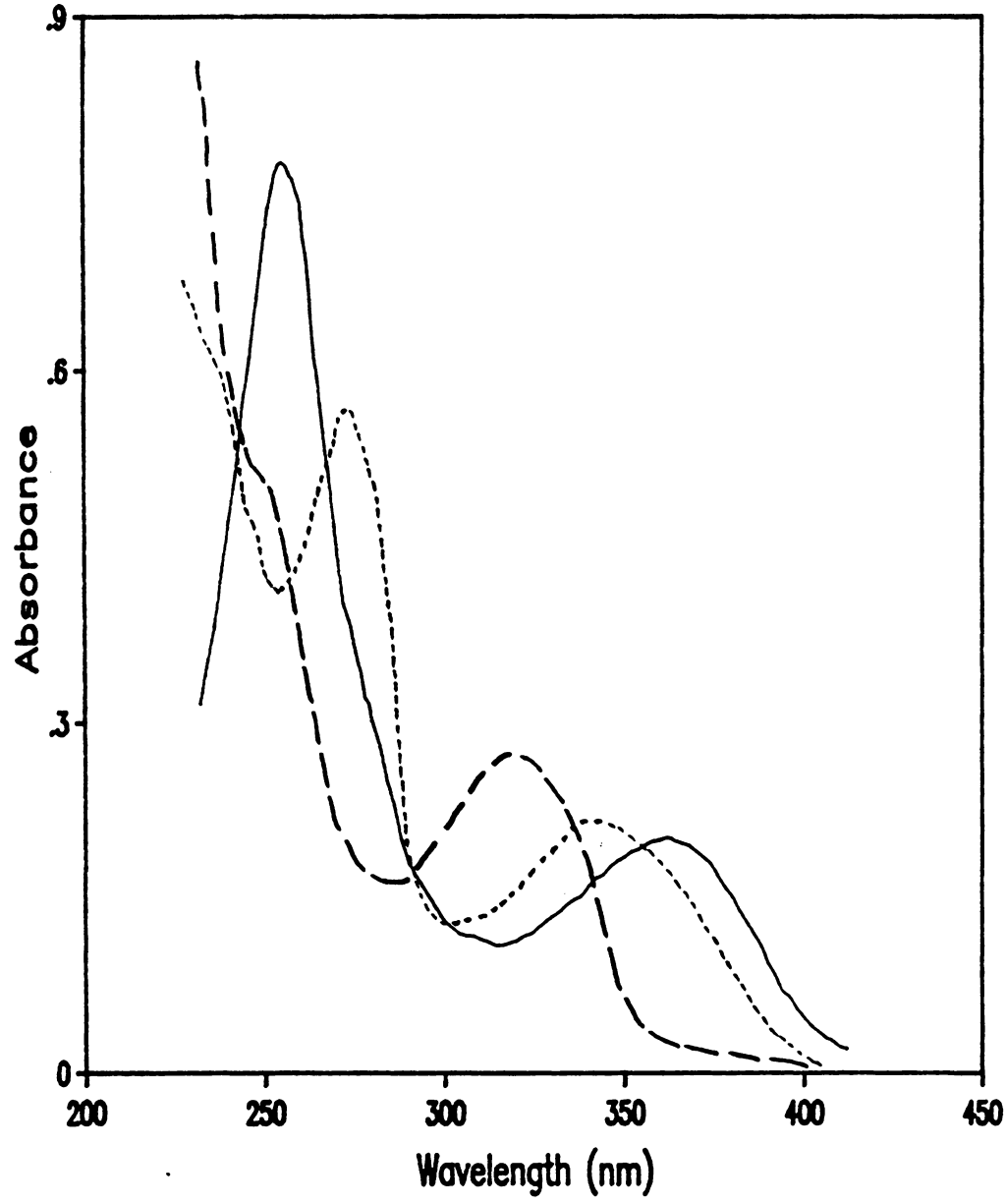


Figure 4. UV-visible spectra of SHP-2: The spectra were recorded with a Varian Cary 219 double beam spectrophotometer at different pH. (—) ,pH 1.5; (----), pH 4.8; (—), pH 11. The spectra are the same as those recorded for neopterin under the same conditions.

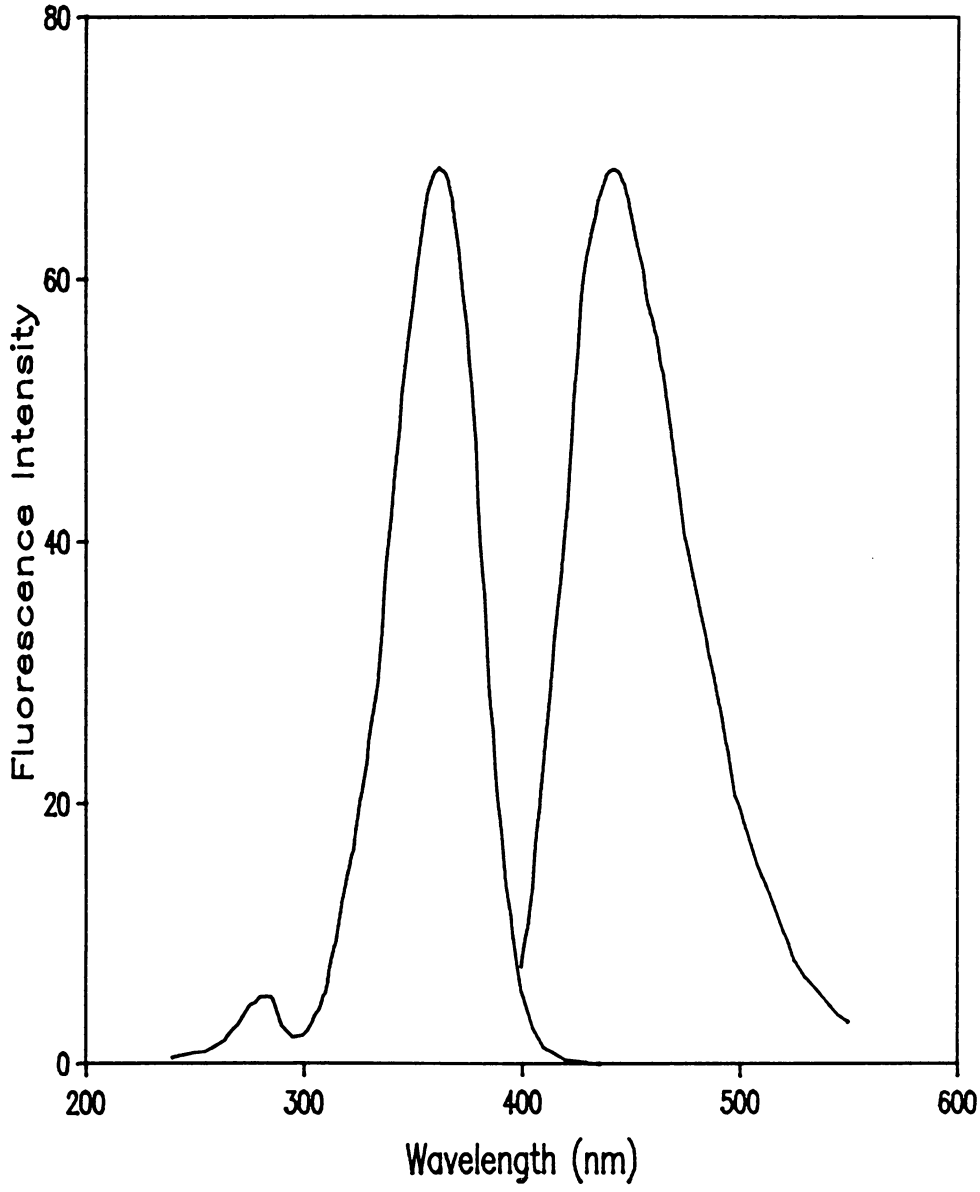


Figure 5. Fluorescence excitation and emission spectra of SHP-2: The spectra were recorded at pH 5.3 in 0.1 M phosphate buffer with a Perkin-Elmer 650-40 fluorescence spectrophotometer. The spectra are identical to those of neopterin when recorded under the same conditions. The fluorescence excitation spectrum (emission wavelenth at 445 nm) is in the left hand side of the figure and the emission spectrum (excitation wavelenth at 362 nm) is in the right hand side of the figure.

dihydropterins have λ_{max} at 332 nm in basic solution (Pfleiderer, 1985). In the same solution, the λ_{max} for oxidized pterins is approximately 350 nm.

A QAE-Sephadex column chromatography of an anaerobic extract from *H. marismortui* is shown in Figure 7 on page 43. The profile basically shows that no oxidized forms of pterins are present in vivo (—▽—, A_{350}). There is some absorbance for the dihydroSHP-2 from fractions 65 - 80 (—□—, A_{332}) which means that there is a small percentage (< 10% of that of the tetrahydroform) of SHP-2 in dihydroform (SHP-2 was found to elute between fraction 65 and 80). The tetrahydroSHP-2 is the major form present in *H. marismortui* in vivo (—×—, A_{287}, fractions 65 - 80). There could be compounds other than tetrahydroSHP-2 in fractions 65-80. However, the presence of tetrahydroSHP-2 was proved by the recovery SHP-2 after the fractions were oxidized. The other A_{287} absorbance peaks are not tetrahydropterins. It is known that pterin cofactors such as biopterin and folic acid are present in the tetrahydro form in vivo (for review, see Wachter et al., 1985; Blakley and Benkovic, 1984, 1985) so tetrahydroSHP-2 could also function in the reduced form in halobacteria.

3.3.3 Oxidation of TetrahydroSHP-2 in Acid or Base

As shown above, SHP-2 is in the tetrahydro form inside the cells. Since SHP-2 is isolated in the oxidized form, this means that SHP-2 resulted from the autooxidation of tetrahydroSHP-2 during the isolation process. This raised the question of whether or not the tetrahydroSHP-2 is degraded when it is oxidized. This is an important question since it is well-known that tetrahydroneopterin and tetrahydrobiopterin are degraded to pterin when oxidized in basic solutions (Fukushima and Nixon, 1980). If this is also true for tetrahydroSHP-2, it could mean that the amount of tetrahydroSHP-2 in the cells was larger than that measured by the recovery of SHP-2. This question was checked by reducing the SHP-2 to tetrahydroSHP-2 and then reoxidizing it back to SHP-2 with iodine in either 0.1 M HCl or 0.1 M NaOH as described in "Reduction and Reoxidation of SHP-2" on page 35. The results of the reduction and reoxidation of SHP-2 and a

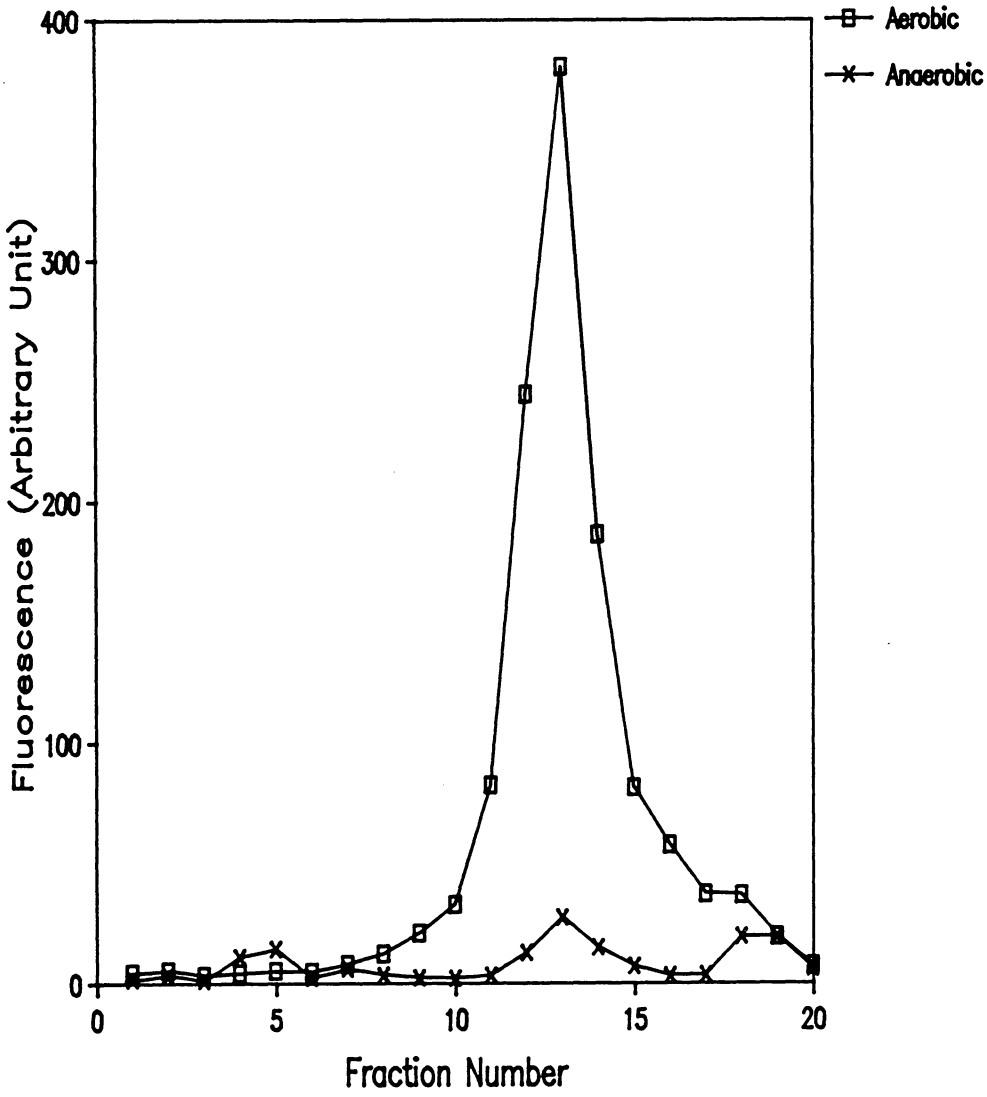


Figure 6. Bio-Gel P-2 chromatography of H. marismortui extracts: Elution profile of a Bio-Gel P-2 column (1.5 x 29 cm, 2.3 mL of solvent was collected in each fraction) of aerobic and anaerobic extracts from *H. marismortui*. The extraction and chromatography procedures are as described in "Materials and Methods" on page 18 except that 40 mL of a 5-day-old culture was used and 0.1 M of β-mercaptoethanol was included in all of the procedures in order to keep the extracts anaerobic. The eluting solvent used for both procedures was a 0.1 M β-mercaptoethanol water solution.

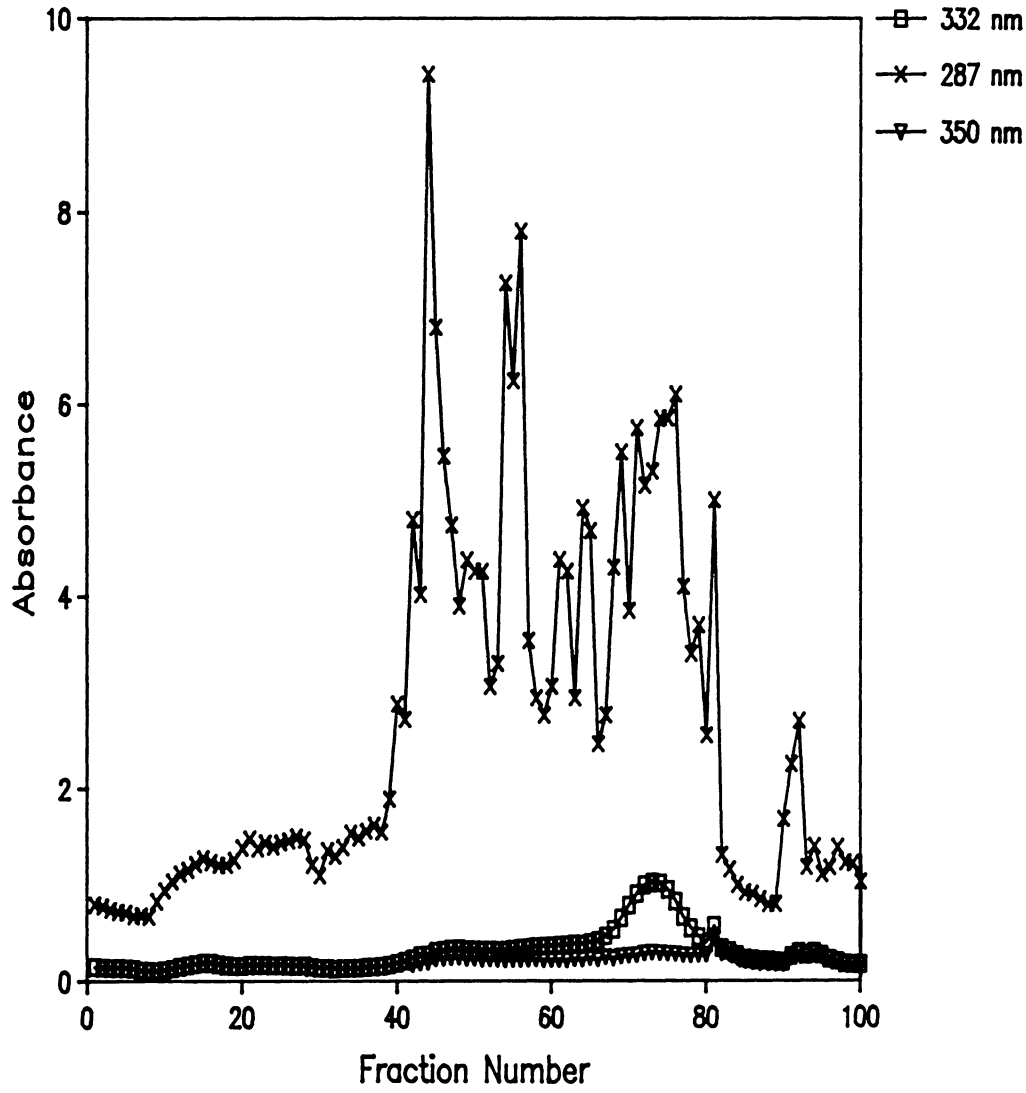


Figure 7. Column chromatography of anaerobic extracts from H. marismortui: Elution profiles of a QAE-Sephadex column (3.5 x 23 cm) of an anaerobic extract from 6.6 L of *H. marismortui* (5-days growth). The experimental procedures are the same as those described in "Purification of SHP-2" on page 31 except that 0.1 M of β-mercaptoethanol was included in all of the procedures. Fractions 66-83 were the position of elution of tetrahydroSHP-2.

controlled reaction with neopterin are shown in Figure 8 on page 45. The figure clearly shows that tetrahydroneopterin was cleaved to pterin when oxidized in base (lane 3) but that tetrahydroSHP-2 was not cleaved under the same conditions. This means that SHP-2 and neopterin have some fundamental difference in their side chain structures. This will be discussed latter. This difference is good since the tetrahydroSHP-2 inside the cells will oxidize to SHP-2 without degradation regardless of the isolation procedure used.

3.3.4 Acid Hydrolysis of SHP-2

A negatively charged side chain was recognized for SHP-2 from paper electrophoresis data (Figure 1 on page 21) and by the fact that it is absorbed on a QAE-Sephadex column. The time-dependent removal of this charge by acid hydrolysis of SHP-2 is complex and two major intermediates (intermediate-1 and intermediate-2) and two hydrolysis products (HP-2-I and HP-2-II) have been identified as shown in Figure 9 on page 46. A detailed discussion and explanations of the hydrolysis will be given in "Acid Hydrolysis Patterns of SHP-2" on page 77.

3.3.5 Sulfate Analysis of SHP-2

As shown in Figure 9, SHP-2 is very unstable in acid. In fact it started to hydrolyze as soon as it dissolved in 1 M HCl. This is a characteristic of sulfate esters which have a half life of less than 1 h in 0.25 N HCl at 100°C (Rees, 1963) or of phosphate esters which have a half life of 30 min in 1 M HCl at 100°C (Robbins and Lipmann, 1956). Both phosphate and sulfate assays were performed as described in " Sulfate and Phosphate Assay" on page 33. The results of the phosphate assay showed that less than 2 nmol of phosphate were present in 45 nmol of SHP-2. This clearly ruled out the possibility of phosphate esters in SHP-2 being responsible for the negative charge. With the sulfate assay method, an average value of 23 ± 3 nmol of sulfate was found from three

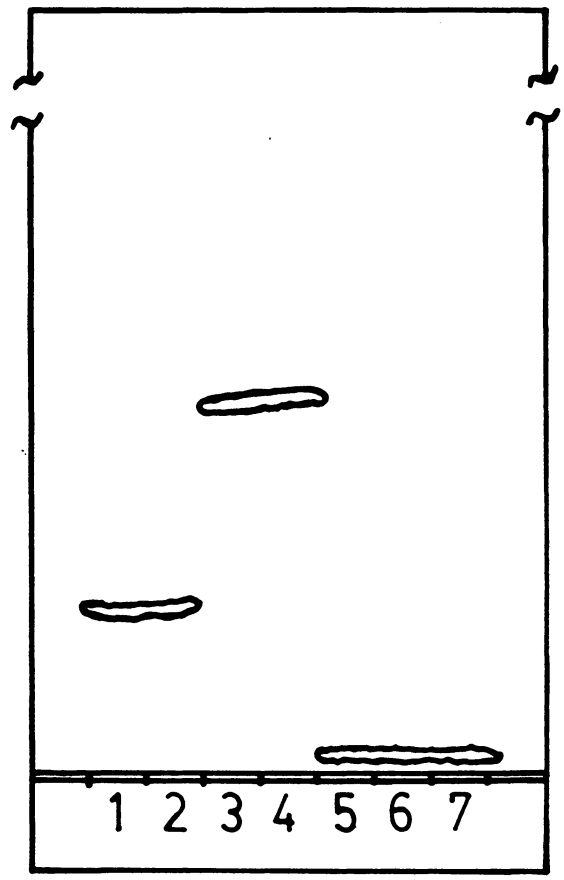


Figure 8. TLC of the oxidation products from tetrahydroSHP-2: Reduction (to H4-pterin) and reoxidation procedures have been described in "Reduction and Reoxidation of SHP-2" on page 35. The solvent used for TLC was S1. Lane 1, standard neopterin; lane 2, H4-neopterin oxidized with I_2 in 0.1 N HCl; lane 3, H4-neopterin oxidized with I_2 in 0.1 N NaOH; lane 4, standard pterin; lane 5, tetrahydroSHP-2 oxidized with I_2 in 0.1 N NaOH; lane 6, tetrahydroSHP-2 oxidized with I_2 in 0.1 N HCl; lane 7, standard SHP-2.

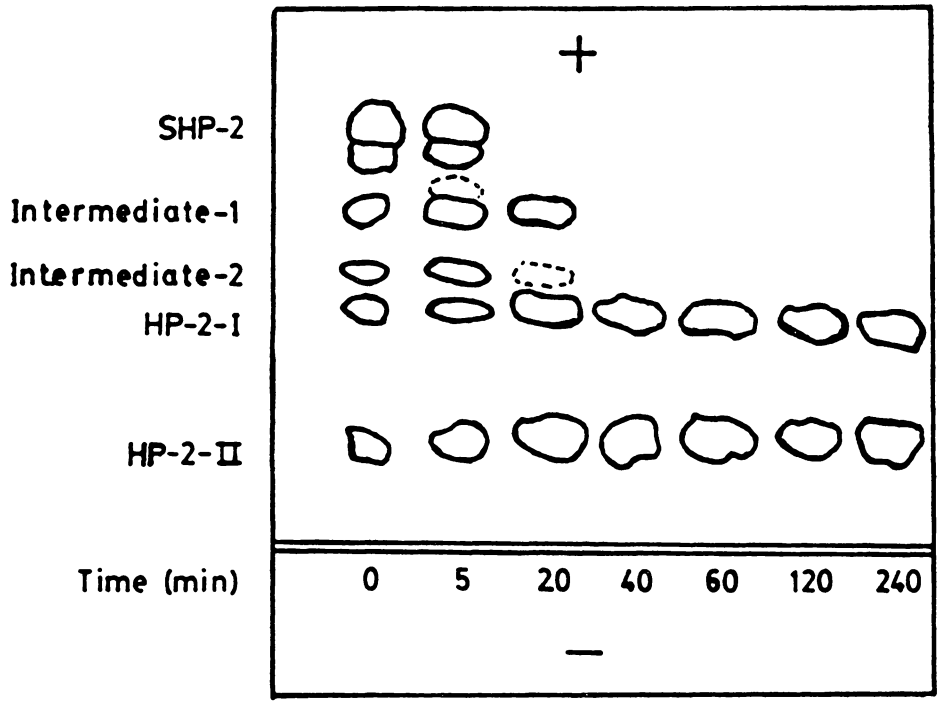


Figure 9. Paper electrophoresis of the time dependent acid hydrolysis of SHP-2: Paper electrophoresis was done as previously described (Figure 1 on page 21). The hydrolysis was done in 1 M HCl at 100°C. Samples were hydrolyzed for the times indicated in the figure and then evaporated in vacuo. The dried samples were redissolved in water and applied to paper. The spots recorded are the fluorescent spots observed by exposing the paper to UV light.

samples containing 24 nmol of SHP-2. This result clearly indicated that one sulfate ester was present for each pterin in the SHP-2. (SHP-2 was quantitated by pterin absorbance. As will be shown latter, there are two pterins in each molecule of SHP-2.) The presence of a sulfate ester in SHP-2 was also proven by the in vivo radioactive sulfate labeling experiment and by its IR spectrum as will be shown latter.

