Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1973), *Biochemistry 12,* 2637.
Muller, P., and Rudin, D. O. (1968), *Nature (London) 217,* 713.
Payne, J. W., Jakes, R., and Hartley, B. S. (1970), *Biochem. J. 117,* 757.
Penkett, S. A., Flook, A. G., and Chapman, D. (1968), *Chem. Phys. Lipids 2,* 273.
Petersen, N. O., Lichtenberg, D., and Chan, S. I. (1974), manuscript in preparation.
Pressman, B. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27,* 1281.
Prestegard, J. H., and Fellmeth, B. (1974), *Biochemistry 13,* 1122.
Robles, E. C., and Van den Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.
Seelig, J., and Seelig, A. (1974), *Biochem. Biophys. Res. Commun. 57,* 406.
Seiter, C. H. A., and Chan, S. I. (1973), *J. Amer. Chem. Soc. 95,* 7541.
Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.

Studies on Motional Characteristics and Distribution of Protonated and Anionic Forms of Spin-Labeled 2,4-Dinitrophenol in Phospholipid Bilayer Membranes†

W. L. Chen and J. C. Hsia*

ABSTRACT: Spin-labeled 2,4-dinitrophenol (Dnp) compounds were used to study the mechanism of action of uncouplers. From an analysis of the electron spin resonance (esr) spectra, it is possible to distinguish and measure the protonated and anionic forms of spin-labeled Dnp in phospholipid bilayer membranes. The distribution studies indicate that both forms of the uncoupler are predominately localized at the polar head-group regions of the membrane. The relevance of these findings to Mitchell's chemiosmotic coupling hypothesis of oxidative phosphorylation is discussed.

Several hypotheses have been proposed to account for the uncoupling of mitochondrial oxidative phosphorylation by various uncouplers, and they vary depending on the mechanisms of oxidative phosphorylation the authors favor (Mitchell, 1968; Van Dam and Slater, 1967; Green and Baum, 1970; Wilson *et al.*, 1971; Weinbach and Garbus, 1969). According to the chemiosmotic hypothesis proposed by Mitchell (1968), the electron transport along the respiratory chain generates a transmembrane proton gradient, which is the driving force for the enzymatic synthesis of ATP. Uncoupling agents such as Dnp[1]act as lipid-soluble proton donor–acceptor systems that dissolve in the lipid phase of the membrane and conduct backdiffusion of protons, thus collapsing the proton gradient across the inner mitochondrial membrane. Supporting evidence for such a mechanism is mainly derived from the fact that usually there is a good correlation between the ability of compounds to uncouple and their effectiveness in increasing proton conductance in phospholipid bilayer membranes (Liberman *et al.*, 1969; Hopfer *et al.*, 1968).

However, in order to provide for direct molecular evidence and to evaluate Mitchell's uncoupling hypothesis more fully, one would like to be able to measure the amounts of protonated and anionic forms of the uncoupler present in the lipid phase of the membrane. Furthermore, one would like to know the distribution and diffusibility of protonated and ionized forms of the uncoupler across the phospholipid bilayers. Toward these goals we have prepared several spin-labeled Dnp compounds so that the behavior of the uncoupler within the membrane can be directly monitored (Hsia *et al.*, 1972a,b). In the present communication, data are presented showing that it is possible to distinguish and measure the protonated and anionic forms of spin-labeled Dnp in phospholipid bilayer membranes. The distribution studies indicate that both forms of the uncoupler are predominately localized at the polar head-group regions of the membrane.