3.3.6 Incorporation of ^{35}S Sulfate into SHP-2 and Characterization of Its Cleavage Products

Acid hydrolysis of SHP-2 (1 M HCl, 100 °C, 1 h) produced two pterins (HP-2) as shown in Figure 9 on page 46, one negatively charged and the other neutral. The charged HP-2 (HP-2-I) could not be further hydrolyzed to uncharged HP-2 (HP-2-II) in acid with longer heating time but the uncharged HP-2 could be changed to charged HP-2 when heated in base or maintained for a long enough period of time in water (data not shown). This means that if HP-2-I is an acid, then HP-2-II is a lactone of the acid. The charge on HP-2-I could be from a sulfonate on the side chain like $R\text{-}CH_2\text{-}SO_3^-$ or a carboxylate like $R\text{-}CH(OH)\text{-}CO_2^-$. The former was checked by an in vivo ^{35}S-labeling experiment and the latter was proved by IR spectroscopy and GC-MS as will be discussed latter. The results of the ^{35}S-labeling experiment follow.

The influence of sulfate on *H. marismortui* growth was studied for the reason mentioned in " ^{35}S-Labeling Experiment" on page 34. It was a surprise to find out that the commonly used medium (CM) is not the optimal medium for the the growth of halobacterial cells (Figure 10 on page 49). While the cells grew well in the normal CM medium (81 mM sulfate), they grew even better when the sulfate concentration in the medium was reduced from 81 to 8.1 and even to 0.81 mM sulfate. This means that a high sulfate concentration actually inhibits cell growth. The medium containing 8.1 mM sulfate was finally selected in the radioisotope experiment because it was found (from paper electrophoresis) that when cells were grown in 0.81 mM sulfate, the SHP-2 yield was

decreased and another negatively charged pterin was produced. [This pterin was shown to be phosphohalopterin-1 based on its identity in paper electrophoresis with a known sample of PHP-1, the generation of the same acid hydrolysis product as PHP-1 and its resistance to periodate cleavage like PHP-1 (data not shown).]

The procedures for the ^{35}S-labeling experiment have been previously described in " ^{35}S-Labeling Experiment" on page 34. The crude extract from the ^{35}S-labeled cells was first purified by paper chromatography, which partially separated SHP-2 from the labeled sulfate, and then purified two times with the same paper electrophoresis system. As shown in Figure 11 on page 50, SHP-2 was partially separated from sulfate by the first paper electrophoresis and fairly well separated by paper electrophoresis with the second run. The figure also clearly shows that SHP-2 was labeled by $^{35}SO_4^-$.

The radioactive SHP-2 was further purified on a Bio-Gel P-2 column (Figure 12 on page 51) and the radioactivity coeluted with the SHP-2 peak indicating that the SHP-2 is labeled with $^{35}SO_4^-$.

The SHP-2-containing fractions were collected from the above Bio-Gel P-2 column and subjected to acid hydrolysis. If the charge of the acid-hydrolyzed sample shown in Figure 9 on page 46 is from sulfonate like $R\text{-}CH_2\text{-}SO_3^-$, then the acid hydrolyzed product of ^{35}S-SHP-2 (HP-2) must still be radioactive because, biosynthetically, the sulfonate group must be from sulfate. The results represented in Figure 13 on page 52 clearly show that no radioactivity was found in either the charged HP-2 (6.2 cm bar) or the uncharged HP-2 (2.8 cm bar). This meant that the charge on HP-2 was not from a sulfonate, which suggested the possibility that the charge was from the carboxylate group on the side chain of HP-2. The IR spectral data, which will be presented later, prove the presence of the carboxylate group.

Another important result of the radiolabeling experiment comes from the data on the partial hydrolysis of ^{35}S-SHP-2. Figure 9 on page 46 shows at least two intermediates arising during the short term hydrolysis of SHP-2 to HP-2 (5 min). The labeling patterns of these two intermediates are shown in Figure 14 on page 54. The figure shows approximately one sulfate per pterin for intact SHP-2 (12 cm bar). More interestingly, this figure shows that the two intermediates (10 cm and 8

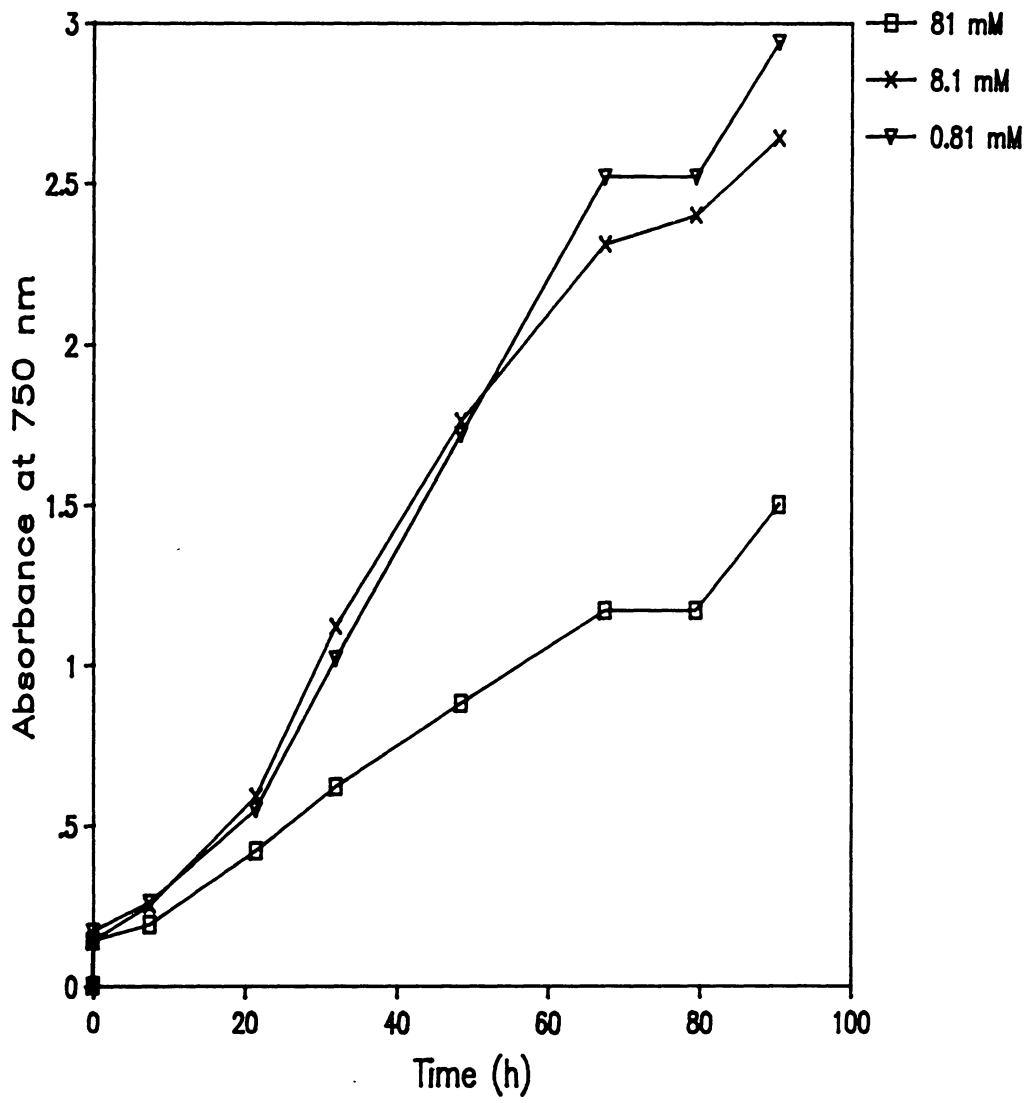


Figure 10. **Influence of sulfate on the growth of H. marismortui;** The growth media and conditions have been described in "Materials and Methods" on page 18. The mM sign in the upper right margin indicates the sulfate concentration in the growth media. The sulfate concentration in the normal CM medium is 81 mM.

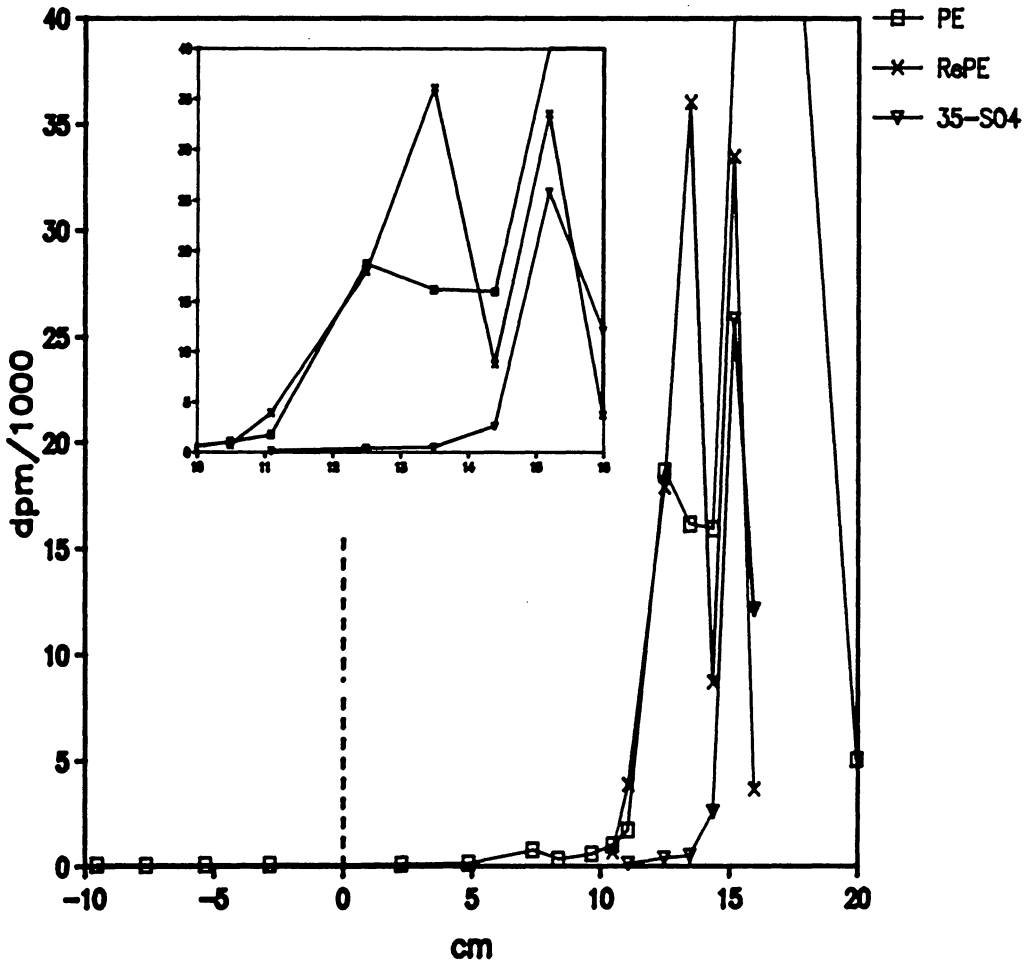


Figure 11. Purification of ^{35}S-SHP-2 by paper electrophoresis: Paper electrophoresis was performed as described in Figure 1 on page 21 and a 0.5-cm wide piece of paper was cut off from cathode (negative numbers, left side of the figure) to anode (positive numbers, right side of the figure). Starting at the origin and proceding both to the cathode and anode, lengths of paper were removed from the strip and counted for radioactivity. The value of radioactivity recorded in the figure represent the total radioactivity in the paper from one distance to the next i.e. the value recorded at 7.4 cm represents the radioactivity from 7.4 - 8.4 cm. The peak at about 13 cm was SHP-2 as detected by fluorescence and the peak at 15-20 cm was sulfate as detected by using a known sample of radioactive sulfate. The upper right sign: PE, first paper electrophoresis purification; RePE, second paper electrophoresis purification; 35-SO4, standard $^{35}SO_4^{=}$. The upper left portion of the figure is an extension from 10-16.

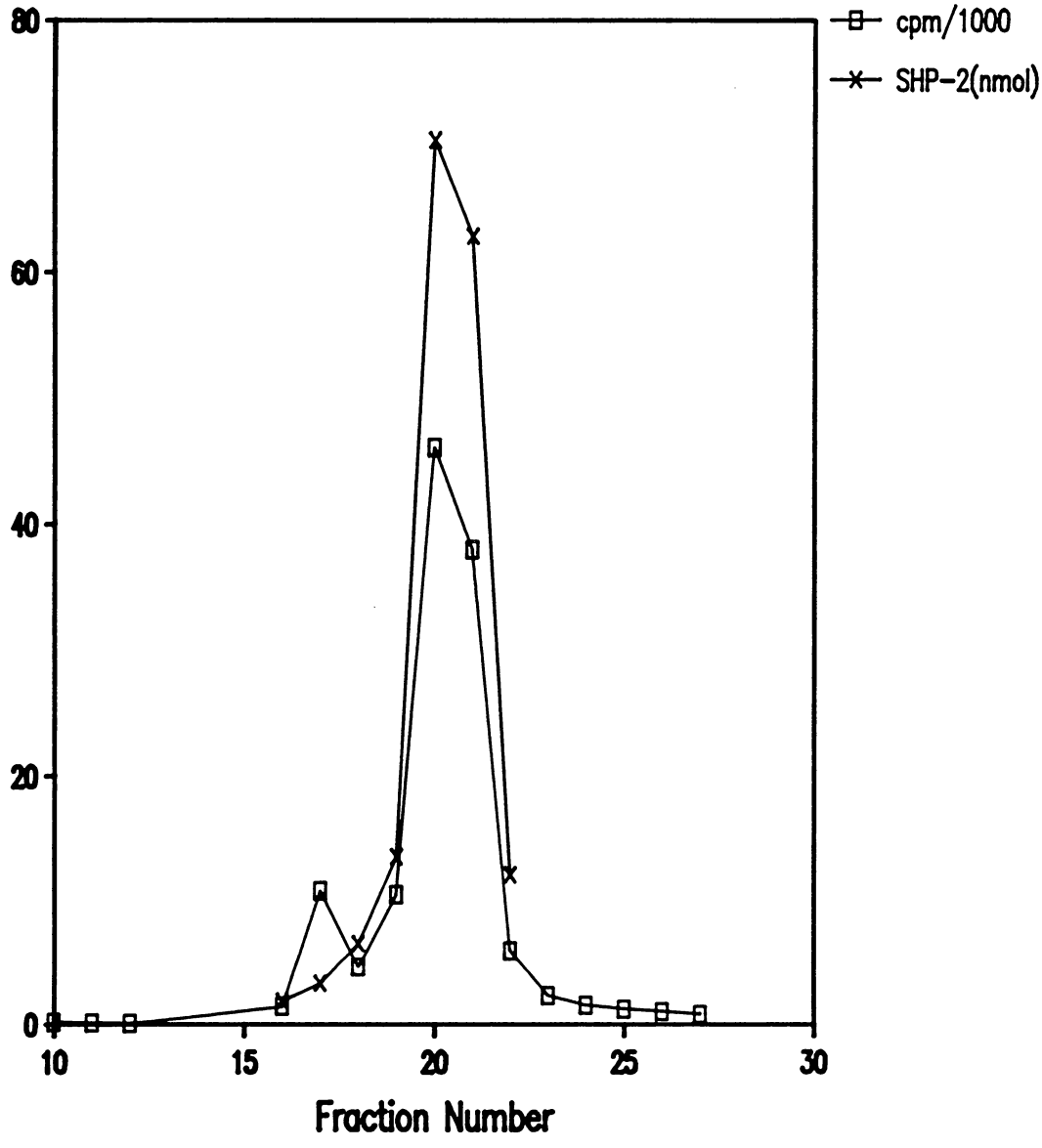


Figure 12. Bio-Gel P-2 purification of ^{35}S-SHP-2: The experimental procedures have been given in " ^{35}S-Labeling Experiment" on page 34. Fractions (2.3 mL) were collected and SHP-2 was quantitated by absorbance.

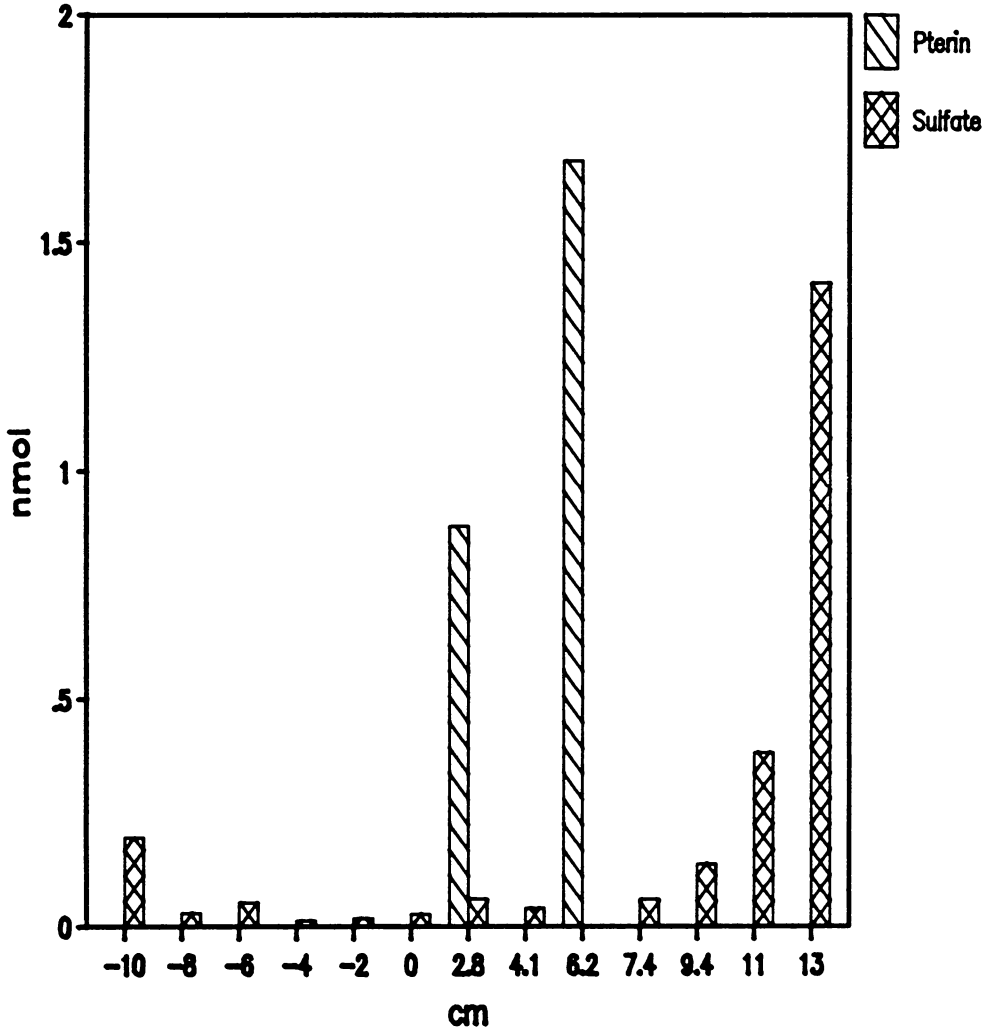


Figure 13. **Paper electrophoresis of the acid-hydrolyzed ^{35}S-SHP-2:** ^{35}S-SHP-2 was hydrolyzed in 1 M HCl at 100 °C for 1 h and then directly applied to paper for the paper electrophoresis. The amount of pterin (nmol) was calculated from the fluorescence and the sulfate (nmol) was calculated from the specific activity of the sulfate. The individual bar is located by the number in the X-axis [from Cathode (left) to Anode (right)] which represent the starting point at which the paper was cut and the next number represents the end of a particular cutting. For example, the first left bar was cut from -10 - -8 cm and the second left bar was cut from -8 - -6 cm. The last left bar (or the first right bar) was cut from 13 - 16.6 cm. The width of the paper cutting was decided on by the band width of the fluorescent band. In this case, the width of the paper cutting is 3.4 cm. The bar at 6.2 cm represents HP-2-I and the bar at 2.8 represents HP-2-II.

cm bars) each contain only half a sulfate per pterin. This is best explained by the pterin dimer hypothesis which proposed that there are two sulfates in the pterin dimer (one sulfate per pterin) and that if the dimer lost one of its sulfates, there would be one sulfate per dimer or half a sulfate per pterin (see "Acid Hydrolysis Patterns of SHP-2" on page 77 for a detailed explanation).

The reaction of SHP-2 with periodate was also checked with radiolabeled SHP-2. Figure 15 on page 55 clearly shows that no radioactivity remained in the pterin portion of the cleaved products and that the pterin was no longer charged after its cleavage. This chemistry will be explained later.

To sum up, the in vivo ^{35}S-labeling experiment has clearly established a sulfate ester as a component of SHP-2. In addition, the possibility of the presence of a sulfonic acid group on the side chain of HP-2 has been ruled out. This experiment has also identified two intermediates in the short-term (5 min) acid hydrolysis of ^{35}S-SHP-2, each of which shows half a sulfate per pterin.

3.3.7 Infrared Spectrum of SHP-2

The above radio-labeled experiment forced the consideration of the presence of a carboxylate group on the SHP-2 side chain. The IR spectrum shown in Figure 16 on page 56 clearly shows three functional groups in SHP-2, the pterin portion of the molecule, the sulfate ester, and the carboxylate group.

3.3.8 CMR Spectra of SHP-2

The assignments of pterin ring carbons in the CMR spectra were according to Ewers et al. (1973, 1974a, 1974b), Geerts et al. (1976), Schircks et al. (1976) and Tobias et al. (1985).

The assignments of the chemical shifts of the side chain carbons of SHP-2 as polyol carbons was according to Koerner et al. (1973), O'Connor et al. (1979) and Bock and Thøgersen (1982).