Experimental Section

Materials. Spin-labeled 2,4-dinitrophenol compounds [1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4-dinitrobenzene, Dnp-SL(5); and 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminomethylpyrrolidinyl)-2,4-dinitrobenzene; Dnp-methylene-SL(5)] were prepared as described (Hsia *et al.*, 1972b). The chemical structures are shown in Figure 1. The p*K* values were determined by a spectrophotometric method and found to be 4.8 for Dnp-SL(5) and 5.6 for Dnp-methylene-SL(5) (Hsia *et al.*, 1972b). The spin-labeled Dnp molecules possess full activity in the uncoupling of oxidative phosphorylation in rat-liver mitochondria (Hsia *et al.*, 1972b). Egg lecithin was purchased from Pierce Chemical Co. The phospholipid was

† From the Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 1A8. *Received May 20, 1974.*

[1] Abbreviations used are: Dnp, 2,4-dinitrophenol; Dnp-SL(5), 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4-dinitrobenzene; Dnp-methylene-SL(5), 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminomethylpyrrolidinyl)-2,4-dinitrobenzene.

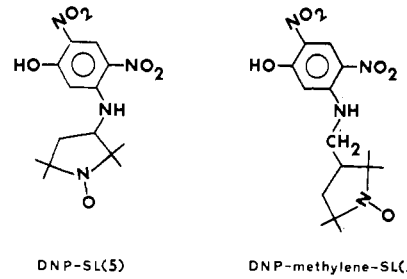

FIGURE 1: The structures of spin-labeled Dnp compounds.

pure by the criterion of thin-layer chromatography and was used without further purification.

Methods. Planar phospholipid multibilayers containing Dnp-SL(5) or Dnp-methylene-SL(5) (phospholipid to spin-label ratio of 150:1) were prepared on the inner surfaces of a flat quartz cell, dimensions 4 cm × 1 cm × 0.25 mm (Hsia *et al.*, 1970). The multibilayers were hydrated with isotonic buffer, pH 2.2 and 7.4 (Long, 1961). Liposomes were prepared by hand dispersion of a dry film of phospholipid and Dnp-SL(5) in isotonic buffer (pH 2.2 and 7.4) to final concentrations of 15 mM and 50 μM, respectively (Hsia *et al.*, 1972a).

For electron spin resonance measurements, 50 μl of each liposome preparation was taken up in a disposable glass micropipet at 22°. All spectra were recorded on a Varian X-band E-6 esr spectrometer.

Results

Resonance Spectra of Dnp-SL(5) and Dnp-methylene-SL(5) in Phospholipid Bilayer Membrane. In the previous publication (Hsia *et al.*, 1972a) we have shown that spin-labeled Dnp is localized in the polar head-group regions of phospholipid bilayers and exhibits rapid anisotropic motion. Furthermore, similar motion of the spin-labeled Dnp in intact mitochondria and the inner mitochondrial membrane was detected (Hsia *et al.*, 1972a). To further understand the orientation of spin-labeled Dnp in membranes, we have measured the resonance spectra of Dnp-SL(5) and Dnp-methylene-SL(5) in ordered phospholipid bilayers. The spectra of Dnp-SL(5) in hydrated phosphatidylcholine multibilayers (excess buffer phase removed) are shown in Figure 2A. A detailed analysis and discussion of these spectra have been previously reported (Hsia *et al.*, 1972a). The maximum (minimum) hyperfine splitting was observed when the normal to the plane of the bilayers was parallel (perpendicular) to the laboratory magnetic field direction, the values being $T_{\parallel}' = 23.0$ G and $T_{\perp}' = 10.9$ G, respectively. These results establish that the label undergoes rapid anisotropic motion in the bilayers and that the plane of the nitroxide pyrrolidine ring (McConnell and McFarland, 1970) is preferentially aligned parallel to the plane of the bilayers, since the largest hyperfine splitting is observed along this direction. In order to establish whether the observed motional characteristics of Dnp-SL(5) in membrane is due to the orientation of the Dnp or the nitroxide moiety in the bilayers, we have measured the resonance spectra of Dnp-methylene-SL(5) in planar multibilayers shown in Figure 2B. The values of $T_{\parallel}'$ and $T_{\perp}'$ obtained from spectra of Dnp-methylene-SL(5) were 16.9 and 14.6 G, respectively. The esr spectral data of Dnp-SL(5) and Dnp-methylene-SL(5) in phospholipid multibilayers are summarized in Table I. The derived order parameters, $S_3 = 0.09$ for Dnp-methylene-SL(5) as contrasted to $S_3 = 0.45$ for Dnp-

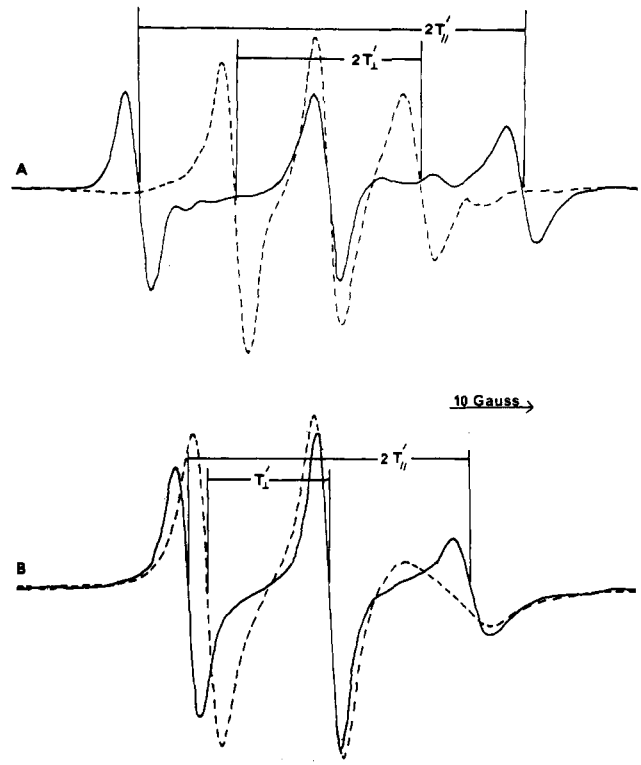

FIGURE 2: Esr spectra of Dnp-SL(5) (A) and Dnp-methylene-SL(5) (B) in hydrated planar phosphatidylcholine multibilayers, after draining the aqueous phase. Spectra were recorded with the normal of the plane of the bilayers parallel (—) and perpendicular (- - -) to the applied magnetic field.

SL(5), indicate that the nitroxide of Dnp-methylene-SL-(5) is poorly oriented in the bilayers, while the label of Dnp-SL(5) is very well oriented. We attribute the observed differences to the rigidity of the linkages between Dnp and the spin-label. In the case of Dnp-SL(5), the amine linkage between Dnp and the nitroxide exhibits some "double-bond" character, thus holding the Dnp and the label rigidly to each other. However, for Dnp-methylene-SL(5), the label can freely rotate with respect to Dnp due to the presence of the methylene group. This implies that the preferential orientation of Dnp-SL(5) in the membrane is due to the anchoring of the Dnp in the polar head-group region of the bilayers and not because of the spin-label moiety *per se*.

The Motional Characteristics of Protonated and Anionic Forms of Dnp-SL(5) in Lipid Membranes. To understand the motional characteristics and distribution of protonated and anionic forms of the uncoupler in the membrane, we

TABLE I: Esr Spectral Parameters of Dnp-SL(5) and Dnp-methylene-SL(5) in Planar Phosphatidylcholine Bilayers at pH 7.4.

Spin-Label	$T_{\parallel}'$	$T_{\perp}'$	S_3[a]
Dnp-SL(5)	23.0	10.9	0.45
Dnp-methylene-SL(5)	16.9	14.6	0.09

[a] The degree of anisotropic motion of the nitroxide may be described in terms of an order parameter S_3 (Seelig, 1970; Hubbell and McConnell, 1971) $S_3 = (T_{\parallel}' - T_{\perp}')/(T_{zz} - T_{xx})$, where T_{zz} and T_{xx} are the hyperfine tensor components of the nitroxide calculated as described by Seelig (1970).