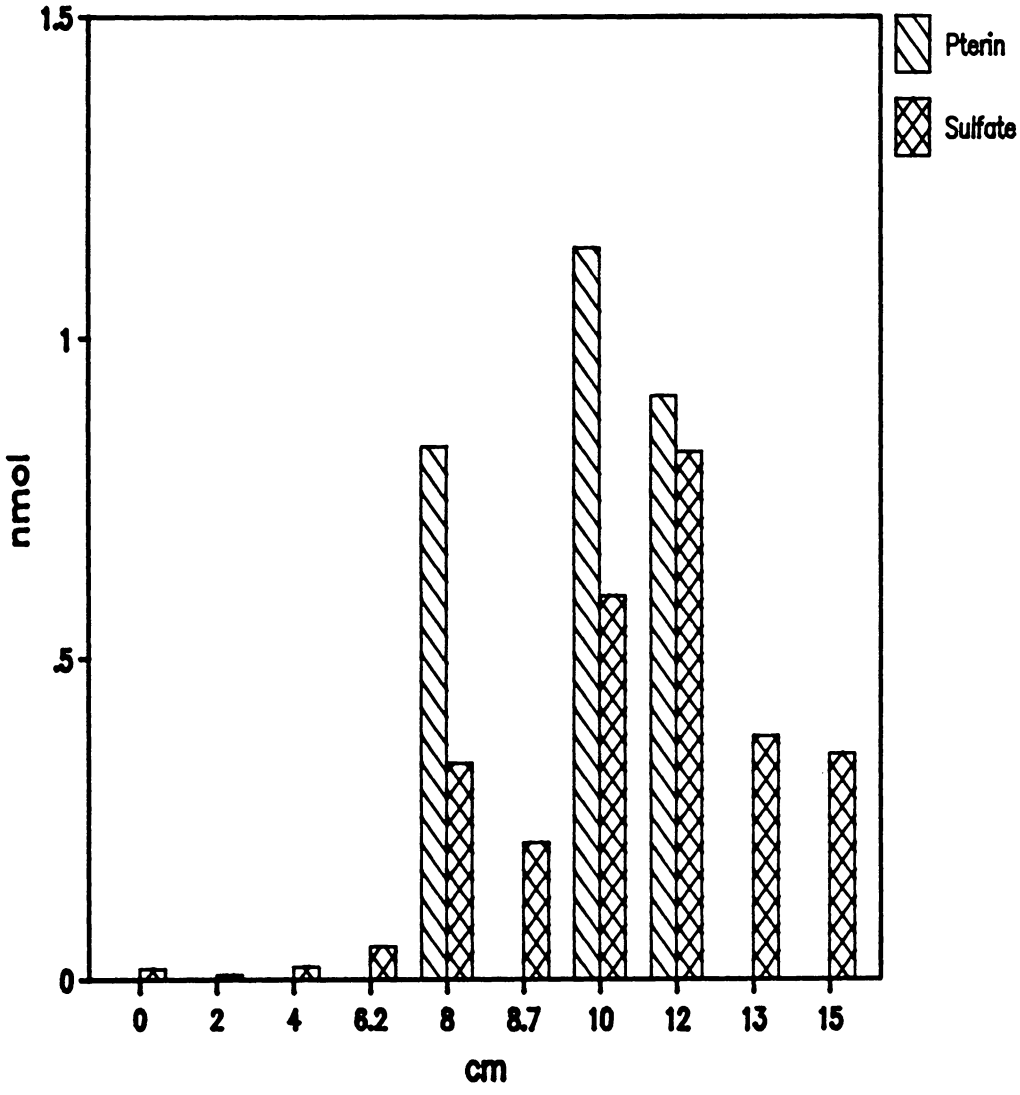


Figure 14. Paper electrophoresis of the partially hydrolyzed ^{35}S-SHP-2: ^{35}S-SHP-2 was hydrolyzed in 1 M HCl for 5 min. This figure is presented in the same manner as Figure 13 except that the last bar represents the cutting from 15 - 17 cm and the width of the cutting is 3.0 cm. The bar at 12 cm is SHP-2, at 10 cm is intermediate-1 and at 8 cm is intermediate-2.

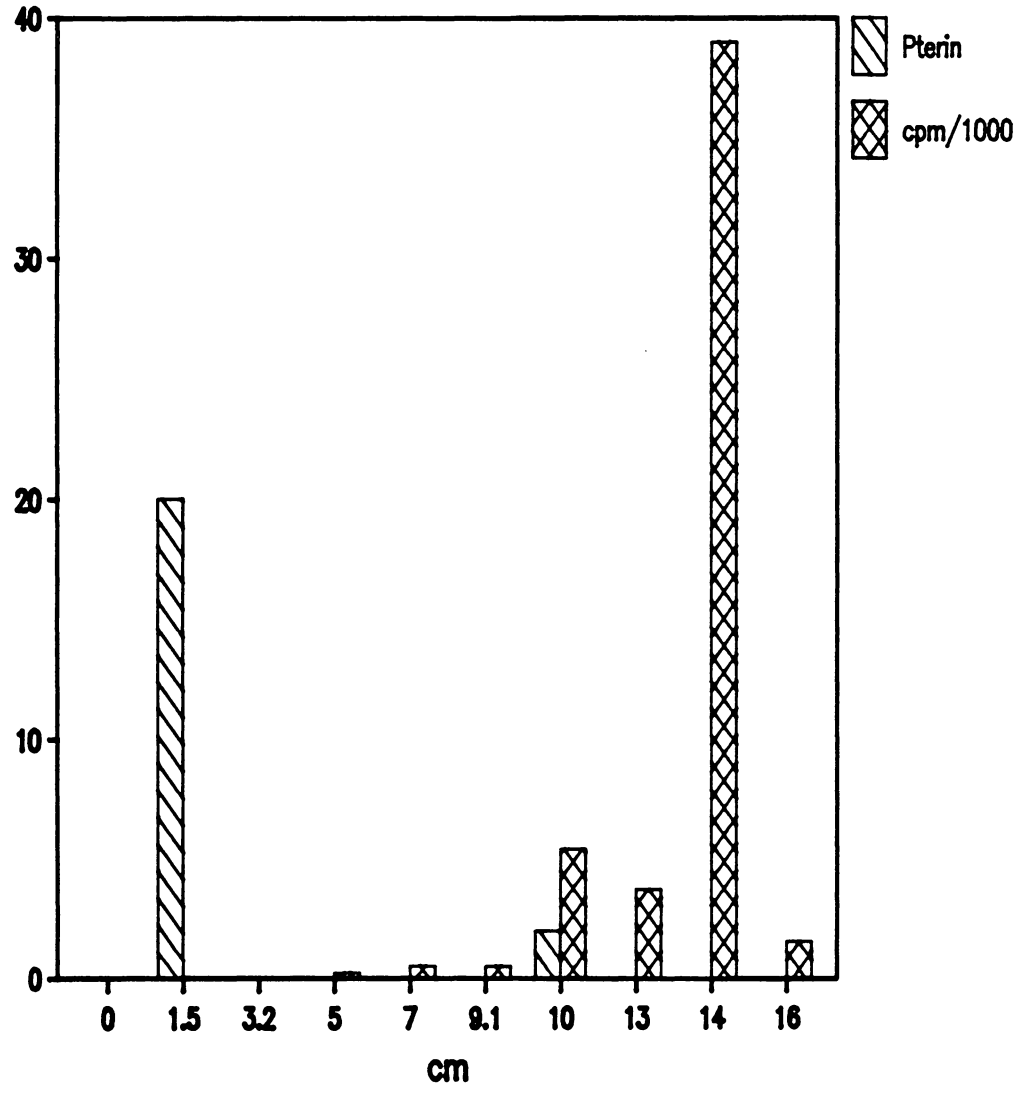


Figure 15. Paper electrophoresis of periodate-cleaved ^{35}S-SHP-2: ^{35}S-SHP-2 was reacted with ten equivalents of sodium periodate and then directly applied to paper for the paper electrophoresis. This figure is presented in the same manner as Figure 13 except that the last bar represents the cutting from 16 - 18 cm and the width of the cutting was 4.1 cm. The bar at 1.5 cm represents the uncharged pterin (fluorescence in an arbitrary unit) resulting from the periodate cleavage of SHP-2.

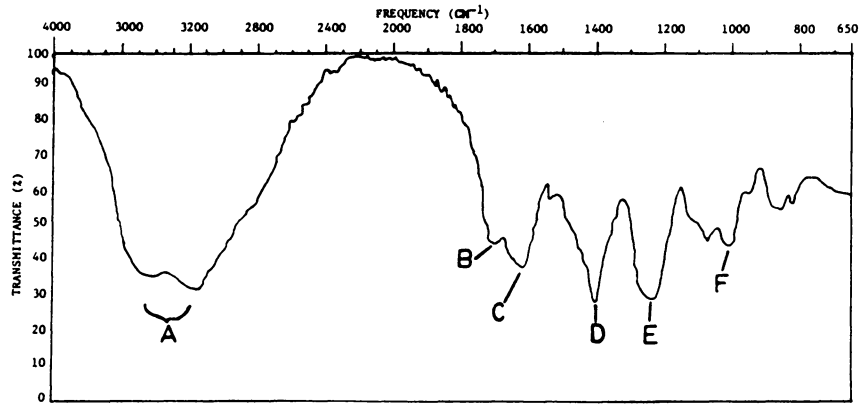


Figure 16. Infrared spectrum of SHP-2: Approximately 2 mg of SHP-2 in 80 mg of dried KBr was used to make a 7 mm window. The assignments are according to Silverstein et al. (1974) and the IR spectra of standard pterin, neopterin, and glucose-6-sulfate recorded under the same conditions using the same instrument. A: broad O-H, N-H, and C-H stretch, 3800-2500 cm^{-1}. B: pterin C=O stretch, 1700 cm^{-1} . C: asymmetric carboxylate anion stretch, 1620 cm^{-1}. D: symmetric carboxylate anion stretch, 1400 cm^{-1}. E: asymmetric $S(=O)_2$ stretch, 1240 cm^{-1}. F: symmetric $S(=O)_2$ stretch, 1020 cm^{-1}.

The assignment of the methylene carbon adjacent to the pterin ring at the 6- or 7- position was according to the spectrum of a synthetic 2′,3′,4′-trihydroxybutylpterin shown in Figure 17 on page 58. Ewers et al. (1978) have pointed out that it is possible to distinguish between 6- and 7-substituted pterins on the basis of the vicinal $^{13}C,^{1}H$ coupling that leads to a doublet splitting for the C-4a in the case of 7-substituted pterin and for the C-8a in the case of 6-substituted pterin. The C-4a doublet in Figure 17 on page 58 clearly shows that the synthetic pterin is consists largely of 7-substituted pterin (the ratio of 7- to 6- substitution is approximately 7 to 1 as will seen from the proton NMR of the same pterin). A bigger splitting constant is the ^{13}C-6-^{1}H-6 coupling which is about 200 Hz (to be shown later). Figure 17 also shows the $^{13}C,^{1}H$ coupling of the side chain carbons. These chemical shifts are good references for CMR of SHP-2 as both were performed in the same solvent.

Figure 18 on page 59 shows the proton-coupled CMR spectrum of SHP-2 which was purified as described in "Purification of SHP-2" on page 31. In the aromatic region of the spectrum (130-180 ppm), the figure clearly shows the splitting of the C-7 carbon resonance into two peaks which has a coupling constant of ~ 200 Hz just like the splitting of the C-6 carbon observed in Figure 17. This is very clear evidence that SHP-2 is a 6-substituted pterin like almost all of the other naturally occurring pterins (see Table 1 on page 9). The polyol region of the spectrum (60-80 ppm) shows six carbons. One of these carbons (75.6 ppm) is a methylene carbon which is connected to C-6 of the pterin of SHP-2. The other five carbons are methyl carbons which have a proton and a hydroxyl group on each of the carbons. The structural features of SHP-2, as summarized from the data so far, are also shown in Figure 18.

Figure 19 on page 61 shows the proton-decoupled CMR spectrum of SHP-2. A comparison of the polyol carbon resonances of the proton-coupled (Figure 18 on page 59) and proton-decoupled (Figure 19 on page 61) ^{13}C-NMR of SHP-2 is as follows: the 82.0 ppm, 80.6 ppm, and 72.9 ppm peaks are the same in these two spectra; the 75.6 ppm peak in Figure 18 corresponds to the 75.5 ppm peak in Figure 19(methylene carbon); and the two peaks, 73.5 ppm and 73.4 ppm, in Figure 18 have fused to one peak (73.5 ppm) in Figure 19. Actually, it is not uncommon that

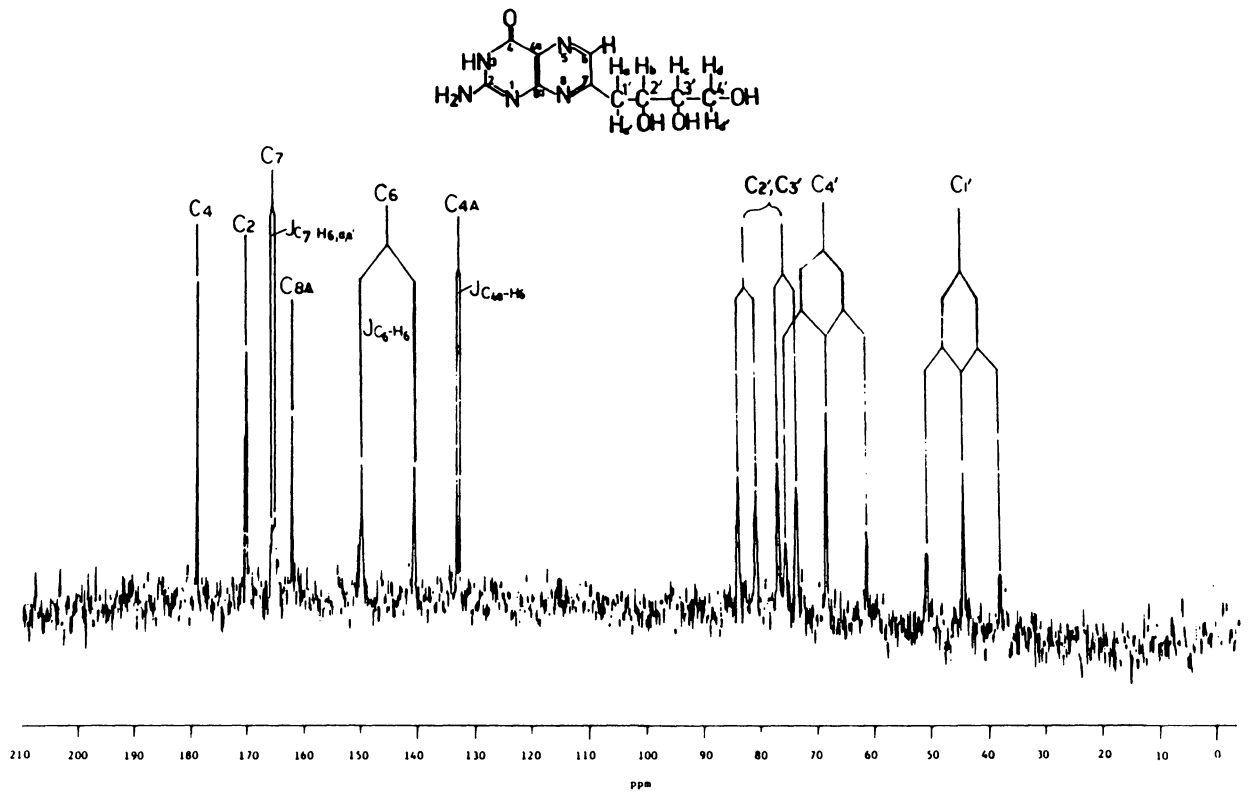


Figure 17. The 20 MHz Fourier Transform ^{13}C NMR spectrum of 2',3',4'-trihydroxybutylpterin: A saturated solution of the sample was dissolved in 1 M deuterated ammonium hydroxide in deuterated water. Resonances are reported in parts per million from internal sodium 3-trimethylsilylpropionate-2,2,3,3-d (TSP).

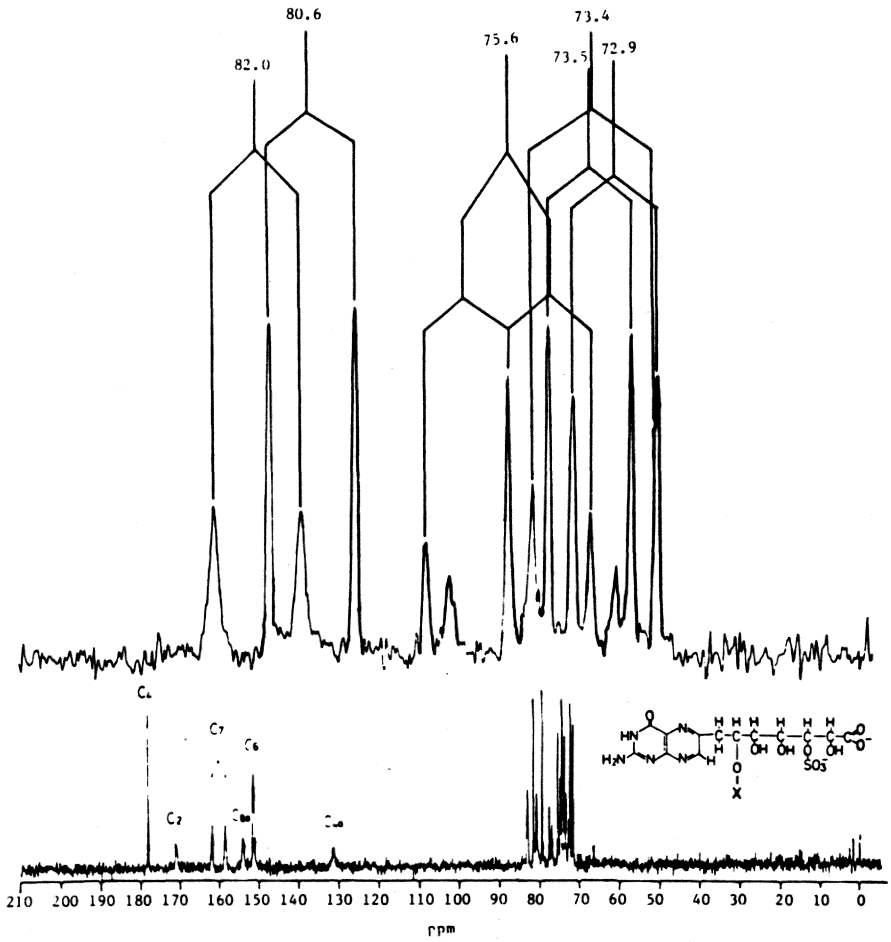


Figure 18. **The 67.9 MHz Fourier Transform CMR spectrum of SHP-2:** SHP-2 (39 μmol) was dissolved in ~ 0.5 mL of ~ 1 M ND_4OD in D_2O in a 5-mm NMR tube. The spectrum was recorded at room temperature with TSP as the internal standard. The X in the formula represents an undefined group.

two separate carbons in the spectrum of polyol display one peak in CMR spectra (Koerner et al., 1973; O'Connor et al., 1979; Bock and Thøgersen, 1982).

By using a technique called insensitive nucleus enhancement through polarization transfer (INEPT), it is possible to distinguish between carbons that contain one or three protons from those that contain two protons. Carbons with one or three protons are found to produce a positive signal, whereas those with two protons produce a negative carbon signal. For the SHP-2 spectrum, no $-CH_3$ carbons can have chemical shifts between 60-80 ppm so that only CH and CH_2 carbons are present. The INEPT spectrum of the proton-decoupled C-13 of SHP-2 showed that the carbon at 75.5 ppm is a methylene carbon and that the other carbons contain only one proton. This is consistent with the proton-coupled C-13 NMR of SHP-2 (Figure 18).

When SHP-2 was hydrolyzed with acid and the resulting HP-2 was purified, a very good proton-NMR spectrum was obtained. As shown in Figure 20 on page 62, a total of seven protons were assigned. The coupling patterns shown in the figure were supported by spin-spin decoupling experiments with the same sample. In a typical experiment, irradiation of H_e changed H_f from a doublet to a singlet and H_d from quartet to doublet.

The coupling parameters observed in the NMR of HP-2 are shown in Figure 20 on page 62 and summarized in table 4 on page 64.

The PMR of SHP-2 was basically the same as the PMR of HP-2. Proton spin-decoupling experiments performed with the SHP-2 sample are summarized in Table 5 on page 65. The data in the table are in agreement with the coupling patterns shown in Figure 20 on page 62 and support the proposed structure.

A PMR spectrum of a sample of 2',3',4'-trihydroxybutylpterin is shown in Figure 21 on page 63 for a comparison with that of SHP-2. As shown in the figure, the methylene protons adjacent to the pterin fall into about the same region of the spectrum as the polyol protons.

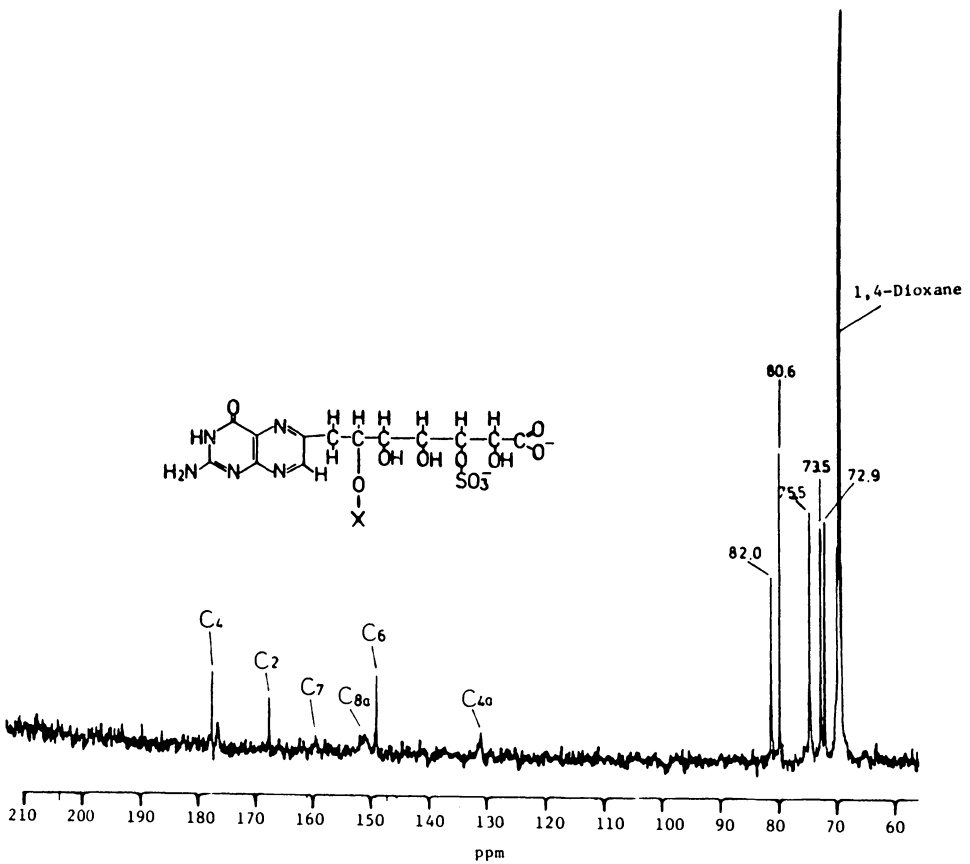


Figure 19. The 67.9 MHz Fourier Transform proton-decoupled ^{13}C NMR spectrum of SHP-2: SHP-2 (19 μmol) was dissolved in about 0.5 mL of 1 M deuterated ammonium hydroxide in deuterated water in a 5-mm NMR tube. The spectrum was recorded at room temperature with dioxane as the internal standard and the chemical shift was then corrected to TSP.