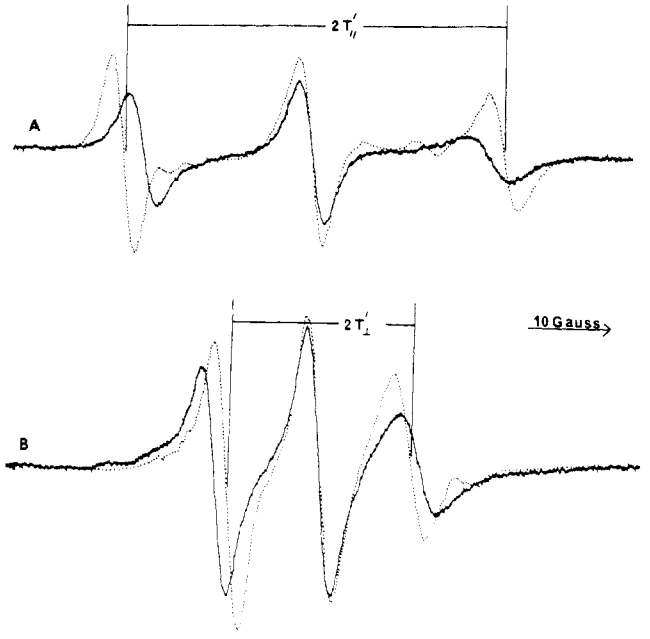

FIGURE 3: Esr spectra of protonated and anionic forms of Dnp-SL(5) in hydrated planar phosphatidylcholine multibilayers, after draining the aqueous phase. Spectra were recorded with the normal of the plane of the bilayers parallel (A) and perpendicular (B) to the applied magnetic field. At pH 2.2 (—) and pH 7.4 (. . .).

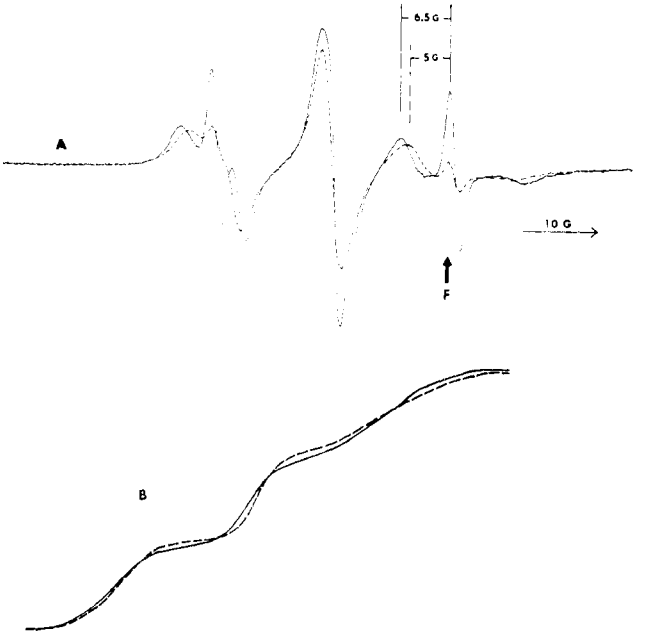

FIGURE 4: (A) Esr spectra of protonated and anionic forms of Dnp-SL(5) in an aqueous dispersion of phosphatidylcholine liposomes. At pH 2.2 (- - -) and pH 7.4 (—). (B) Total spin density from double integration of curves (A). At pH 2.2 (- - -) and pH 7.4 (—).

have determined the resonance spectra of Dnp-SL(5) in phospholipid bilayers and liposome preparations at pH 2.2 and 7.4. The pK value of the phenolic group of Dnp-SL(5) has been previously determined to be 4.8 (Hsia *et al.*, 1972b). Thus at pH 2.2 and 7.4, the spin-labeled Dnp compound was predominately in the protonated and the anionic forms, respectively. The spectra of protonated and anionic forms of Dnp-SL(5) in hydrated phosphatidylcholine multibilayers (excess buffer phase removed) are shown in Figure 3 and the esr parameters are summarized in Table II. The results clearly demonstrate that the hyperfine splittings $T_{\parallel}'$ and $T_{\perp}'$ of protonated and anionic forms of Dnp-SL(5) in phospholipid bilayers are different. The derived order parameters $S_3 = 0.33$ for the protonated form and 0.45 for the anionic form of Dnp-SL(5) indicate that there is more motional restriction of the ionized uncoupler at the polar head-group region of the phospholipid bilayers, presumably due to the anchoring effect of the anionic phenolic group at the membrane–water interface. We attribute the observed changes in hyperfine splittings to the degree of ionization of the uncoupler and not to the ordering of phosphatidylcholine because we have previously shown that pH ranging from 2.2 to 9.7 has no effect on the orientation of 3-doxylcholestane in phosphatidylcholine bilayers (Hsia and Boggs, 1972).

TABLE II: Esr Spectral Parameters of Dnp-SL(5) in Phosphatidylcholine Multibilayers at pH 2.2 and 7.4.

Dnp-SL(5)	T'	$T_{\perp}'$	S_3
Protonated form (pH 2.2)	20.9	12.4	0.33
Anionic form (pH 7.4)	23.0	10.9	0.45

Distribution of Protonated and Anionic Forms of Dnp-SL(5) in Lipid Membranes. In order to determine the distribution of the two forms of Dnp-SL(5) in the phospholipid bilayers, we have measured the resonance spectra of Dnp-SL(5) in egg phosphatidylcholine liposomes suspended in buffer at pH 2.2 and 7.4 (Figure 4A). A detailed analysis of the spectra has been discussed in an earlier publication (Hsia *et al.*, 1972a). The "free" spin-label in the buffer phase (indicated by F) is four times greater when the uncoupler changes from the protonated to the anionic form. This change in the partition of Dnp-SL(5) between lipid membrane and the buffer phase is anticipated since the anionic form with its negative charge would become more soluble in the aqueous phase and less in the membrane phase. We have double integrated the two Dnp-SL(5) spectra (Figure 4A) to ensure that the total spin density solubilized in the membrane and the buffer phase is the same for the two forms of the uncoupler (Figure 4B). Since the high-field line of Dnp-SL(5) in the aqueous phase is distinctly separated from the membrane-bound peaks (Figure 4A), we have used its intensity in the presence and absence of membrane phase to estimate the partition coefficient of Dnp-SL(5). The measured partition coefficients for Dnp-SL(5) between phosphatidylcholine and buffer are 2000 for the protonated form and 475 for the anionic form at 22°. These partition coefficients suggest that the protonated form of Dnp-SL(5) is four times more soluble in the membrane phase than the anionic form. To discern the distribution of the two forms of Dnp-SL(5) between the polar and apolar regions of the phospholipid bilayers, we measured the partition coefficients of Dnp-SL(5) between *n*-hexane and buffer (pH 2.2 and 7.4), assuming that the apolar regions of the bilayers have the same hydrophobic characteristics as hexane. The partition coefficients of Dnp-SL(5) between buffer and *n*-hexane are 5.6 for protonated form and 0.05 for the anionic form as determined from the decrease in esr signal intensity in the buffer phase after equilibration with equal volume of *n*-hexane. The high solubility

of the anionic form of Dnp-SL(5) in hexane, *i.e.*, partition coefficient of 0.05 as compared to the calculated value of approximately 0.01, may be due to the presence of a secondary amine group on Dnp-SL(5) which can serve as a lipid soluble countercation. Nevertheless, the difference in the partition coefficients in phospholipids and *n*-hexane suggests that at any given time more than 95% of both forms of the membrane-soluble Dnp-SL(5) is located in the polar region of the phospholipid bilayers, presumably due to favorable dipole–dipole interactions between Dnp and the polar head groups of the phospholipids.