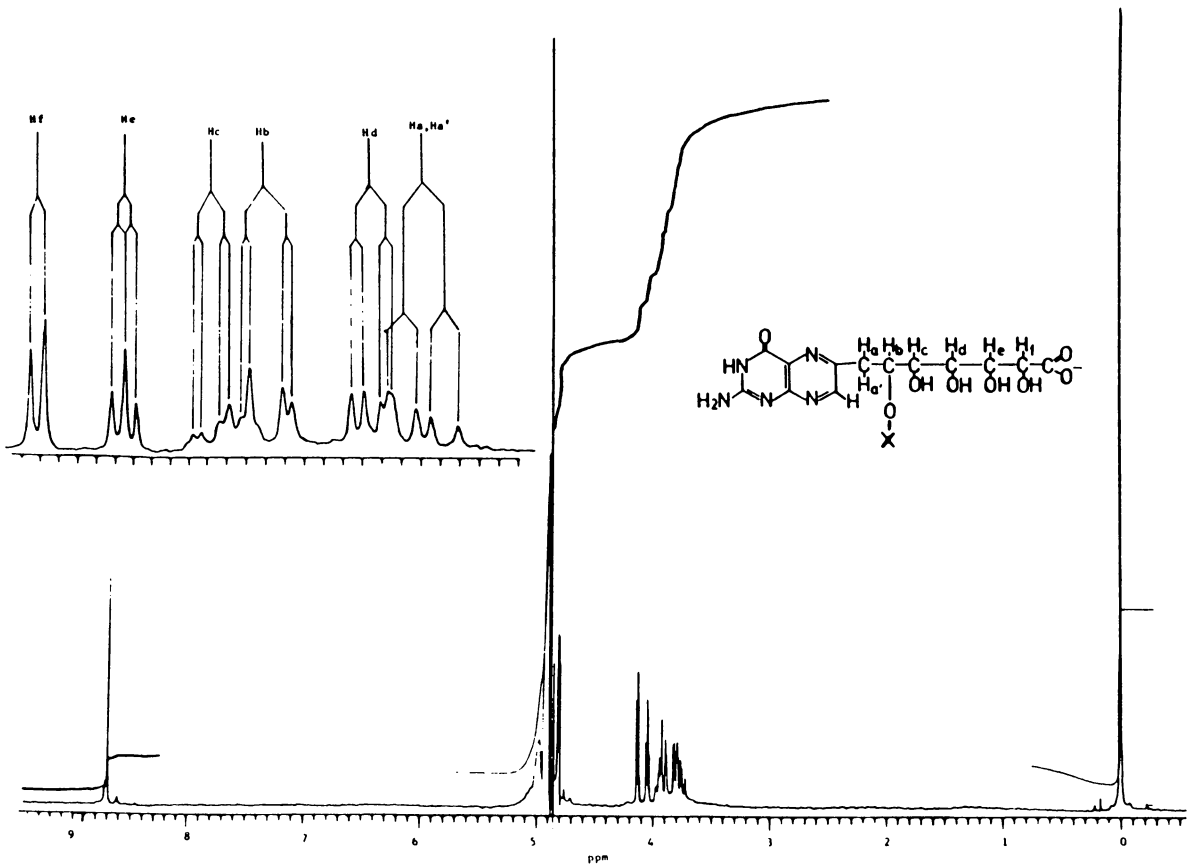


Figure 20. The 270 MHz Fourier Transform PMR spectrum of HP-2: HP-2 (4 μmol) was dissolved in about 0.5 ml of 1 M deuterated ammonium hydroxide in deuterated water in a 5-mm NMR tube. The spectrum was recorded at room temperature with TSP as the internal standard.

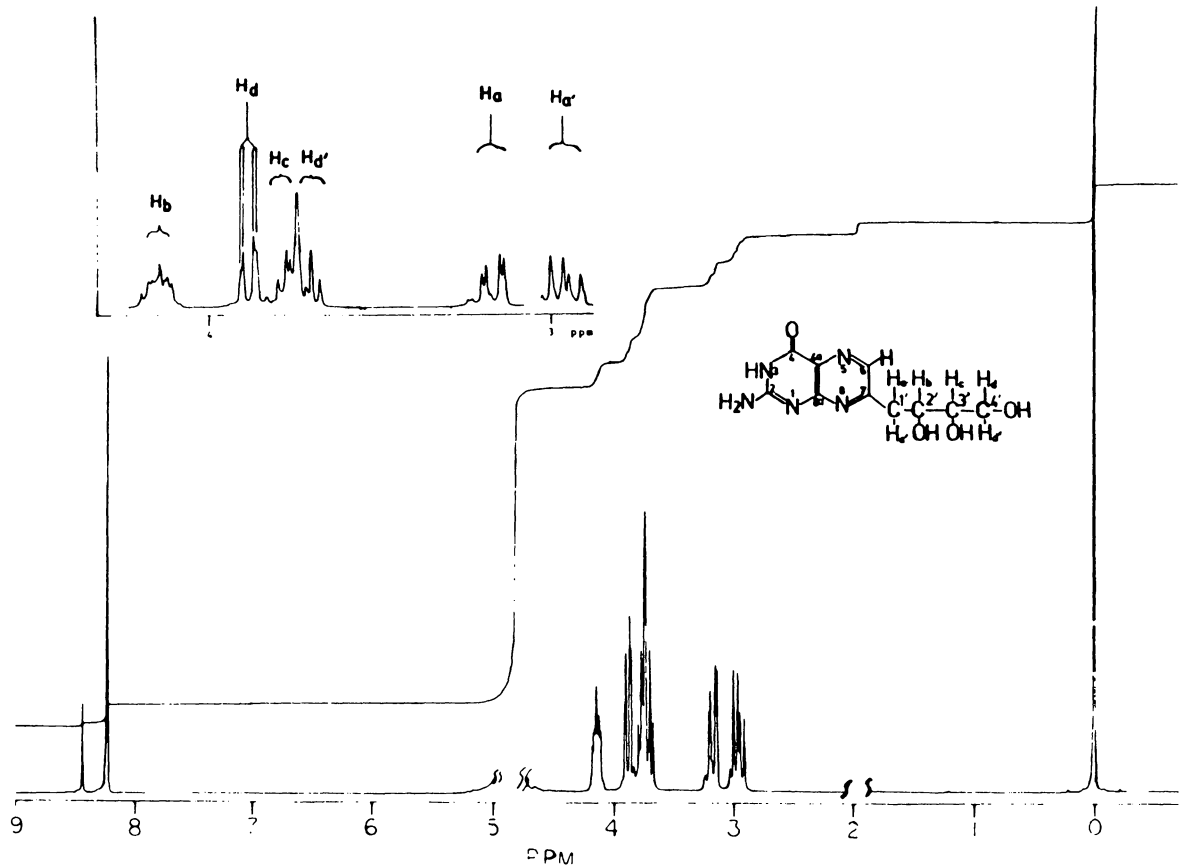


Figure 21. The 270 MHz Fourier Transform PMR Spectrum of 2',3',4'-trihydroxybutylpterin: A saturated solution of the sample was prepared in about 1 mL of 1 M deuterated ammonium hydroxide in deuterated water in a 5-mm NMR tube. The spectrum was obtained at room temperature with TSP as the internal standard.

Table 4. Proton Magnetic Resonance Parameters for HP-2

	δ (ppm from TSP)	J (Hertz)	
δa [1]	3.715	Jaa' 7.13	Jab 10.81
δa'	3.715	Ja'a 7.13	Ja'b 10.81
δb	3.866	Jbc 2.22	Jba 10.75
δc	3.918	Jcb 2.35	Jcd 7.34
δd	3.764	Jde 3.16	Jdc 7.51
δe	4.006	Jed 3.45	Jef 3.45
δf	4.092		Jfe 3.74

[1] The letters (a, a', b, c, d, e, f) indicate the position of protons in the structural unit as indicated by the formula in Figure 20 on page 62 .

Table 5. Spin Decoupling Experiment on SHP-2

Irradiated Protons	Ha,Ha'	Hb	Hc	Hd	He	Hf
Hb	q[1]→c[2]		q→d[3]	nd[4]	uc[5]	uc
Hc	uc	ch[6]		ch	uc	uc
Hd	uc	uc	ch		t[7]→d	uc
He	uc	uc	uc	q→d		d→s[8]
Hf	uc	uc	uc	uc	t→d	uc

[1] q: quartet. [2] c: complex. [3] d: doublet. [4] nd: not determined.
[5] uc: unchanged. [6] ch: change. [7] t: triplet. [8] s: singlet.

3.3.9 Circular Dichroism (CD) Spectrum of SHP-2

If a hydroxyl group is adjacent to a pterin as it is in biopterin and neopterin, the asymmetric center must then be reflected in the CD spectrum of the pterin. Figure 22 on page 67 shows the CD spectra of biopterin and neopterin which have mirror image spectra because they have opposite stereochemistry at the 1′-carbon. The figure also shows that SHP-2 has essentially no CD-active bands which supports the idea that SHP-2 has no asymmetric center at the 1′-carbon. These data support the proposed structure of SHP-2 with 1′-methylene carbon.

3.3.10 Characterization of the Pterin Portion of SHP-2

As shown in Figure 1 on page 21, the pterin which results from the periodate cleavage of SHP-2 is not charged. Periodate cleavage of HP-2 gives the same pterin product confirmed by TLC (data not shown). In order to test for any functionality at the 1′-carbon, SHP-2 was cleaved with periodate and a UV-visible spectrum was recorded before and after the reaction. The idea being that if SHP-2 has any structural features similar to those of neopterin, then the aldehyde produced by periodate cleavage would conjugate with the aromatic system of the pterin ring and the UV-visible spectrum would change. The experimental procedures have already been outlined in "Periodate Oxidation of HP-2 and Its Spectrum Change" on page 36. As a control, neopterin was subjected to the same reaction procedures as were HP-2-I and HP-2-II. As shown in Table 6 on page 68, after reaction with periodate, there is a 7 nm red shift for neopterin because of the conjugation of the resulting aldehyde with the aromatic ring of the pterin, however no shift was observed for HP-2. This means that the aldehyde produced by the periodate cleavage of HP-2 is not conjugated with the pterin. This is as expected if the 1′-carbon of HP-2 is a methylene as discussed before and if the HP-2 is a dimer pterin as will be discussed later.

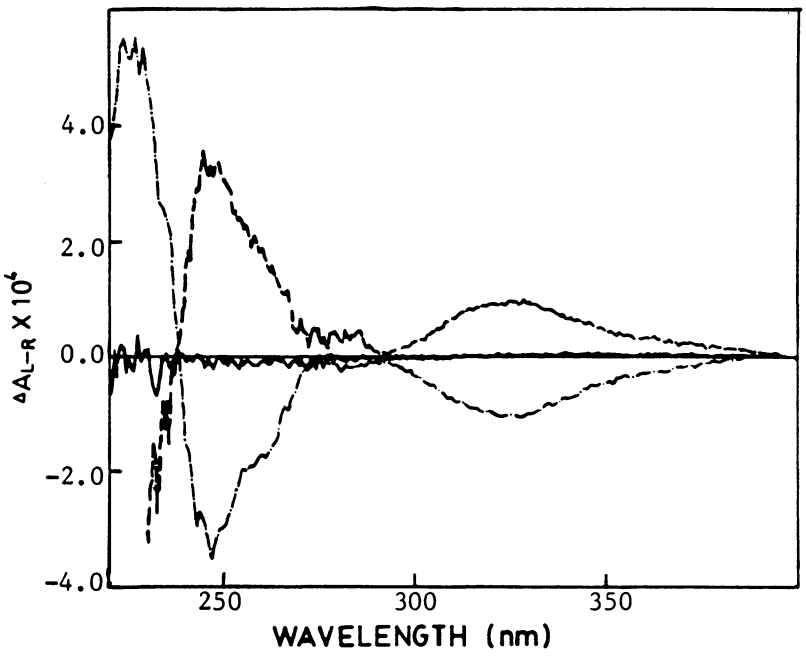


Figure 22. CD spectrum of SHP-2: SHP-2 and standard biopterin and neopterin was dissolved in water with a concentration of ~ 0.1 M. (----), neopterin; (·---), biopterin; (—), SHP-2.

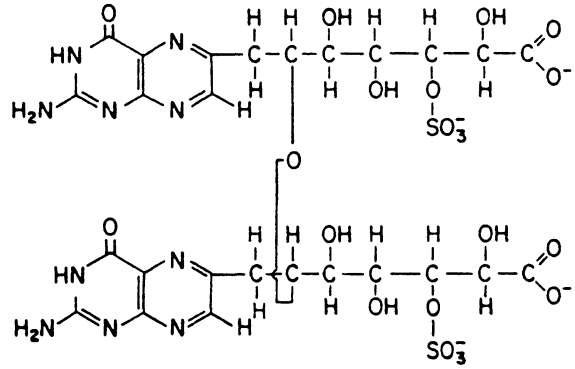
Table 6. Spectra Change of HP-2 upon Periodate Cleavage

Pterin	λmax(nm) before cleavage	λmax(nm) after cleavage	λ(a) - λ(b) [1]
Neopterin	362	369	7
HP-2-I	362	362	0
HP-2-II	362	362	0

[1] The λmax after cleavage minus λmax before cleavage.

If the X group of SHP-2 in the formula in Figure 18 on page 59 is a proton, that is, if the pterin is a monomer pterin like the "normal" naturally occurring pterins in Table 1 on page 9, then cleaving SHP-2 with periodate and then reducing the resulting aldehyde by borohydride (the pterin ring is not reduced by borohydride) should result in the product of 2′-hydroxyethylpterin as the pterin portion (SHP-OR). Figure 23 on page 70 shows that this is clearly not the case. As shown in the figure, 2′-hydroxyethylpterin (prepared by the oxidation of 2′,3′,4′-trihydroxybutylpterin with periodate followed by the reduction of the resulting aldehyde by borohydride) is less polar than 6-hydroxymethylpterin (from Sigma) so that it runs faster than the latter in the TLC system. On the other hand, SHP-OR runs slower than 6-hydroxymethyl-pterin, which means that the former is more polar than the latter. So, the X group in Figure 18 on page 59 must not be a proton.

If the X group is not a proton, then there is hardly any other structure that would not show resonance in either PMR or CMR. The only explanation is that the pterin is a symmetrical dimer as shown in the following formula:



If this structure is correct, then the pterin produced by periodate oxidation followed by borohydride reduction of SHP-2 would be as shown in Figure 24 on page 71. The pterin portion of the products (SHP-OR) can easily be identified by its fluorescence and it can be purified as described in "Oxidation of SHP-2 with Periodate and Reduction of the Resulting Aldehyde" on page 35. For mass spectrometric analysis, 0.1 μmol of purified SHP-OR was dried in a 0.2 mL bottle and then 20 μL of pyridine:hexamethyldisilazane:chlorotrimethylsilane (9:3:1 v/v/v) was added to the bottle. The resulting sample was heated at 110 °C for ~ 15 min and then directly placed into a MS probe (2 μL) for mass spectrometric analysis. Figure 25 on page 73 shows the molecular

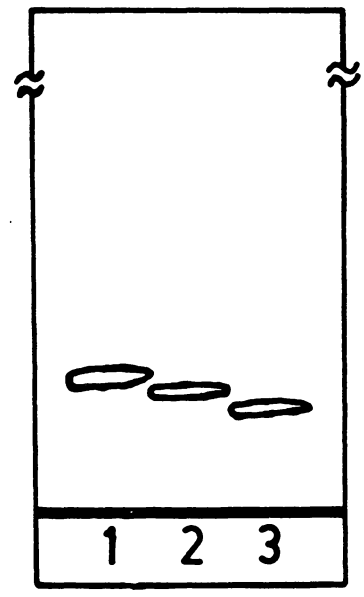


Figure 23. **TLC of SHP-OR:** Lane 1. 7-(2′-hydroxyethyl)pterin; lane 2. 6-hydroxymethylpterin; lane 3. SHP-OR. The samples were applied to a thin-layer plate and developed with S1 as described in Figure 2 on page 23.

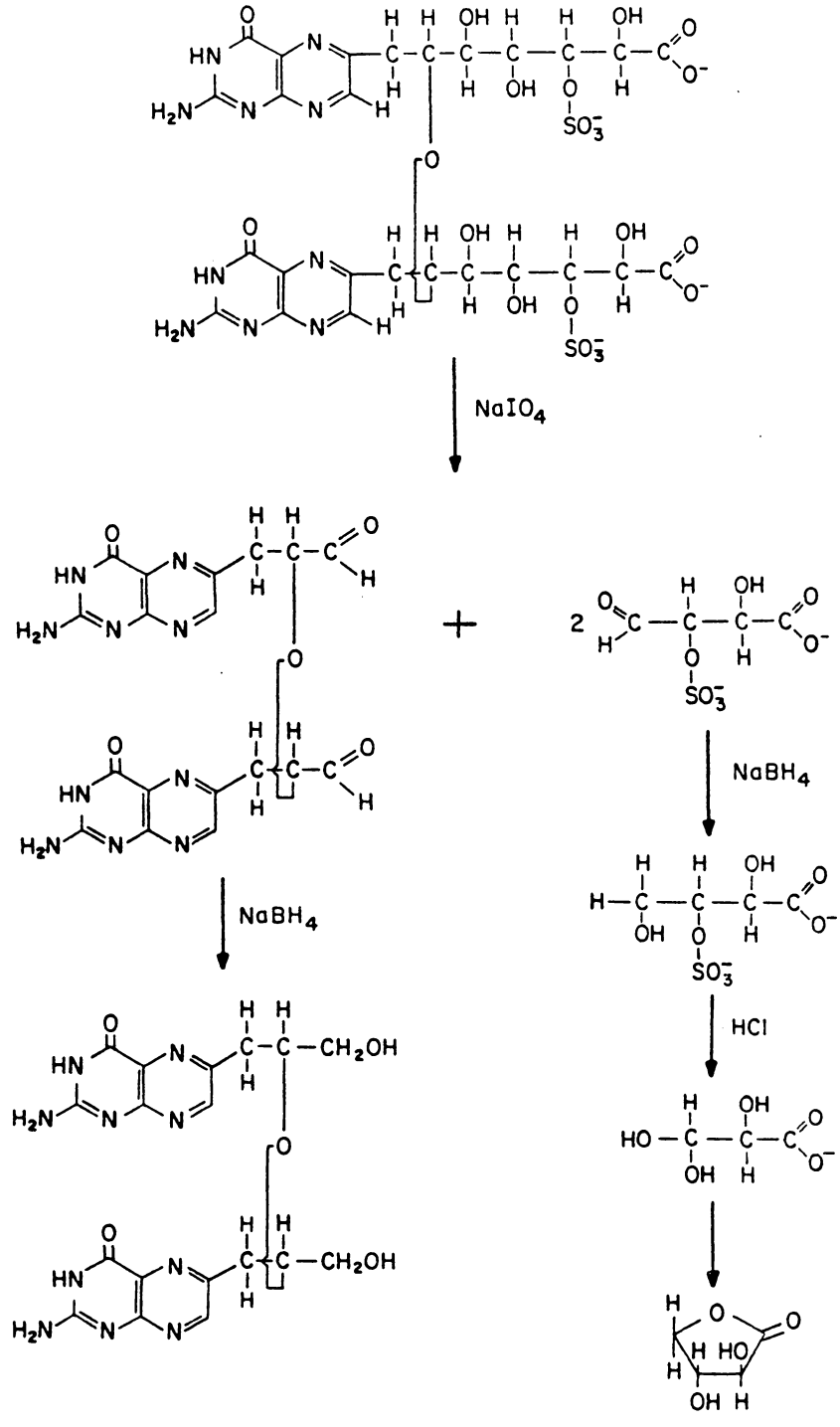


Figure 24. Reaction sequence of periodate oxidation and the borohydride reduction of SHP-2

formula and the mass spectrum of the resulting TMS derivative. The spectra shows a M^+ - $[CH_3]$ ion at m/z 873, M^+ - $[Si(CH_3)_3 + 2H]$ ion at m/z 813, M^+ - $[OSi(CH_3)_3]$ at m/z 799, M^+ - $[CH_2OSi(CH_3)_3]$ ion at m/z 785, M^+ - [2 x $Si(CH_3)_3$ + H] at m/z 741, M^+ - $[OSi(CH_3)_3$ + $Si(CH_3)_3$ + H] at m/z 725, M^+ - $[OSi(CH_3)_3$ + $NHSi(CH_3)_3]$ at m/z 711, M^+ - $[NHSi(CH_3)_3$ + $CH_2OSi(CH_3)_3]$ at m/z 697, M^+ - [2 x CH_3 + + 2 x $CH_2OSi(CH_3)_3$ + H] at m/z 651, M^+ - [2 x $Si(CH_3)_3$ + 2H + $CH_2OSi(CH_3)_3]$ at m/z 637, the m/z 623 is 637 - 14 (CH_2), M^+ - $[Si(CH_3)_3$ + 2 x $CH_2OSi(CH_3)_3]$ at m/z 609, M^+ - $[OSi(CH_3)_3$ + 2H + 2 x $CH_2OSi(CH_3)_3]$ at m/z 591, the m/z 577 is 591 - 14 (CH_2), and M^+ - [2 x $Si(CH_3)_3$ + 2H + $NHSi(CH_3)_3$ + $OSi(CH_3)_3]$ at m/z 563.

3.3.11 Isolation and Identification of Threonic Acid from SHP-2

The threonic acid (the stereochemistry of this acid will be discussed later in this section) produced by periodate oxidation and the reduction of SHP-2 as shown in Figure 24 on page 71 is hard to identify in micro quantities. In order to follow this molecule, reactions similar to those shown in Figure 24 were performed except that $NaBT_4$ was used instead of $NaBH_4$ in the reduction step (see "Sodium Borotritide Reduction Experiment" on page 36 for the experimental procedures). With one of the γ protons of the resulting threonic acid replaced with tritium and the threonic acid and its derivatives can now be detected by radioactivity. As shown in Figure 26 on page 75, paper electrophoresis of the periodate oxidation of SHP-2 and borotritide reduction of the resulting aldehyde produced two radioactive peaks, the dominant one is threonic acid-3-sulfate (—□—,peak maximum at fraction 21), (see Figure 24 for the predicted reactions), and the other one (peak maximum at fraction 15) is threonic acid which is produced by the hydrolysis of threonic acid-3-sulfate during the reaction process. The threonic acid-3-sulfate was almost completely hydrolyzed to threonic acid during the acid hydrolysis as shown in the figure (—✕—, peak maximum at fraction 15). Fractions 12-17 in the acid hydrolysis sample (Figure 26 on page 75, —✕—) were cut off and the tritium-labeled threonic acid was eluted off with water. The sample was dried, dissolved

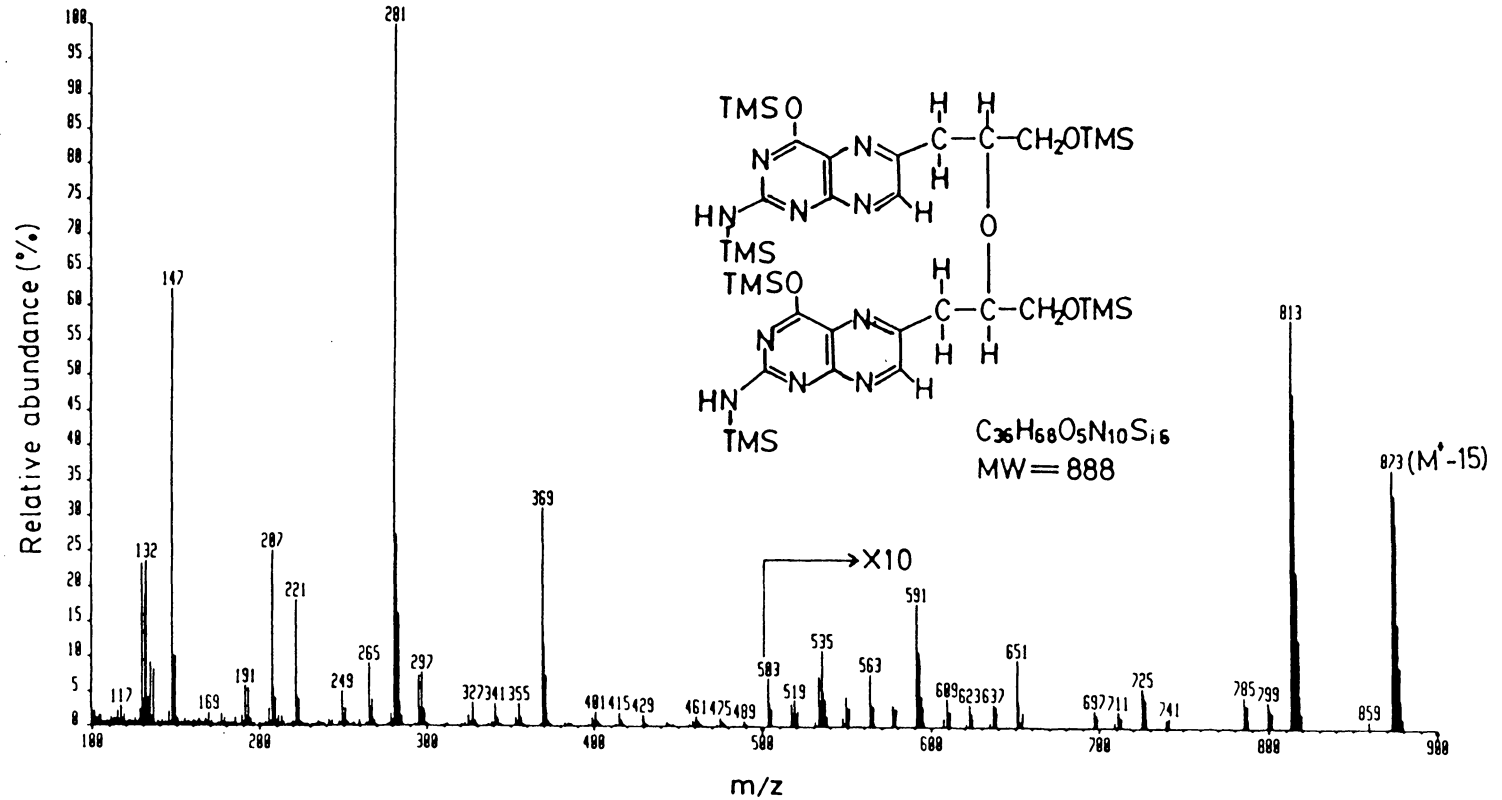


Figure 25. Electron impact mass spectrum of (TMS) -SHP-OR

in ~ 0.5 mL of water, and passed through a small Dowex 50W-8X H^+ (0.7 x 1.0 cm) column. The resulting elution was then dried in a small bottle (1 x 3 cm) and sealed with an aluminum sealer and rubber septum. The resulting lactone (the Dowex column chromatography step converts the majority of the threonic acid into threono-1,4-lactone) was derivatized by adding 50 µL of BSTFA (bis(trimethylsilyl)trifluoroacetamide) and then heated at 60 °C for 1 h (Izumi et al, 1979). Two microliters of sample were injected into the GC with the TMS derivatives of threonic acid, threono-1,4-lactone, erythronic acid, and erythrono-1,4-lactone as standard (for synthesis of the standards, see "Preparation of Threonic Acid and Erythronic Acid" on page 37).

The GC trace of the sample is shown in Figure 27A. The GC fractions were collected at the position indicated in the figure and the radioactivity of each was measured. As shown in the figure, the major radioactive peak corresponds to $(TMS)_2$ derivative of threono-1,4-lactone (peak 210); there is also a small peak which corresponds to the $(TMS)_4$ derivative of threonic acid (peak 266). Mass spectra B and C in Figure 27 on page 76 are the same with published mass spectra (Petersson, 1970; Thompson et al., 1975) thereby proving the proposed structure.

The stereochemistry of the threono-1,4-lactone from SHP-2 was determined by comparing its retention time with those of standard samples with a 10% SP-2100 GC column programmed from 100 - 300 °C at 10 °C/min. The preparation of the standard acids and their lactones have been previously described in "Preparation of Threonic Acid and Erythronic Acid" on page 37. The preparation of the TMS derivatives of these compounds were the same with the tritium-labeled sample as described above. The TMS derivative of the threono-1,4-lactone eluted before erythrono-1,4-lactone under these GC conditions and the TMS derivatives of the tritium-labeled lactone and acid from SHP-2 coeluted with the TMS derivative of threono-1,4-lactone and threonic acid. This stereochemistry fits the idea that the sidechain of SHP-2 is partially biosynthesized from gluconic acid. The biosynthesis and the cleavage pattern can be explained by the following reaction sequence:

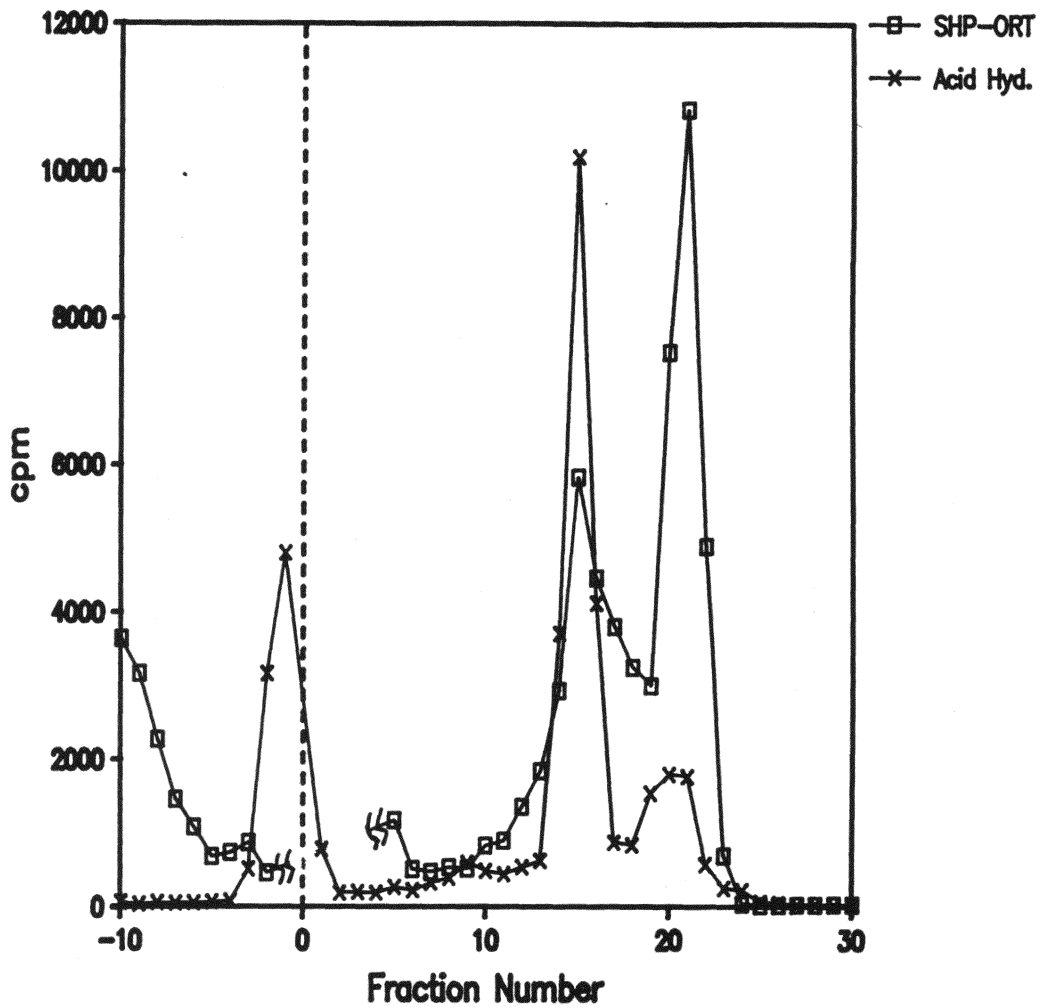


Figure 26. **Paper electrophoresis of tritium-labeled SHP-OR (SHP-ORT):** SHP-ORT was prepared as described in "Sodium Borotritide Reduction Experiment" on page 36. Paper electrophoresis was performed as described in Figure 1 on page 21 and a 0.5-cm wide piece of paper was cut off from cathode (negative numbers, left side of the figure) to anode (positive numbers, right side of the figure). Starting at the origin and preceding both to the cathode and anode, 0.5 cm lengths of paper were removed from the strip and counted for radioactivity. The result is represented in the figure by —□— (SHP-ORT). Then the paper corresponding to fractions 10-23 was cut off, the radioactive sample was eluted off with water, dried, dissolved in 1 M HCl and heated at 110 °C for 3 h. The acid-hydrolyzed sample was then dried, dissolved in 2 M NH_4OH, and heated at 110 °C for 1 h. The resulting sample was applied to the paper, run and counted as described above to generate the acid hydrolysis data (—×—)

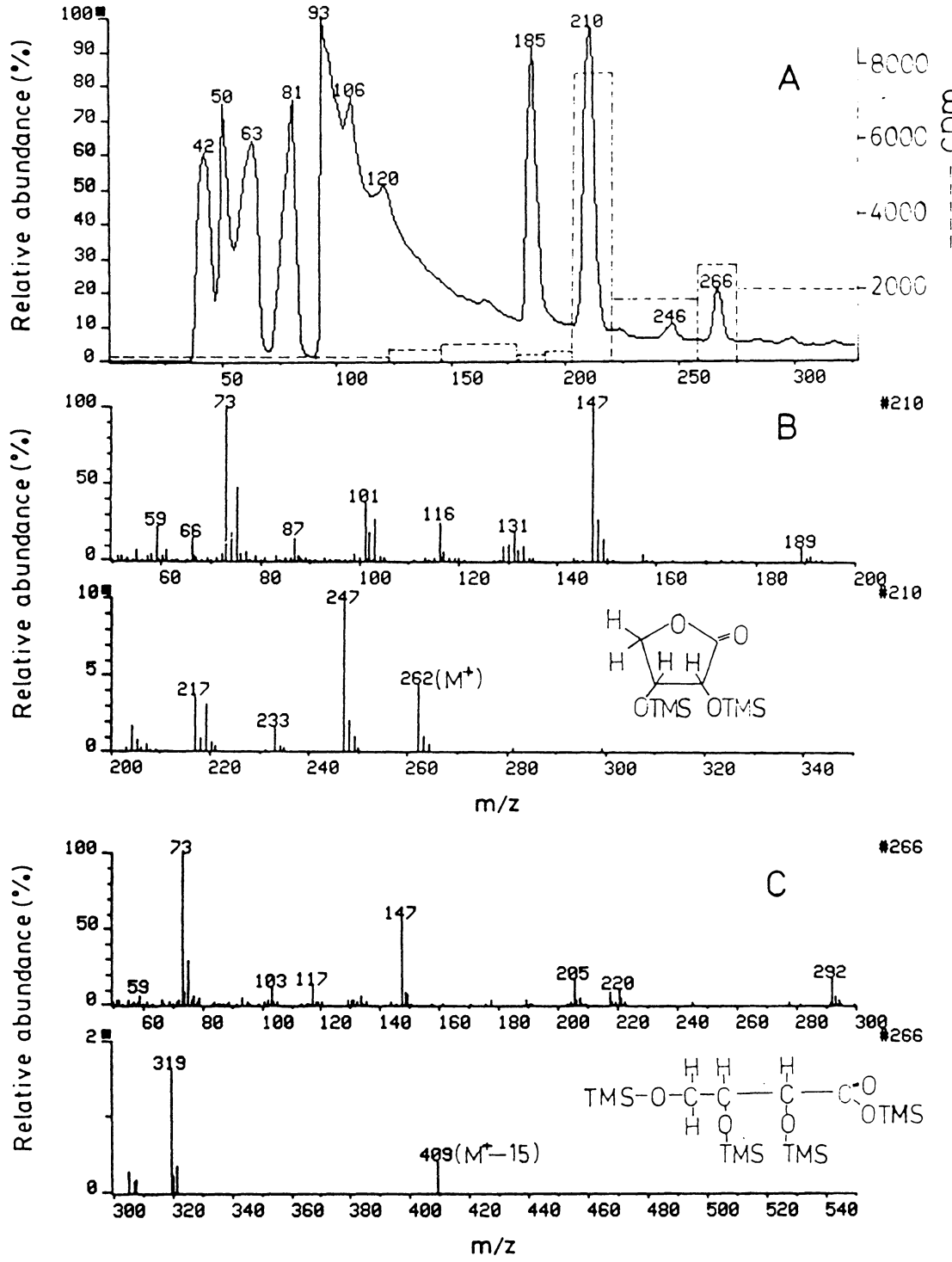
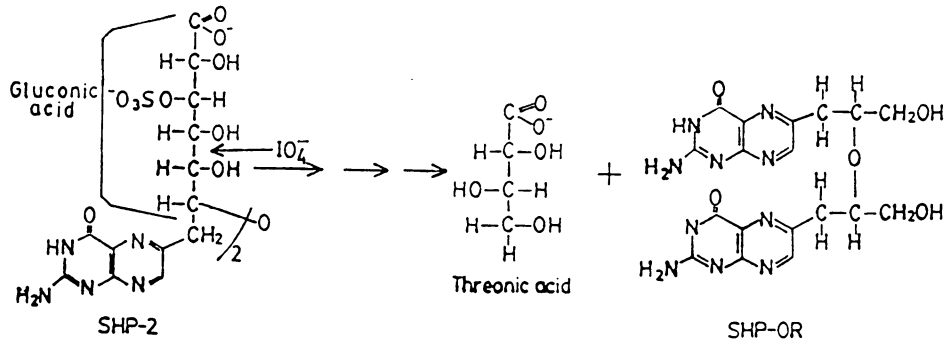


Figure 27. GC-MS of the TMS derivatives of threono-1,4-lactone and threonic acid from SHP-ORT: A: GC trace of the GC-MS data. B: mass spectrum of $(TMS)_2$ derivative of Threono-1,4-lactone. C: mass spectrum of the $(TMS)_4$ derivative of Threonic acid.



3.3.12 Acid Hydrolysis Patterns of SHP-2

As shown in Figure 9 on page 46, at least two intermediates from SHP-2 to HP-2-I and HP-2-II were detected by paper electrophoresis when SHP-2 was subjected to a short-term acid hydrolysis. These intermediates can be explained by the formulas shown in Figure 28 on page 78. The figure shows three of several possible intermediates from SHP-2 to HP-2-I and HP-2-II. The actual reaction course could be much more complex than what is shown in the figure, however, since not all the possible intermediates are shown, nor can they all be separated in the paper electrophoresis system used. Two of the intermediates shown in Figure 28 have one sulfate for each pterin dimer and one of these has three negative charges and the other has two negative charges. These two intermediates could be separated in the paper electrophoresis according to the charge differences and detected in the ^{35}S-labeling experiment shown in Figure 14 on page 54 as the two intermediates which have half a sulfate for each pterin (or one sulfate for each pterin dimer).

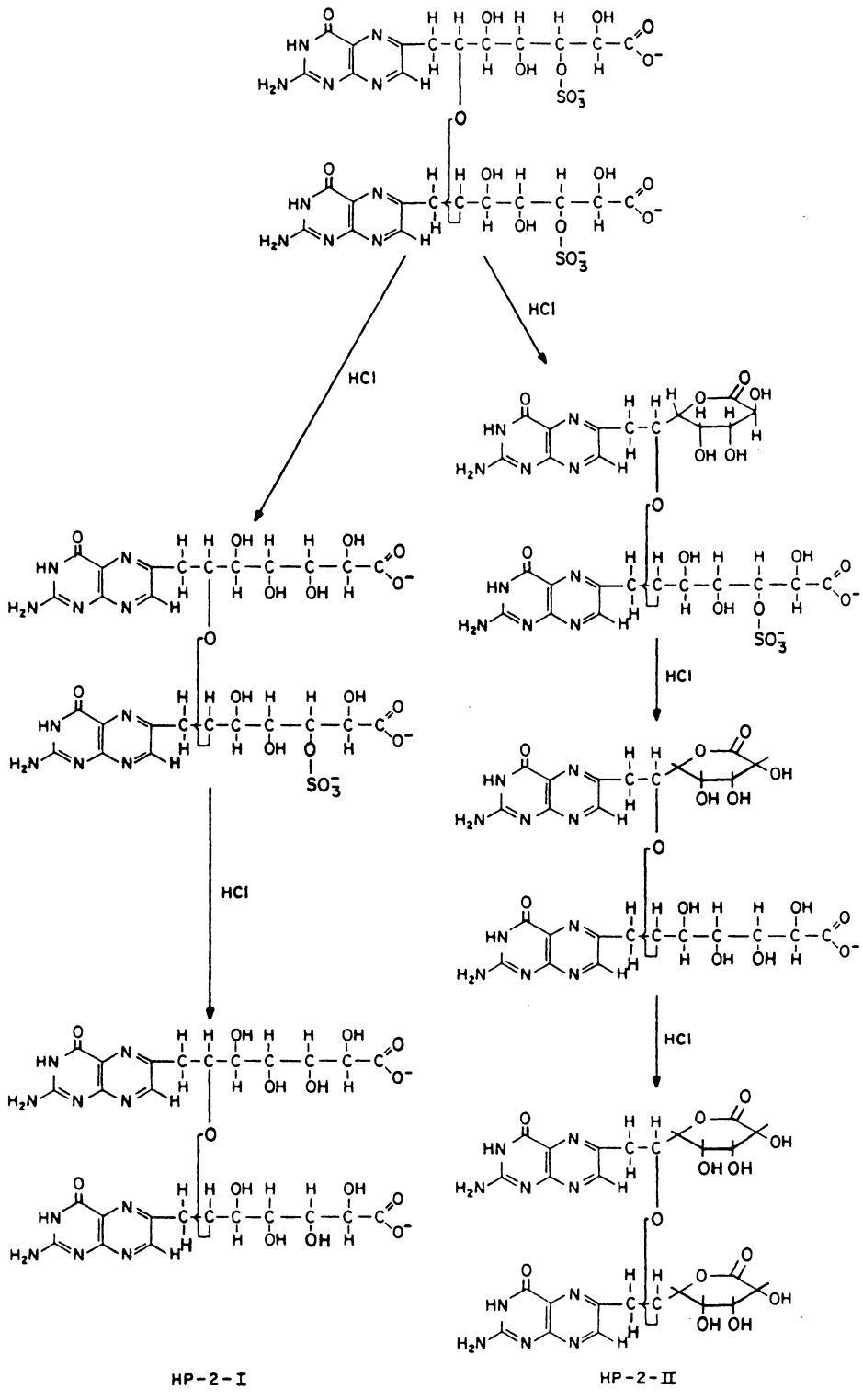


Figure 28. Acid hydrolysis products of SHP-2

Chapter IV

Isolation and Characterization of Phosphohalopterin-1 from Halobacterium salinarium

4.1 Abstract

Phosphohalopterin-1 (3.1 μmol) was isolated from 433 g (wet weight) of *H. salinarium* cells from 40 L of a fully grown culture; in contrast, 39 μmol of SHP-2 was isolated from 40 L of *H. marismortui.* UV-visible and fluorescence spectra of the phosphohalopterin-1 (PHP-1) showed that it is an unconjugated pterin. The pterin portion of the compound was also proved by the titration of the pterin in both acid and base. Phosphate analysis of PHP-1 proved that there are two bound phosphate residues per pterin. One of the phosphates is hydrolyzed by acid phosphatase while the other is not. The proton NMR of PHP-1 proved that the pterin has a polyol side chain similar to that of SHP-2. Periodate cleavage produced the same pterin aldehyde as derived from SHP-2 indicating that PHP-1 is also a pterin dimer.

4.2 *Materials and Methods*

H. salinarium was grown under the same conditions as *H. marismortui* which are described in Chapter III, "Purification of SHP-2" on page 31. The centrifugation and extraction procedures were also the same as in "Purification of SHP-2" on page 31. The procedures for the purification of phosphohalopterin-1 (PHP-1) were different from those used with SHP-2 because PHP-1, from the crude cell extract, was not absorbed by the QAE-Sephadex column. Norite (100 g) was added to the water extract of the cells and mixed well. The solvent was filtered off and the Norite was then washed with about 1 L of distilled water. The material that absorbed to the Norite was then eluted with 2% NH_4OH in 50% ethanol. The resulting eluent was evaporated, dissolved in a minimum amount of water, and applied to Whatman no. 3 paper (23 x 55 cm) for paper chromatography. The paper chromatography was developed by solvent system S1, and the most intense lowest R_f blue fluorescent band (PHP-1) was cut off and eluted from the paper with water. The water solution was evaporated, dissolved in a minimum amount of water, and repurified by paper chromatography using solvent system S2 consisting of MeOH:H_2O:pyridine:n-butanol:88% formic acid (8:2:6:4:1 v/v/v/v/v). The PHP-2 band was then isolated as described above and further purified by paper electrophoresis. The PHP-1 was passed through a Bio-Gel P-2 column developed with water as described in "Purification of SHP-2" on page 31 (yielding 3.9 μmol). The PHP-1 was then dissolved in 0.1 M HCl and passed through a Dowex-50 (0.7 x 5 cm) column which was preequilibrated with 0.1 M HCl. The column was washed with 0.1 M HCl and the PHP-1 was eluted with water (yielding 3.1 μmol of PHP-1 which was used for NMR and subsequent analysis).

Acid phosphatase, a type I phosphatase from wheat germ containing 0.36 units of activity / mg solid (1 unit will hydrolyze 1.0 μmol of p-nitrophenol phosphate / min. at pH 4.8 at 37 °C), was obtained from Sigma Chemical Co.. The phosphate assay was previously described in Chapter III, " Sulfate and Phosphate Assay" on page 33. The phosphatase cleavage reaction was done by dissolving 1 mg of acid phosphatase in 0.1 mL of 0.1 M NH_4OAc buffer (pH 4.8) and adding about 0.1 μmol of PHP-2. The mixture was then incubated at 37 °C for 1 h. The resulting

phosphohalopterin-1 phosphatase cleaved product (PHP-E) was purified by paper chromatography, quantitated by absorbance at 342 nm, and made ready for phosphate and other analyses.

4.3 Results and Discussion

PHP-1, the first halopterin discovered in this work, was found accidentally during an effort to identify coenzyme A in halobacteria. The CoA present in the cell extracts was derivatized by mBBr (monobromobimane) to form a yellow fluorescent adduct (Fahey and Newton, 1983) which would allow for the identification and isolation of CoA by paper chromatography. As shown in Figure 29 on page 82 (lane 2), CoA-mBBr was identified from the *E. coli* extract (later proved by proton NMR, data not shown) by this method. CoA-mBBr was not found in *H. salinarium* by this method, however, another blue fluorescent compound was found on the paper (Figure 29, lane 4) which was not present in *E. coli* (Figure 29, lane 2). It was soon found that this compound is also absent in yeast, methanogens, and other nonhalobacteria (see Chapter II, "Results and Discussion" on page 20).

This blue fluorescent compound was later named phosphohalopterin-1 because it was the first halopterin found and because there are two phosphate esters per pterin in the molecule, as will be discussed later. The fluorescence excitation and emission spectra were the same as in Figure 5 on page 40 and the UV-visible spectra in different pH were the same as in Figure 4 on page 39. Both of these observations prove that the chromophore of PHP-1 is a typical, unconjugated pterin. In order to determine the pKa's of the chromophore of this compound, a titration curve was done and the result is shown in Figure 30 on page 84. Two pKa's were identified in this experiment, pK_1 = 2.6 and pK_2 = 8.1. These values are typical of previously reported values for unconjugated pterins (Pfleiderer, 1985). For example, biopterin has pK_1 = 2.25 and pK_2 = 7.98, neopterin has pK_1 = 2.23 and pK_2 = 7.97, and 6-methylpterin has pK_1 = 2.31 and pK_2 = 8.10. A very in-

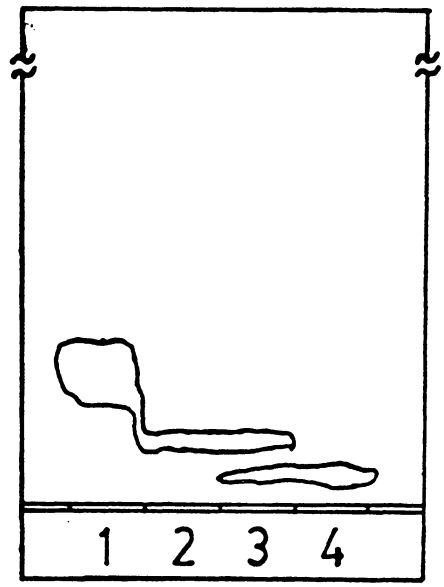


Figure 29. Paper chromatography of CoA-mBBr and halobacterial extract: The cell extracts were derivatized by adjusting to pH 8 and adding 2 mM of mBBr in 1 mM EDTA, 10 mM NH_4HCO_3, pH 8 buffer and incubating at 65 °C for 10 min. The resulting mix was applied directly to the paper and the paper was developed by solvent system S2. The bands were identified by the fluorescence of the compounds by using a fluorescent light (long wavelength) held above the paper in the dark. Lane 1 is standard CoA-mBBr. Lane 2 is *E. coli* extract-mBBr. Lane 3 is a mixture of *E. coli* extract-mBBr and *H. salinarium* extract-mBBr. Lane 4 is *H. salinarium* extract-mBBr. The CoA-mBBr band in lanes 2 and 3 runs lower and narrower than the standard in lane 1 because of the high salt and protein concentrations in the crude cell extracts.

teresting point is that the pKa's of PHP-1 are closer to those of 6-methylpterin than to those of biopterin or neopterin. This fact supports the idea that the 1'-carbon of PHP-2 is a methylene carbon like SHP-2 rather than a hydroxymethylcarbon like biopterin and neopterin.