Discussion

The object of the present research is to provide a molecular description of uncoupling by Dnp as proposed by Mitchell (1968). According to the chemiosmotic hypothesis, uncouplers such as Dnp act as lipid-soluble proton donor–acceptor systems that dissolve in the lipid phase of the membrane and conduct backdiffusion of protons. In order to examine this model closely, we would like to know the amounts of protonated *vs.* anionic forms of the uncoupler in the lipid region of the mitochondrial membrane prior and after energization. Furthermore, we would like to know the distribution and diffusibility of protonated and anionic forms of Dnp in polar and apolar regions of phospholipid membrane. This communication describes in detail the distribution and motional characteristics of protonated and anionic forms of spin-labeled Dnp in phospholipid bilayer membranes.

The resonance spectra of Dnp-SL(5) in planar phosphatidylcholine multibilayers (Figure 2A) indicate that the nitroxide moiety of the label (and probably the entire molecule) undergoes rapid anisotropic motion in the bilayers and that the plane of the nitroxide pyrrolidine ring is preferentially parallel to the plane of the bilayers. In order to find out whether the observed orientation is due to Dnp or the nitroxide moiety, we have measured the motional characteristics of Dnp-methylene-SL(5) in bilayers. The results show that the nitroxide of Dnp-methylene-SL(5) is poorly oriented in the bilayers (Figure 2B), presumably due to lack of rigidity of the linkage between Dnp and the spin-label moiety. These results establish that the orientation of the label is largely due to (a) the localization of the Dnp in the order polar head-group region of the phosphatidylcholine bilayer and (b) the rigidity of the intramolecular linkage of Dnp-SL(5).

The motional characteristics of protonated and anionic forms of Dnp-SL(5) in phospholipid bilayers and liposome suspensions were subsequently investigated. The results demonstrate that the orientation of Dnp-SL(5) in the membrane is dependent on the degree of ionization of the phenolic group of Dnp. There appears to be more motional restriction of the anionic uncoupler at the polar head-group region of phospholipid bilayers, presumably due to the anchoring effect of the anionic phenolic group at membrane–water interface and the intrinsic intramolecular configuration of Dnp-SL(5). The partition studies in liposome dispersions also indicate that the membrane-partition of protonated Dnp-SL(5) is fourfold higher than the anionic form. The differences in resonance spectra and partition coefficients of protonated and anionic forms of Dnp-SL(5) in membranes strongly suggest that it is possible to quantitate the two forms of the uncoupler within membranes through an analysis of the esr spectra. This finding is especially relevant to the evaluation of Mitchell's hypothesis. In principle we should be able to observe a change in the resonance spectra of spin-labeled Dnp in the membrane as well as a decrease in the membrane partition upon energization of the mitochondrial membrane consequent to a decrease in pH inside the mitochondria. Indeed it is our goal to make use of the different membrane properties of protonated and anionic forms of spin-labeled Dnp to detect the generation of proton gradient within the membrane[2] by employing the reconstituted oxidative phosphorylation system developed by Racker and coworkers (Racker and Kandrach, 1971; Kagawa and Racker, 1971; Hinkle *et al.*, 1972). In addition, this system will enable us to rigorously test Mitchell's hypothesis of uncoupler action. This research is currently in progress.

The partition studies indicate that both protonated and anionic forms of Dnp-SL(5) are predominately distributed in the polar head-group region of the membrane. This result suggests that Dnp may exert its action mainly at the polar membrane–water interphase. The partition coefficient of 475 deduced for the anionic form into membranes is consistent with previous observations that Dnp adsorbs to phospholipid bilayer membranes and produces a negative surface potential (McLaughlin, 1972). In a previous publication, we have observed that spin-labeled Dnp binds to both mitochondrial membrane protein(s) and phospholipids (Hsia *et al.*, 1972a). The possibility that the uncoupler exerts its action on membrane lipoprotein(s) is currently being investigated.