According to Pfleiderer (1982), the chemistry of the two pKa's in the pterin ring is as shown below:

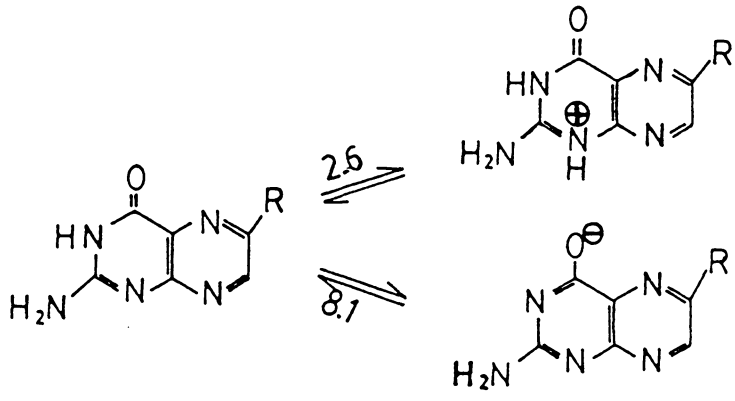


Figure 31A shows the TLCs of PHP-1, PHP-E, and HP-1. The figure clearly shows that PHP-1 is cleaved by acid phosphatase and that the resulting PHP-E is different from the acid hydrolysis product of PHP-1 (HP-1).

In contrast to the charged pterin produced by the acid hydrolysis of SHP-2 (Figure 9 on page 46), the acid hydrolysis product of PHP-1 is not charged (Figure 31B). Acid hydrolysis released two phosphates per pterin as quantitated by the phosphate assay method (11 nmol of PHP-1, as quantitated by pterin extinction coefficient, released 24 nmol of phosphate). As shown in Figure 31, the acid phosphatase cleavage product of PHP-1 (PHP-E) is still negatively charged though less charged than PHP-1 (Figure 31B, lane 3). The PHP-E can be further hydrolyzed by acid and is found to release one phosphate per pterin according to the phosphate assay (13 nmol of PHP-E released 12 nmol of phosphate). This means that there are two phosphate esters per pterin in PHP-1 and that one of the phosphate esters can be hydrolyzed by acid phosphatase and the other one is resistant to acid phosphatase.

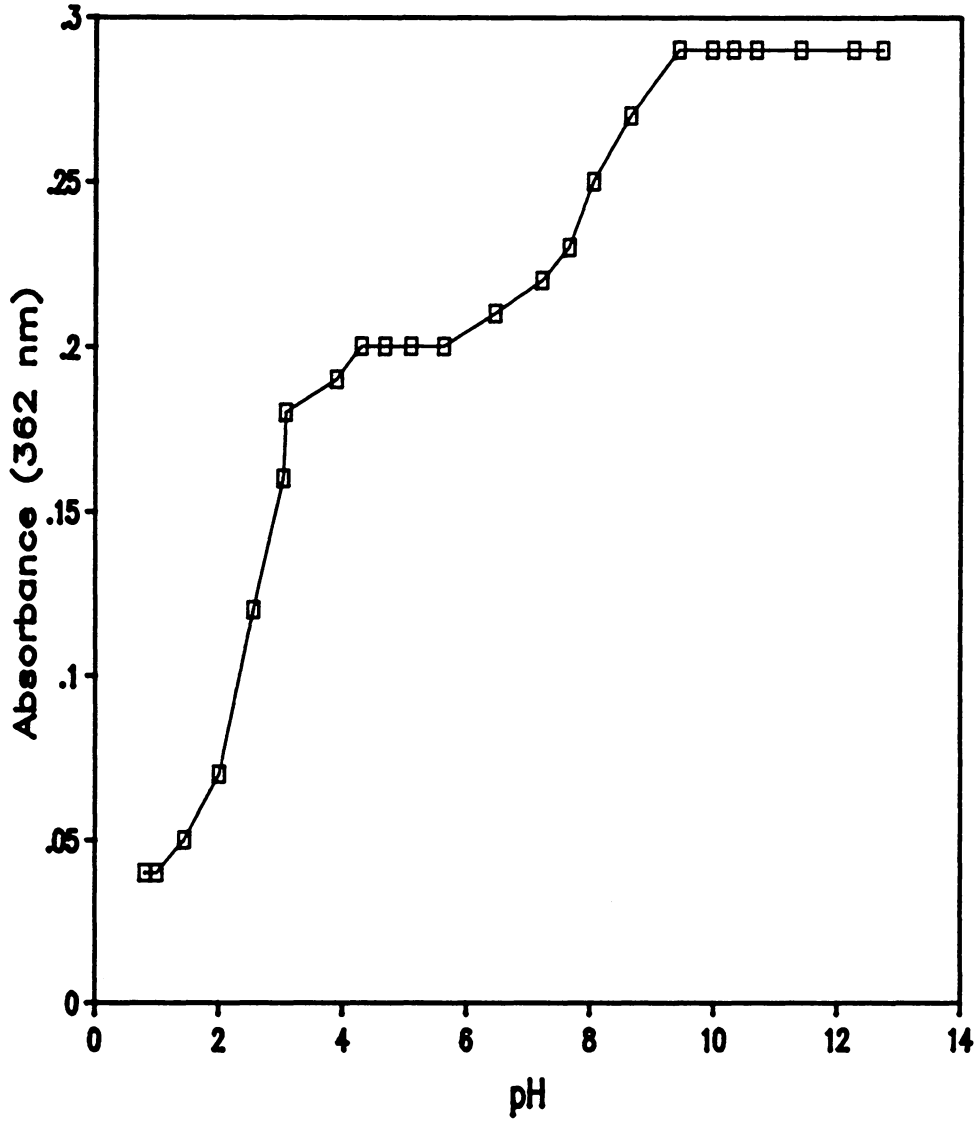


Figure 30. Titration curve of PHP-1: About 0.12 μmol of PHP-1 was dissolved in 3 mL of 0.15 M ethyleneamine-citrate buffer (pH 12.73) and then a UV-Visible spectra from 430-230 nm was recorded using a 1 cm path length cuvet. Concentrated HCl (10 μl alliquats) was then added to the cuvet, mix well and the spectra taken again. The same process was repeated 24 times until a total of 240 μl of concentrated HCl was added and the pH of the cuvet was brought down to 0.81. The pH of the buffer in each spectra was predetermined by using the same amount of buffer and adding the same amount of acid and measuring the pH with a pH meter. pK_1 = 2.6 and pK_2 = 8.1 was read from this curve.

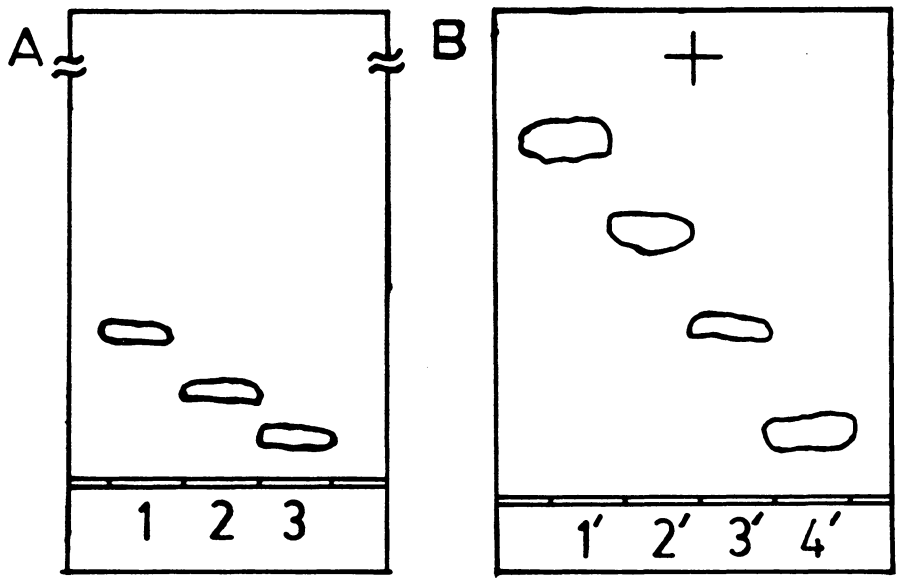
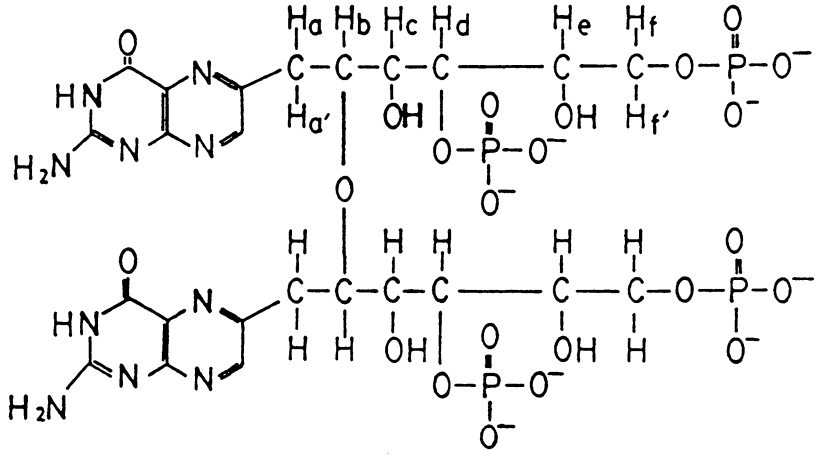


Figure 31. **TLC (A) and paper electrophoresis (B) of PHP-1, PHP-E, and HP-1:** The preparation of PHP-1 and PHP-E was as described in "Materials and Methods". HP-1 was prepared by dissolving PHP-1 in 1 M HCl and then heating at 100 °C for 12 h. A. The TLC was performed as in Figure 2 on page 23 with S1 as solvent. Lane 1 is PHP-1. Lane 2 is PHP-E. Lane 3 is HP-1. B. The paper electrophoresis was the same as in Figure 1 on page 21. Lane 1′ is SHP-2. Lane 2′ is PHP-1. Lane 3′ is PHP-E. Lane 4′ is HP-1.

It has been shown in Figure 1 on page 21 that PHP-1 is not cleaved by periodate in contrast to SHP-2. This means that the phosphate esters and other bonds in the molecule, i.e., an ether bond like SHP-2, must protect the polyol side chain, shown to be present by proton NMR, from periodate cleavage. The acid hydrolysis product of PHP-1 (HP-1) is cleaved by periodate; the TLC of the resulting cleavage product is shown in Figure 32 on page 87. The figure shows that the periodate-cleaved product of HP-1 is different from the pterin-6-aldehyde produced from neopterin, but that it is the same as that of the SHP-2 periodate-cleaved product. This fact tends to support the idea that PHP-1 has a side chain carbon skeleton similar to that of SHP-2.

Considering that PHP-1 and SHP-2 are both negatively charged pterins which have been isolated from halobacteria, that the periodate cleavage of PHP-1 produces the same product as the cleavage of SHP-2, and that the molecular weight of PHP-2 is that expected for a dimer (Chapter VI), the following structure is proposed for PHP-1.



The PMR of PHP-1 is shown in Figure 33 on page 89. The protons were assigned by assuming that the side chain structure of PHP-1 is like the one presented in the figure. The assignments are supported by the spin-spin decoupling experiments which showed that when H_d was irradiated, both H_c and H_e were changed; when H_c was irradiated, both H_d and H_b were changed (H_a and $H_{a'}$ were also changed for an unknown reason); and when H_a and $H_{a'}$ were irradiated, only H_b changed. The coupling between H_d and phosphate was proven by the PMR of HP-1 in Figure 34 on page 90 which shows that, after the phosphate was removed by hydrolysis, the H_d peak changed from quartelet to doublet and its chemical shift shifted to a higher field of about 0.1

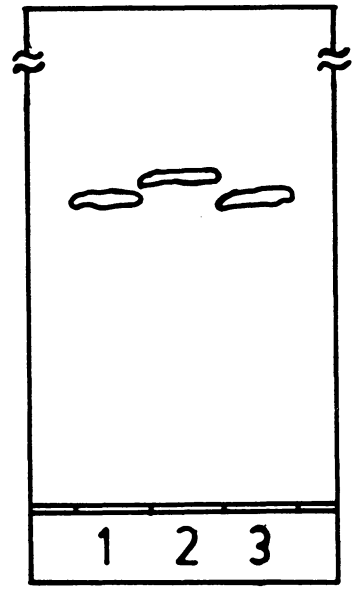


Figure 32. **TLC of periodate-cleaved HP-1:** The TLC was developed by solvent system S1. About 50 nmol of HP-1, neopterin and SHP-2 were each dissolved in about 40 µL of water, and 0.75 µmol of $NaIO_4$ in 10 µL of water was added. The reaction mixture was kept in the dark for ~ 3 h, after which ~ 1 µL of each of the cleaved products was applied to the TLC for analysis. The samples were detected as blue fluorescent bands under fluorescent light in the dark. Lane 1 is SHP-2 cleaved by periodate. Lane 2 is neopterin cleaved by periodate (pterin-6-aldehyde). Lane 3 is HP-1 cleaved by periodate.

ppm which is common for sugar phosphate (Gerlt and Youngblood, 1980). The H_d-^{31}P coupling in this case is 3 Hz and is the same magnitute of coupling (1 - 8 ppm) reported in the literature for H-C(=R,R')-O-P_i (O'Connor et al., 1979).

Above is the preliminary work that has been done on the structural elucidation of PHP-1. Due to the low concentration of PHP-1 present in the cells (see Table 2 on page 25, the PHP-1 concentration in *H. salinarium* is only about 10% of that of SHP-2 in *H. marismortui*) and the fact the PHP-1 is not absorbed by a QAE-Sephadex column when present in crude cell extracts, it is much more difficult to purify PHP-1 than SHP-2. About 400 L of *H. salinarium* is needed to isolate enough PHP-1 to do a ^{13}C-NMR according to the above data. Considering the difficulty of dealing with such a huge volume of cell culture saturated with salt, it would be very difficult to prove the structure of SHP-1. But there may be other approaches to this problem. One of these approaches is to isolate a mutant of *H. salinarium* which would produce large amounts of PHP-1. A second and may be more hopeful approach is to use a sulfate-deficient medium to grow *H. marismortui*; as mentioned in Chapter III, "Incorporation of ^{35}S Sulfate into SHP-2 and Characterization of Its Cleavage Products" on page 47, when *H. marismortui* was grown in 0.81 mM sulfate, PHP-1 was produced in addition to SHP-2. Though no further work has been done to quantitate this result, the PHP-1 produced may be in a comparable amount as that of SHP-2 in *H. marismortui*. If this is proven to be correct, then 40 L of *H. marismortui* grown in sulfate deficient media would be enough to isolate PHP-1 in order to obtain a ^{13}C-NMR and perform other structural analyses.

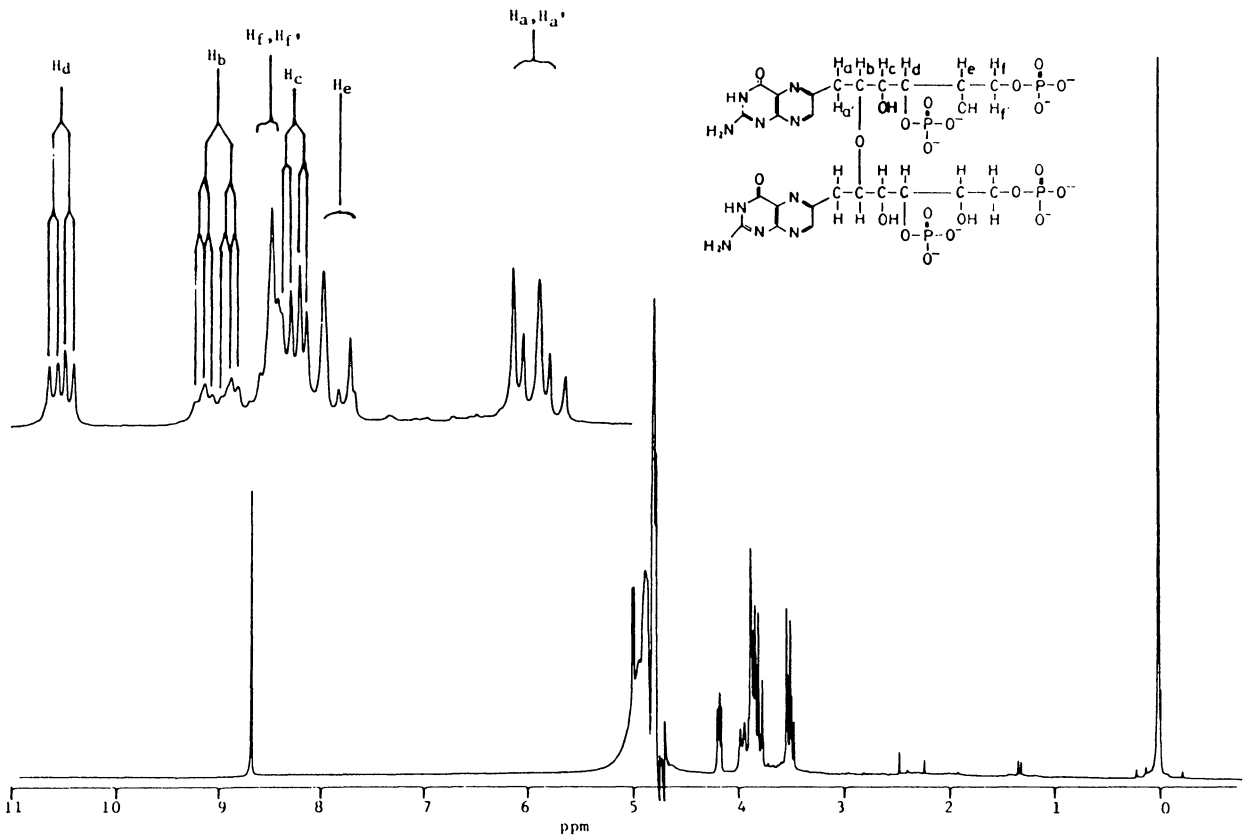


Figure 33. 270 PMR of PHP-1: PHP-1 (3 µmoles) was dissolved in about 10% of concentrated deuterated ammonium hydroxyde in deuterated water with TSP as internal standard in a 5 mm NMR tube. The spectrum was recorded at room temperature.

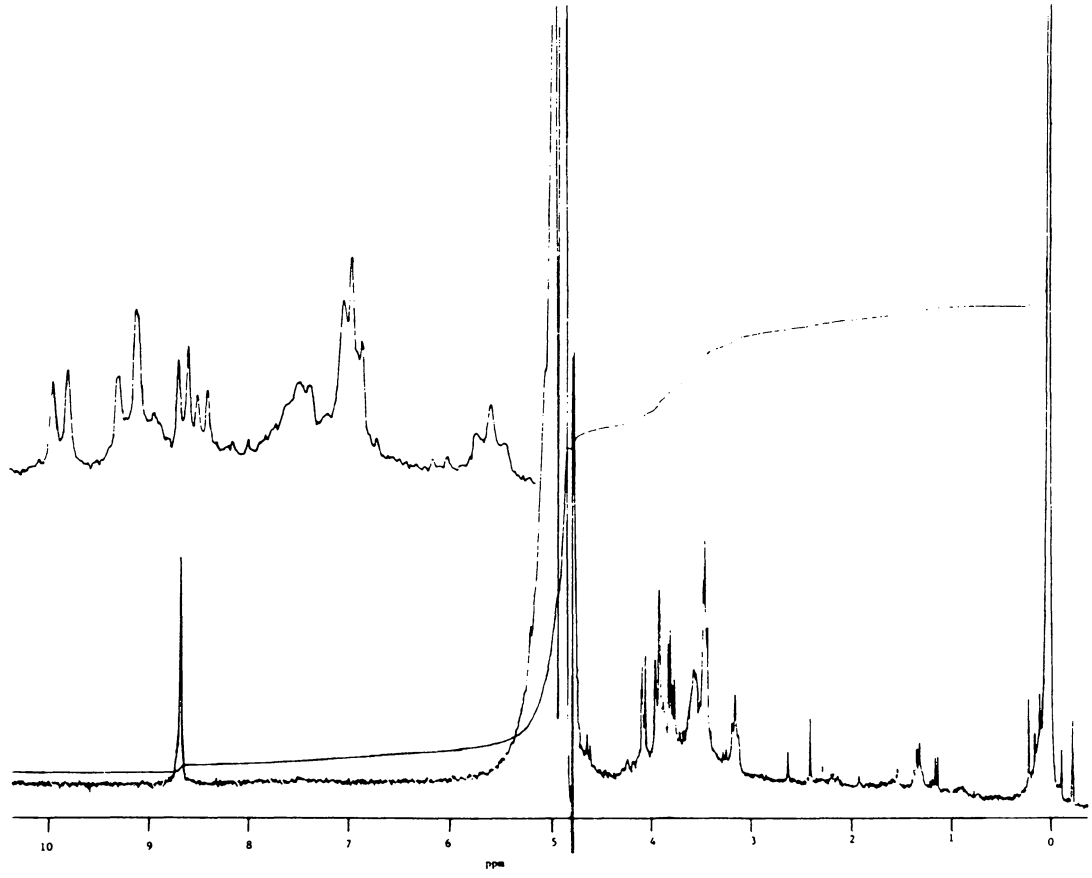


Figure 34. PMR of HP-1: The conditions was the same as in Figure 33 on page 89 except about 0.8 μmol of HP-1 was used.

Chapter V

Isolation and Characterization of Solfapterin from Sulfolobus solfataricus

5.1 Abstract

A positively charged unconjugated pterin was isolated from the thermoacidophilic archaebacterium, *Sulfolobus solfataricus*. This pterin was named solfapterin because of its origin. PMR proved that solfapterin has a polyol side chain like the halopterins. One primary or secondary amine was identified on the side chain of this pterin by selective acetylation. This method also proved that the origin of the positive charge is from this amine. Chemical degradation methods were also used to identify the structure of solfapterin. The data presented below constitute a preliminary report on the structural elucidation of this compound.

5.2 *Materials and Methods*

The growth and harvesting procedures for *S. solfataricus* have been previously described (Chapter II, "Materials and Methods" on page 18). The wet cells (65 g from 80 L of full-growth culture) were lysed by adding 300 mL of 70% EtOH and heated at about 80 °C for ~ 2 h. The mixture was centrifuged, the clear ethanol layer removed, and the pellet repeatidly reextracted (15 times) with 200 mL of 70% ethanol until no more solfapterin remained in the pellet as checked by paper chromatography. The resulting combined, clear supernatants were rotary evaporated at 60 °C at reduced pressure and dissolved in ~ 100 mL of water. Chloroform (100 mL) was added to the water solution and the resulting two phase mixture was mixed well and centrifuged. The clear yellow water layer was removed, evaporated under reduced pressure, dissolved in minimum amount of water, and applied to Whatman no. 3 paper (23 x 37 cm) for paper chromatography with solvent system S1 as solvent as previously described (Chapter II, "Materials and Methods" on page 18). Seven blue fluorescent bands were identified in this paper chromatography system and the lowest blue fluorescent band, the one which containd the solfapterin, was cut off and the pterin eluted from the paper with water, and further purified by paper electrophoresis as described in "Materials and Methods" on page 18. The solfapterin which had been purified by paper electrophoresis was re-purified by paper chromatography developed with solvent system S2. The solfapterin which was purified by paper chromatography was then dissolved in water and applied to a Bio-Gel P-2 column (3.5 x 37 cm) which had been preequilibrated with water. The column was then eluted with ~ 500 mL of water, and solfapterin which was absorbed on the top of the column was eluted with 1% NH_4OH in water. The 3 μmol of solfapterin which was isolated by this procedure was used for NMR and other analyses.

The acetylation of solfapterin was basically according to the methods of Greenstein and Winitz (1961). For a typical procedure, 20 nmol of solfapterin was dissolved in 20 μL of water. The solution was cooled in ice-water, 20 μL of acetic anhydride (Ac_2O) were added and the resulting solution was mixed well. The mixture was allowed to sit in the dark at room temperature for ~ 3 h

after which it was evaporated to dryness in a vacuum. After disolving the sample in 20 μL of water, this process was repeated two more times, using 30 min instead of 3 h reaction time in order to complete the selective acetylation of the side chain amine.

5.3 Results and Discussion

Solfapterin was identified as a positively charged pterin by paper electrophoresis (see Figure 2 on page 23). The fluorescence excitation and emission spectra of solfapterin were almost identical to those recorded for neopterin (spectra not shown), thus confirming that the chromophore of solfapterin is a pterin. The amine in the side chain of solfapterin is very easy to acetylate and the resulting acetylated solfapterin is no longer positively charged (as shown in Figure 35 on page 94).

The selectivity of the acetylation reaction was checked by running a control experiment using neopterin. If the neopterin was not changed when subjected to the same acetylation reactions ("acetylated" neopterin in Figure 36 lane 2, means that an attempt was made to acetylate the neopterin by using the same acetylation procedures as used for solfapterin), it would mean that the functional groups in either the pterin ring or polyol side chain were not acetylated. Three TLC systems were used to verify the results shown in Figure 36 on page 95 which prove that only the amine on the side chain of solfapterin was acetylated under these conditions.

As will be shown later, the PMR of solfapterin proved that it has a polyol side chain similar to that of the halopterins. Also, the side chain polyol of solfapterin is cleaved by periodate as shown in Figure 37, lane 2. If the amine on the side chain of solfapterin is close to the pterin (for example, on C-1′ or C-2′ of the side chain carbon), then the periodate cleavage product of solfapterin-Ac would be different from that of solfapterin. Figure 37 (lane 3) shows that this is clearly not the case. Actually, the figure shows that the periodate cleavage products of solfapterin and

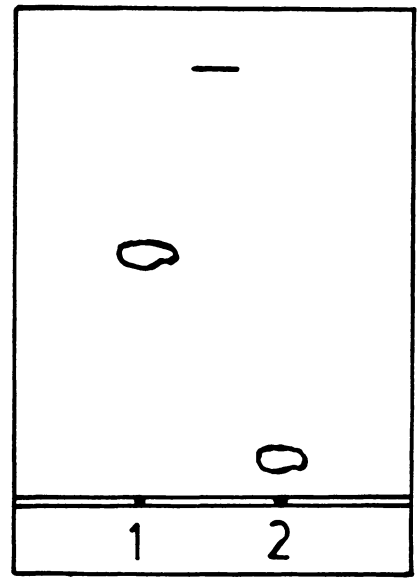


Figure 35. Paper electrophoresis of solfapterin and solfapterin-Ac: The conditions for the paper electrophoresis were the same as described in Figure 1 on page 21. Lane 1, solfapterin. Lane 2, acetylated solfapterin.

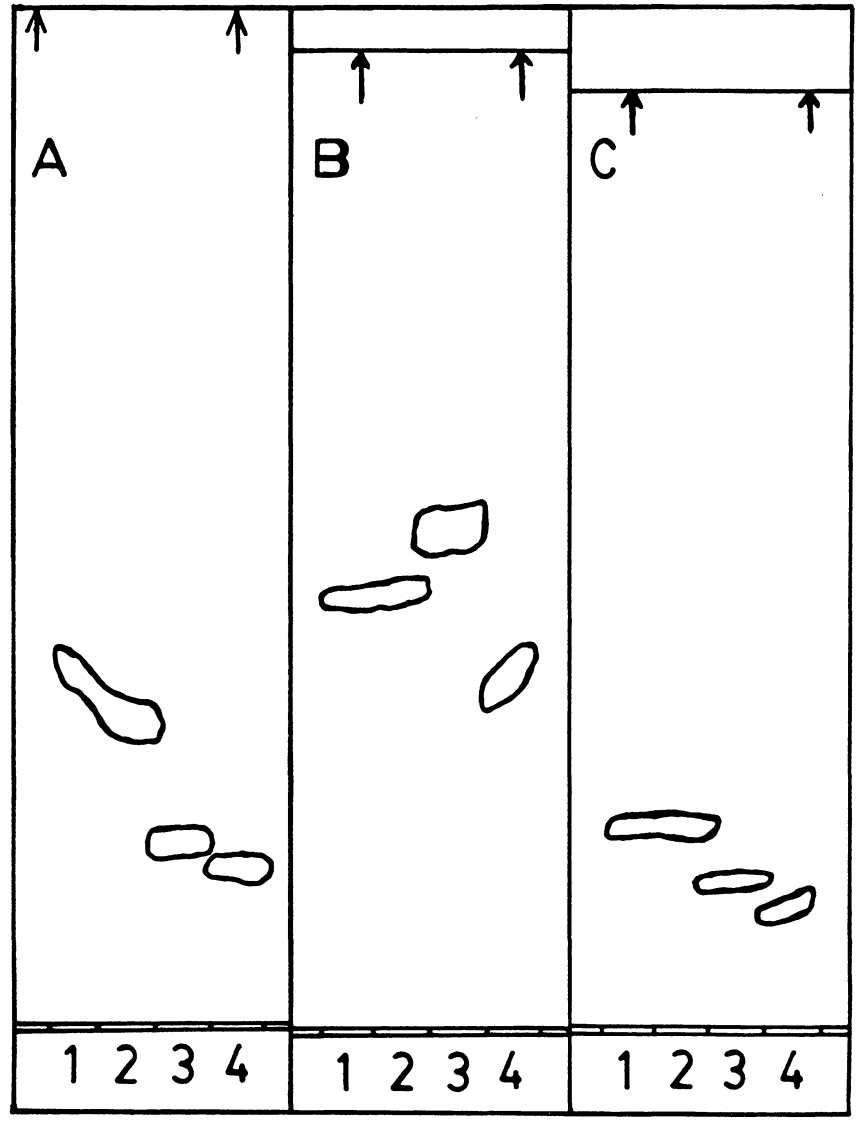


Figure 36. TLC of solfapterin, solfapterin-Ac and neopterin: The conditions for the TLCs were the same as described in Figure 2 on page 23. Lane 1 is neopterin, lane 2 is "acetylated" neopterin, lane 3 is acetylated solfapterin (solfapterin-Ac), and lane 4 is solfapterin. The solvent systems used for the three TLC plates were (A) acetonitrile:H_2O:88% formic acid (8:2:1, v/v/v), (B) pyridine:n-butanol:MeOH:H_2O:NH_4OH (15:10:15:10:1, v/v/v/v/v), (C) n-butanol:AcOH:H_2O (12:3:5, v/v/v).

solfapterin-Ac are identical. This means that the amine is at least three carbons away from the pterin on the polyol side chain.

The PMR of solfapterin is shown in Figure 38 on page 98. Approximately nine to ten side chain protons in the polyol region of the spectrum have been identified based on the integration of the spectrum by setting the sum of the pterin protons at about 8.5 and 8.7 ppm equal to 1. An unusual result from this PMR is the discovery that the solfapterin is in two isomers in the cells. Presumably, the 8.7 ppm peak represents the 6-substituted pterin and the 8.5 ppm peak represents the 7-substituted pterin. The ratio of the six to seven substitution is ~ 6:1 based on the integration of the pterin proton signals. This is unusual in that the cells usually make only one isomer instead a mixture as in this case. The side chain protons in the figure include $H_{1'}$ (1 proton), $H_{2'}$ (1 proton), H_n (4 protons), H_t (2 or 3 protons) and H_a (1 proton). Table 7 on page 100 shows the results of the spin decoupling experiments of these protons. The observed spin decoupling data appear to divide the protons into two separate groups. One group, H_t and H_a, apparently coupled to each other but not to any of the other protons. In the other group, which includes the rest of the protons, $H_{1'}$ is only coupled to $H_{2'}$, $H_{2'}$ is coupled to both $H_{1'}$ and H_n, and H_n is coupled to $H_{2'}$.

Figure 39 on page 99 shows the PMR of solfapterin-Ac. This spectrum clearly shows that H_a shifted from ~ 2.7 ppm to over 3 ppm after the acetylation of the side chain amine. This could mean that H_a shares the same side chain carbon with the amine. No structure has been proposed to date that is consistant with all the data. More work needs to be done to elucidate the structure of solfapterin. An exciting speculation is that this positively charged unconjugated pterin could substitute for folic acid in this thermoacidophilic archaebacterium.

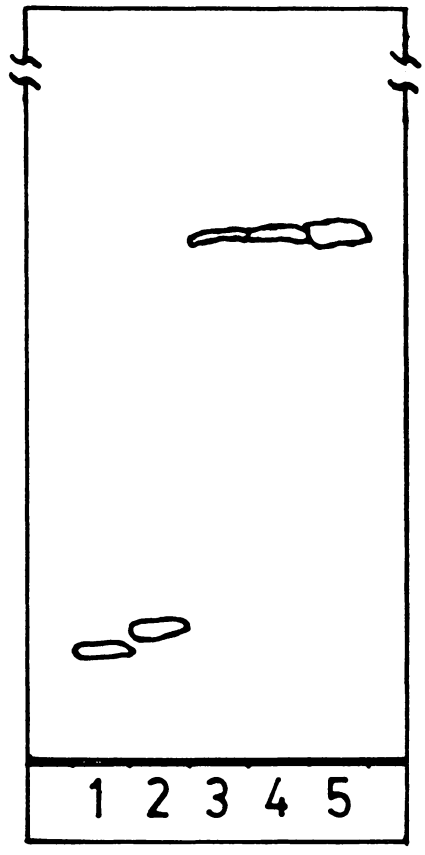


Figure 37. TLC of periodate cleaved solfapterin and solfapterin-Ac: The TLC was developed with solvent system S1. Lane 1 is solfapterin, lane 2 is solfapterin-Ac, lane 3 is solfapterin cleaved by periodate, lane 4 is solfapterin-Ac cleaved by periodate, and lane 5 is neopterin cleaved by periodate.

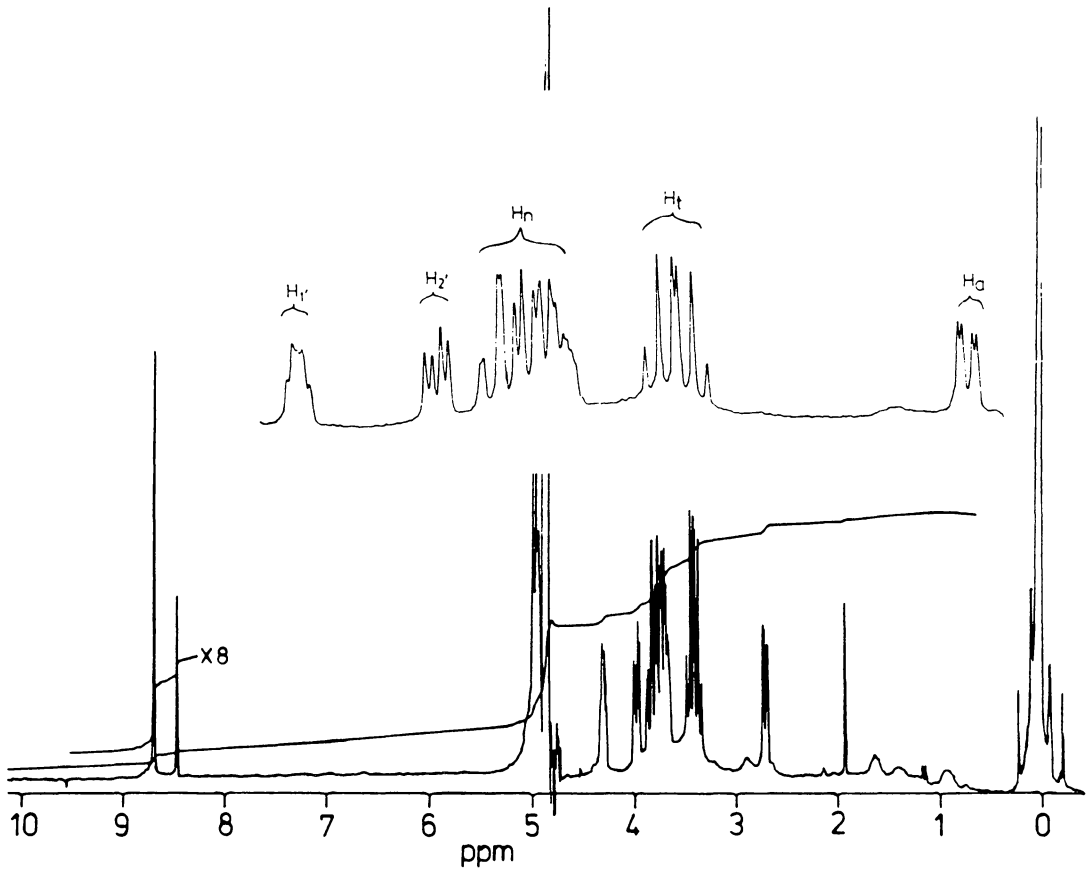


Figure 38. PMR of solfapterin: Solfapterin (3 μmol) was dissolved in 0.5 mL of 10% concentrated ND_4OD in D_2O and the spectrum was recorded at room temperature with TSP as internal standard.

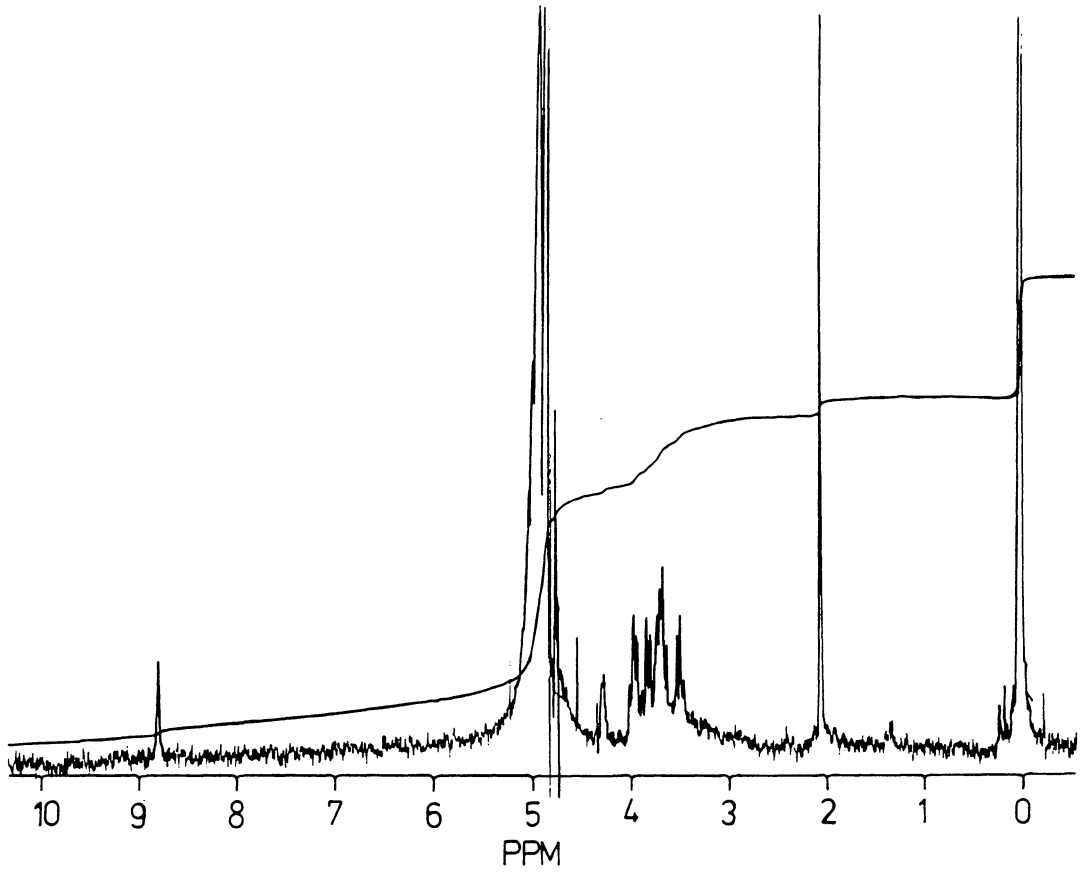


Figure 39. PMR of solfapterin-Ac: Solfapterin-Ac (0.7 μmol) was dissolved in 0.5 mL of 10% concentrated ND_4OD in D_2O and the spectrum was recorded at room temperature with TSP as internal standard.

Table 7. Spin Decoupling Experiment on Solfapterin

Irradiated Protons	Ht	Ha	H-1'	H-2'	Hn
Ht		ch[1]	uc[2]	uc	uc
Ha	ch		uc	uc	uc
H-1'	uc	uc		q[3]→d[4]	uc
H-2'	uc	uc	ch		ch
Hn	uc	uc	uc	q→t[5]	

[1] ch: change. [2] uc: unchanged. [3] q: quartet. [4] d: doublet. [5] t: triplet.

Chapter VI

Gel Filtration of Pterins

6.1 Abstract

A gel filtration procedure was used to determine the molecular weights of the newly discovered pterins which had been isolated from the halobacteria and thermoacidophiles. Using this procedure, it was found that the pterins had gel chromatographic properties which were dependent upon the chemical structure of the side chain which was attached to the 6 or 7 position of the pterin. The molecular weights of our unknown pterins, which were calculated from the gel filtration experiments, are consistent with the proposed structure for these pterins.

6.2 *Materials and Methods*

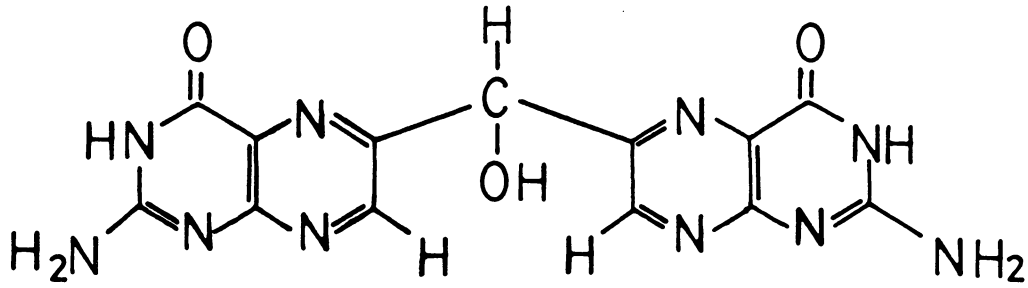
The gel filtration procedures were basically the same as those of Krüger and Meyer (1986). Sephadex G-15-120 (particle size 40 - 120 μ) and guanidine (aminomethanamidine) hydrochloride (Gu•HCl) Grade 1 were from Sigma Chemical Co. The column (1.0 x 59 cm) was equilibrated and eluted with 4 M Gu•HCl in 50 mM KH_2PO_4 buffer (pH 1.2) and 0.75 mL fractions were collected for calibrations.

The chemical synthesis of some standard pterins was done by Dr. R. White and is briefly described below.

6[and 7]-(1',2',3',4'-tetrahydroxybutyl)Pterin was synthesized by condensing glucose with 2,4,5-triamino-6-hydroxypyrimidine as described by Forrest and Walker (1948).

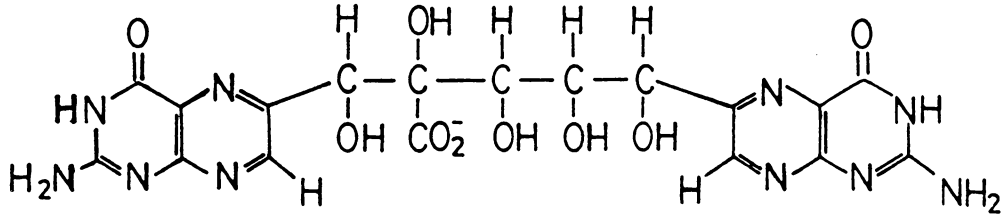
7-(2',3',4'-trihydroxybutyl)Pterin was synthesized by condensing 3-deoxy-D-erythro-hexos-2-ulose with 2,4,5-triamino-6-hydroxypyrimidine as described by Storm (1971). [A 6-substituted pterin was supposed to be produced by this procedure but the 7-isomer was found to be the major product as proven by ^{13}C-NMR (see Figure 17 on page 58).] The 3-deoxy-D-erythro-hexos-2-ulose was prepared by reacting D-glucose with benzoylhydrazine according to the methods of Madson and Feather (1981) and Kucar et al. (1974).

1-Di(2-amino-4-hydroxy-6-pteridinyl)methanol (Pterin dimer-1) was synthesized by condensing 2,3,4-trihydroxy-D-xylo-glutardialdehyde with 2,4,5-triamino-6-hydroxypyrimidine as described by Goto et al. (1965) for the preparation of monosubstituted pterins. The 2,3,4-trihydroxy-D-xylo-glutardialdehyde was synthesized from glucose according to the method of Kiss et al. (1975).



Pterin Dimer-1

1,5-Di(2-amino-4-hydroxy-6-pteridinyl)-1,2,3,4,5-pentahydroxy- 2-carboxypentane (Pterin dimer-2) was synthesized by condensing 9-aldo-4-C-formyl-nonose with 2,4,5-triamino-6-hydroxypyrimidine as described by Goto et al. (1965). The 9-aldo-4-C-formyl-nonose was synthesized from 1,2-isopropylidene glucose according to the method of Schaffer and Isbell (1956, 1959).



Pterin Dimer-2

6.3 Results and Discussion

Krüger and Meyer (1986) used FAD, FMN, riboflavin, and pterin-6-carboxylic acid as standards in order to calibrate their Sephadex G-15 column for the determination of the molecular weight of an unknown pterin that they discovered in the carbon monoxide dehydrogenase from

Pseudomonas carboxydoflava. They called this pterin bactopterin. Using the same standards, a similar calibration line was obtained with the Sephadex column system used in this experiment as is shown in Figure 40 on page 107.

Since our unknown compounds are pterins, we decided to use only known pterins to calibrate our column. The results (shown in Figure 41 on page 108) were unexpected as none of the pterins fit into the standard line shown in Figure 40. Instead, the pterins formed their own line which has a slope much steeper than the FAD, FMN, and riboflavin line, which indicates that the pterins must have some special interactions with the Sephadex gel even in the 4 M Gu•HCl pH 1.2 buffer. These interactions must be very uniform for the pterin monomers used in this experiment in that a good calibration line was obtained (Figure 41). Because our unknown halopterins are dimers, based on the data shown in Chapters III IV, it is possible that pterin dimers would have a calibration line different from the monomers. In order to explore this possibility, two pterin dimers were synthesized and the resulting gel filtration calibration line is shown in Figure 42 on page 109. The figure shows that the pterin dimers do, in fact, behave differently from pterin monomers. A very interesting phenomenon is that the two pterin dimers form a straight line with pterin which signatures the pterin properties of these compounds. The slope of the pterin dimer line is steeper than that of the pterin monomer line just as the slope of the pterin monomer line is steeper than that of the FAD line. Which means that there is a stronger interaction of the dimers with the column.

According to the above experiment, in order to calibrate the molecular weight of an unknown pterin from the gel filtration system we would have to use the correct *pterin* calibration line and we would have to know if the unknown pterin is a *monomer* or a *dimer* in order to decide whether to use the monomer or the dimer pterin line. There is the possibility, however, that an unknown pterin would not fit into any of the above discussed lines because of its special structural properties. In order to determine which line would be suitable for the calculation of the molecular weight of the halopterins, the molecular weight of the halopterin was compared with that of its acid hydrolysis product. The difference between the two molecular weights would be equal to the molecular weight of the groups that have been lost during the acid hydrolysis. This is known because of the structural

analysis of the halopterins. For example, the acid hydrolysis of SHP-2 causes it to lose one SO_3 (mwt 80) per pterin, i.e., mwt of SHP-2 - mwt of HP-2 = 80 if it is a monomer and mwt of SHP-2 - mwt of HP-2 = 160 if it is a dimer. In the case of PHP-1, two PO_3H (mwt 2 x 80 = 160) per pterin were lost during acid hydrolysis and thus the expected difference in molecular weight would be 160 for the monomer and 320 for the dimer. These differences in molecular weight can be used as a criterion for deciding on the suitability of a calibration line for the calculation of the molecular weight of a halopterin. The calculated molecular weights of the unknown pterins according to different calibration lines is summarized in Table 8 on page 112. As shown in the table, if we determine the molecular weight of SHP-2 from the monomer pterin line, its molecular weight would be 380 and the molecular weight of HP-2 would be 352. The difference in weights of 28 is too removed from the expected difference of 80 to be experimental error. This means that the pterin monomer line is not suitable for calibrating the molecular weight of SHP-2. This is also a evidence that SHP-2 is not a monomer. A similar argument can be used against using the monomer pterin line to determine the molecular weight of PHP-1 and its hydrolysis product HP-1. (A difference in molecular weight from the monomer pterin line of 72 is far removed from the expected difference of 160.)

When we used the pterin dimer line instead of the monomer line to calibrate the molecular weights of SHP-2 and HP-2, a difference of 79 resulted, which is also too great a difference from the expected 180. A similar argument can be used against using the pterin dimer line to calibrate the molecular weight of PHP-1 and HP-1 since the difference in molecular weights from the dimer line is 177, which is far from the expected 320.

From the above discussion, it is clear that none of the these pterin calibration lines are suitable for calculating the molecular weight of the unknown halopterins. But we can think about this problem in another way. As mentioned above, it is possible that the unknown halopterins formed their own line due to their special structural properties. Since we have good evidence in supporting of the proposed structures of SHP-2, HP-2, and SHP-OR (Chapter 3), we can calculate the molecular weight of these three compounds and construct a SHP-2 line. This line, which is shown in Figure 43 on page 110, shows that the SHP-2 series of compounds forms a separate straight line,

which, very interestingly, like that of pterin dimer line, intercepts with the pterin point. As mentioned above, a straight line was drawn from the proposed molecular weights of SHP-2, HP-2, and SHP-OR. Since a reasonably straight line was generated by these compounds, based on their proposed molecular weights, this can be used as evidence in support of the proposed structure. The molecular weight calculation for PHP-1 and HP-1 according to the SHP-2 line is shown in Table 8 on page 112. The table shows that the difference in molecular weight between PHP-1 and HP-1, as calculated by the SHP-2 line, is 298, which is very close to the expected value of 320 thereby making this the correct line for the molecular weight determination of PHP-1. The molecular weight of PHP-1 (966) calculated from the SHP-2 line is similar to the molecular weight (956) proposed in Chapter 4.

When all the above four lines are put into one figure (Figure 44 on page 111), we find an increase in the slope in going from the FAD line to the monomer pterin line to the dimer pterin line to the halopterin line. This figure also includes the calculated molecular weights of PHP-1 and HP-1 based on their proposed structures as discussed in Chapter 4. Very interestingly, all the pterin lines intercept at pterin which signatures the pterin nature of these compounds.

Pterins are a very special class of compounds. The uncharged pterins, i.e., neopterin and biopterin do not dissolve well in either water or organic solvents (Pfleiderer, 1985). The proton NMR spectrum of pterin in neutral aqueous solution has very poor resolution (Van Beelen et al., 1984a). The PMR of SHP-2 in D_2O also shows some very broad bands (data not shown) so that the NMR of pterins in D_2O have to be done in either acid or base. It is also hard to derivatize pterin in order to obtain a good mass spectrum (until now, all attempts to obtain a mass spectrum of the TMS derivative of HP-2 have failed). The special behavior of the pterins in gel filtration is another example of the special nature of pterins. The reasons for the formation of special lines by pterin and for the different kinds of pterins forming different lines are not known. However, this research has presented the first evidence of the special behavior of pterins in a Sephadex gel filtration system. The gel filtration experiment also provided support for the proposed structures of halopterins and solfapterins.

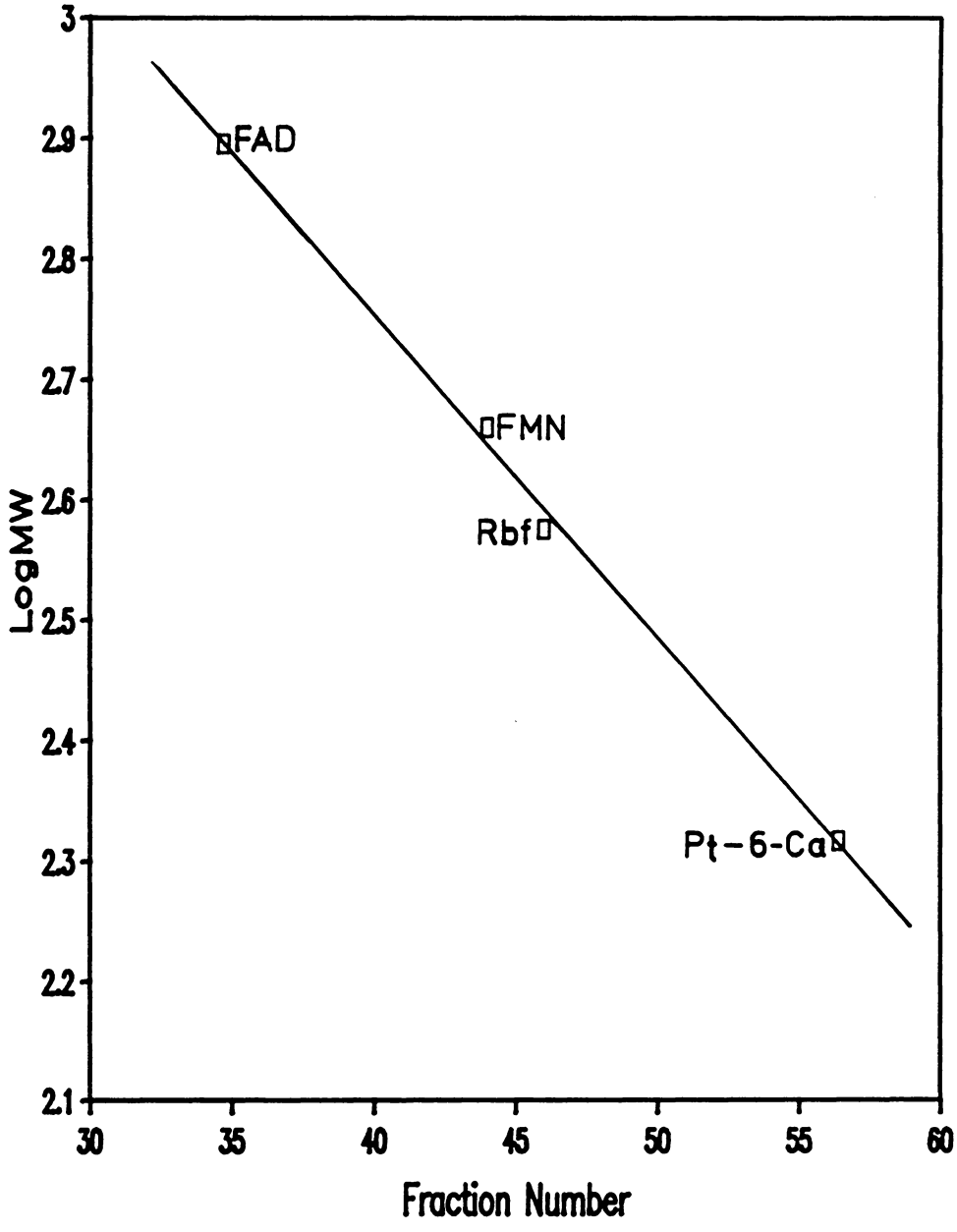


Figure 40. Molecular weights calibration curve of some standard compounds separated by Sephadex G-15-120: the FAD line. FAD, FMN, and riboflavin (Rbf) were monitored by their absorbance at 450 nm. Pterin 6-carboxylic acid (Pt-6-Ca) was monitored by its absorbance at 320 nm.

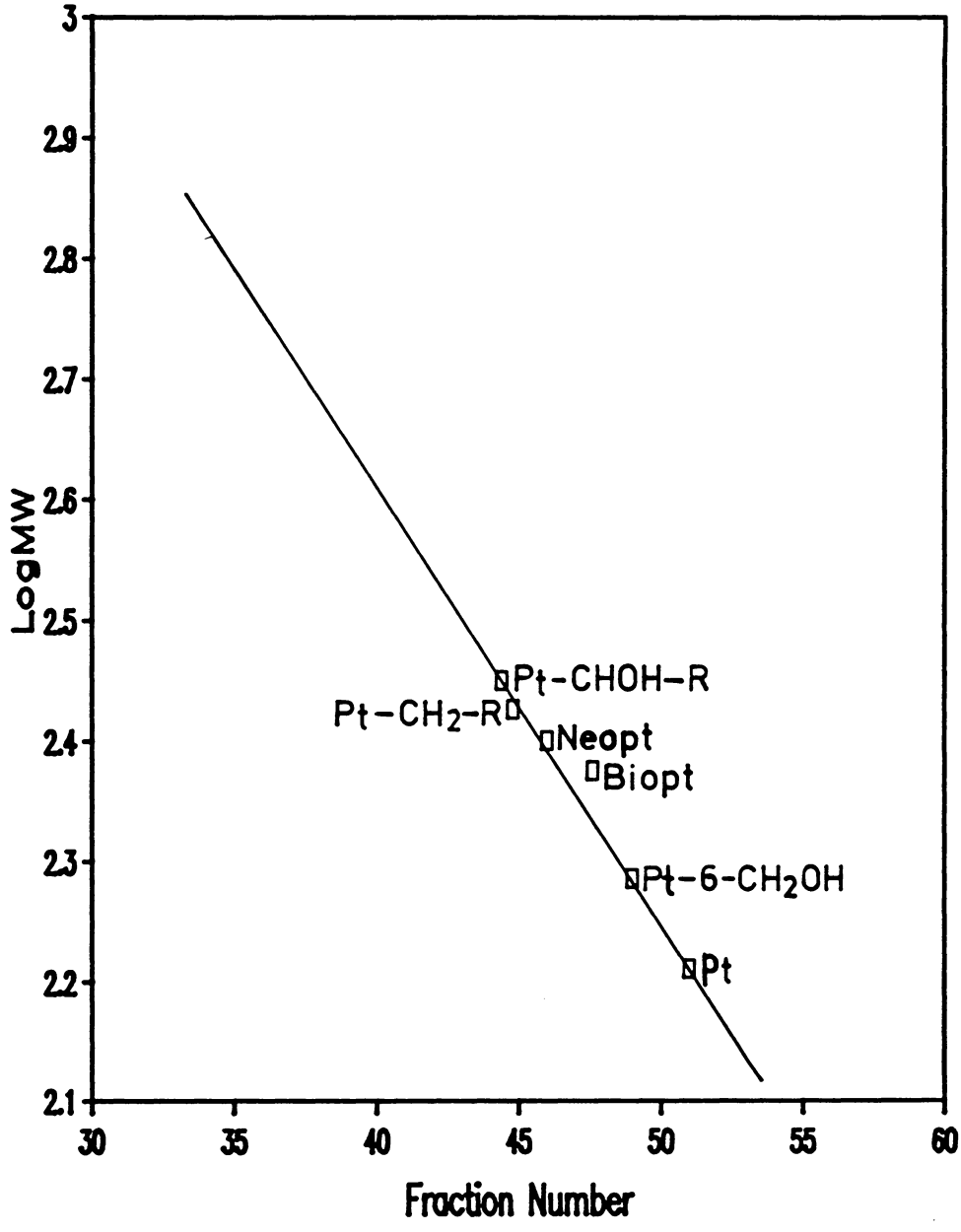


Figure 41. **Calibration curve of some standard pterin monomers:** the pterin monomer line. Pterin (Pt), 6-hydroxymethylpterin (Pt-6-CH_2OH), biopterin (Biopt), neopterin (Neopt), 2′,3′,4′-trihydroxybutylpterin (Pt-CH_2-R), and 1′,2′,3′,4′-tetrahydroxybutylpterin (Pt-CHOH-R) were monitored by their absorbance at 320 nm.

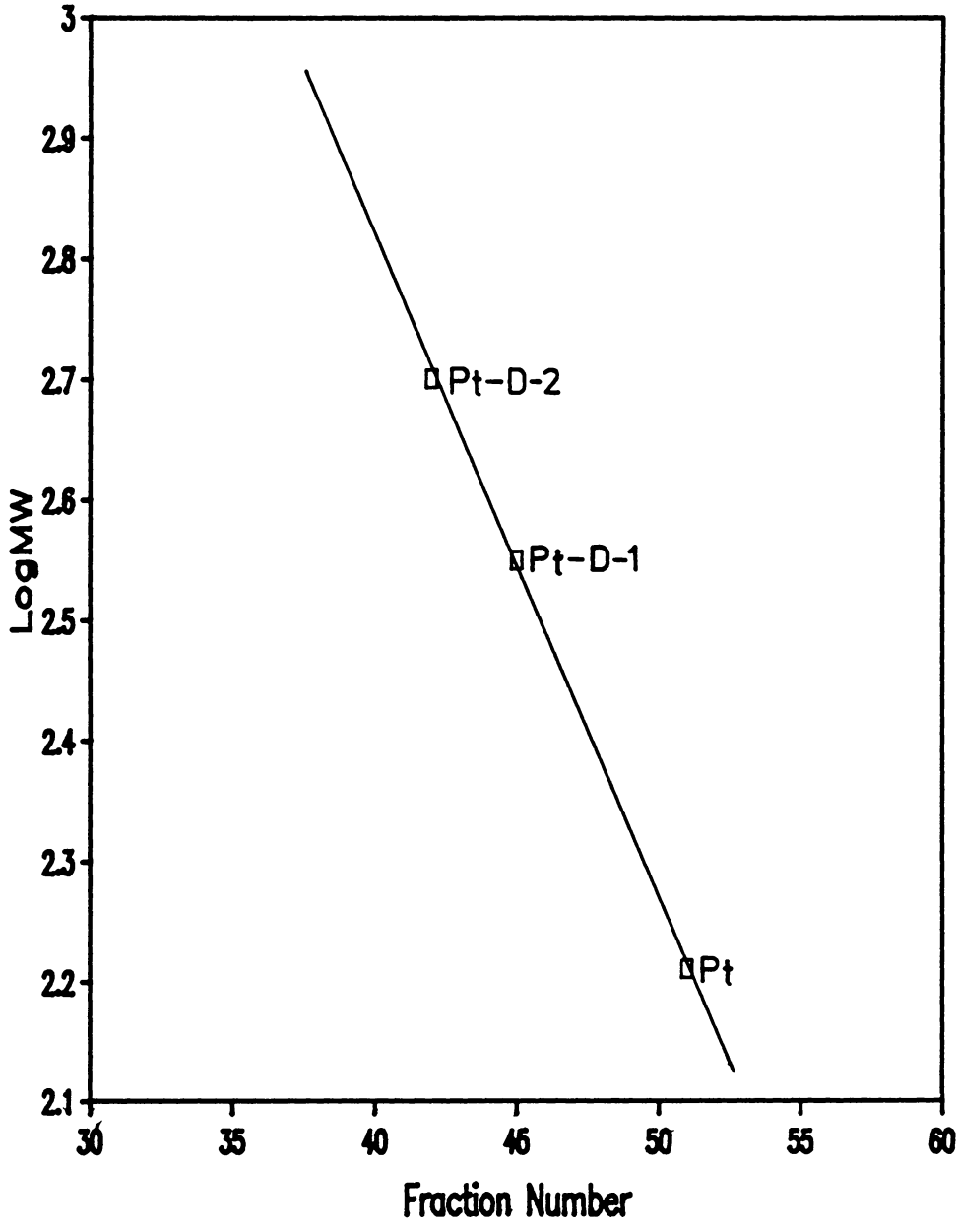


Figure 42. Calibration curve of standard pterin dimers: the pterin dimer line. Pterin (Pt), pterin dimer-1 (Pt-D-1), and pterin dimer-2 (Pt-D-2) were monitored by their absorbance at 320 nm.

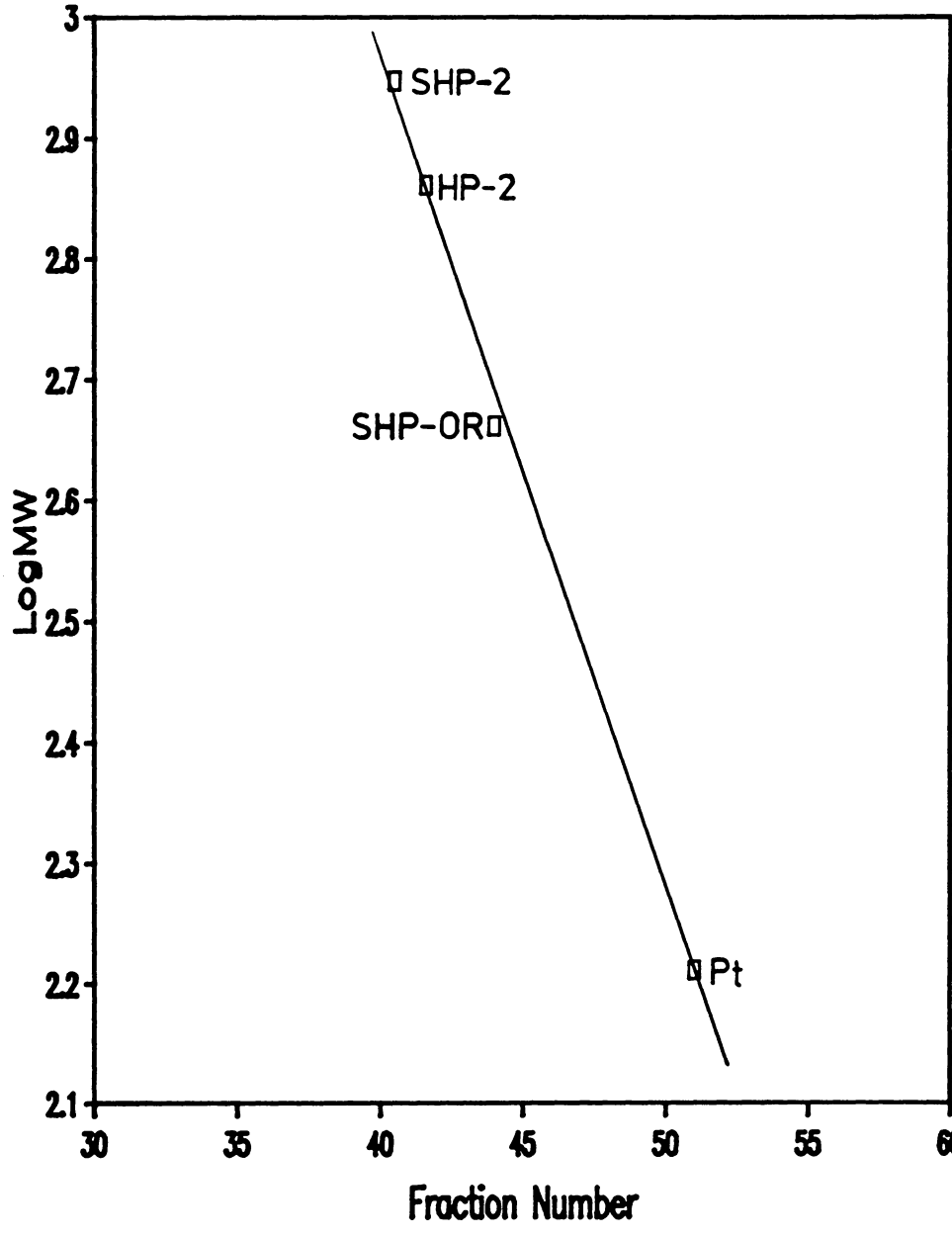


Figure 43. **Calibration curve of sulfohalopterin-2 series:** the SHP-2 line. Pterin (Pt), SHP-2, HP-2 and SHP-OR were monitored by their absorbance at 320 nm.

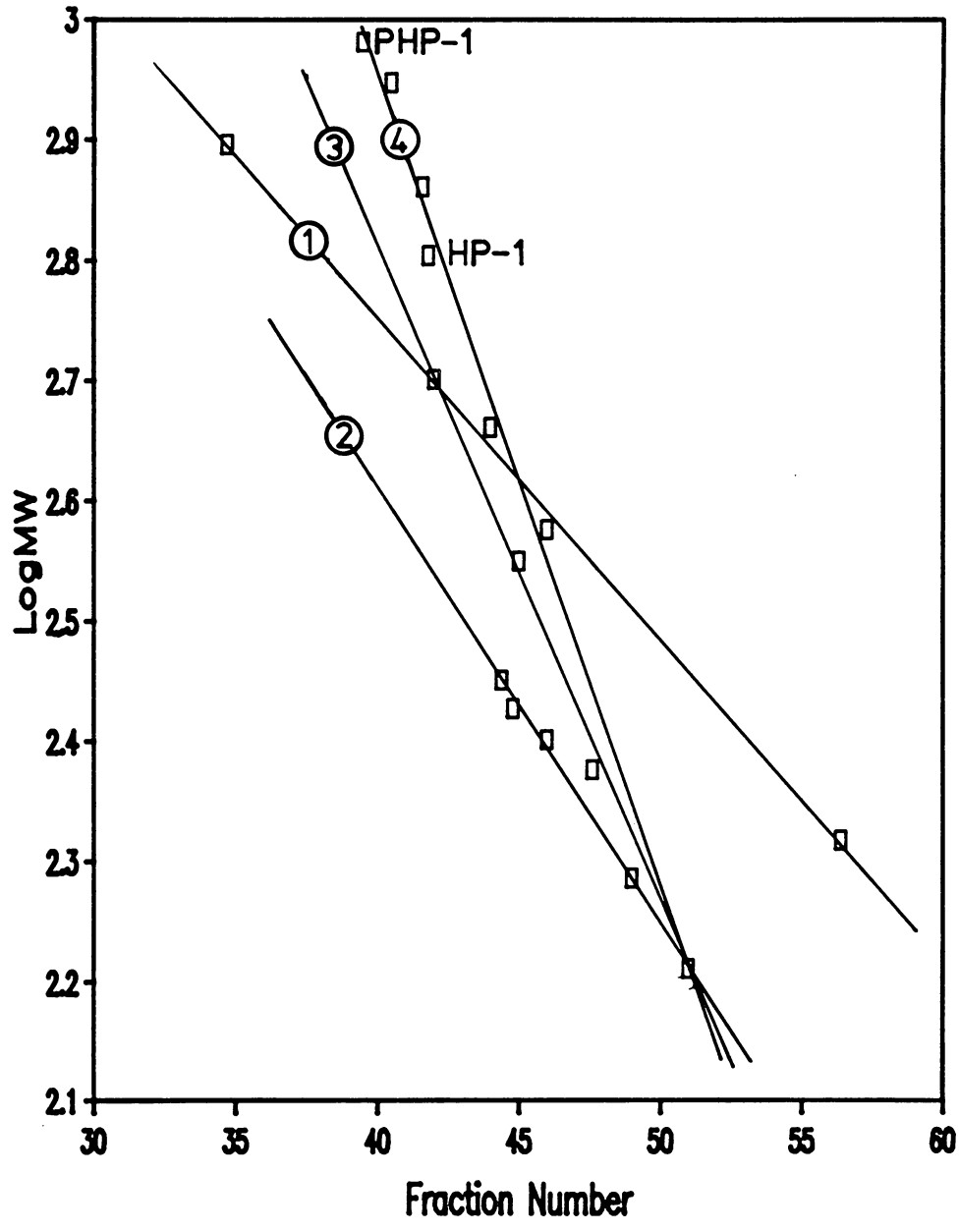


Figure 44. **Calibration curve collections:** (1) the FAD line, (2) the pterin monomer line, (3) the pterin dimer line, and (4) the SHP-2 line. PHP-1 and HP-1 are specifically indicated in the SHP-2 line because they have not appeared in the previous figures.

Table 8. Molecular Weights of Unknown Pterins Calculated from Different Calibration Curves

Name	Fraction Numbers	MW[1] (Monomer)	MW[2] (Dimer)	MW[3] (SHP-2)	MW[4]
SHP-2	40.5	383	611		884
HP-2	41.6	352	532		724
SHP-2 - HP-2		31	79		160
SHP-OR	44.0	290	396		456
PHP-1	39.4	420	698	966	956
HP-1	41.8	348	521	668	636
PHP-1 - HP-1		72	177	298	320
Solfapterin-Ac	39.5	415			

[1] Calculated from the standard pterin monomer calibration curve.

[2] Calibrated from the standard pterin dimer calibration curve.

[3] Calculated from the SHP-2 curve.

[4] Calculated from the proposed structure of the unknown pterins.

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