Acknowledgments

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References

Green, D. E., and Baum, H. (1970), Energy and the Mitochondrion, New York, N. Y., Academic Press.

Hinkle, P. C., Kim, J. J., and Racker, E. (1972), *J. Biol. Chem. 247,* 1338.

Hopfer, U., Lehninger, A. L., and Thompson, T. E. (1968), *Proc. Nat. Acad. Sci. U. S. 59,* 484.

Hsia, J. C., and Boggs, J. M. (1972), *Biochim. Biophys. Acta 266,* 18.

Hsia, J. C., Chen, W. L., Long, R. A., Wong, L. T., and Kalow, W. (1972a), *Proc. Nat. Acad. Sci. U. S. 69,* 3412.

Hsia, J. C., Chen, W. L., Wong, L. T., Long, R. A., and Kalow, W. (1972b), *Biochem. Biophys. Res. Commun. 48,* 1273.

Hsia, J. C., Schneider, H., and Smith, I. C. P. (1970), *Biochim. Biophys. Acta 202,* 399.

Hubbell, W. L., and McConnell, H. M. (1971), *J. Amer. Chem. Soc. 93,* 314.

Kagawa, Y., and Racker, E. (1971), *J. Biol. Chem. 246,* 5477.

Liberman, E. A., Topaly, V. P., Tsofina, L. M., Jasaitis, A. A., and Skulachev, V. P. (1969), *Nature (London) 222,* 1076.

[2] The most crucial objection to the chemiosmotic coupling hypothesis is that there is no evidence for the existence of a proton gradient of sufficient magnitude across the mitochondrial membrane, except in the case where valinomycin is present (Van Dam and Meyer, 1971).

Long, C. (1961), Biochemists Handbook, New York, N. Y., Van Nostrand, pp 30–40.
McConnell, H. M., and McFarland, B. G. (1970), *Quart. Rev. Biophys. 3,* 91.
McLaughlin, S. (1972), *J. Membrane Biol. 8,* 361.
Mitchell, P. (1968), Chemiosmotic Coupling and Energy Transduction, Bodmin, Cornwall, Glynn Res. Ltd.
Racker, E., and Kandrach, A. (1971), *J. Biol. Chem. 246,* 7069.
Seelig, J. (1970), *J. Amer. Chem. Soc. 92* 3881.
Van Dam, K., and Meyer, A. J. (1971), *Annu. Rev. Biochem. 40,* 115.
Van Dam, K., and Slater, E. C. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 2015.
Weinbach, E. C., and Garbus, J. (1969), *Nature (London) 221,* 1016.
Wilson, D. F., Ting, H. P., and Koppelman, M. S. (1971), *Biochemistry 10,* 2897.

The Identification of Poly-γ-glutamyl Chain Lengths in Bacterial Folates†

Charles M. Baugh,* Eleanor Braverman, and M. G. Nair

ABSTRACT: Mild procedures have been developed for the extraction of folyl polyglutamates in 90% yield. These have been applied to three strains of folic acid requiring bacteria (*Lactobacillus casei* 7469, *Streptococcus faecium* 8043, and a methotrexate-resistant strain of 8043) grown on a defined medium supplemented with folic acid labeled in the glutamyl moiety with ^{14}C. To minimize the heterogeneity of the folates conferred by the pteridine ring, the state of reduction, and position or nature of single carbon substituents, the crude extracts have been subjected to a reductive procedure which cleaves the C^9–N^{10} bond. This generated a homologous series of *p*-aminobenzoylglutamyl poly-γ-glutamates which have been identified by DEAE-cellulose cochromatography with authentic compounds synthesized in this laboratory. In summary, the following distributions have been found (the number in parentheses refers to the total number of glutamates): *L. casei:* (1) 3.2%; (2) 0%; (3) 9.3%; (4) 59.4%; (5) 23%; (6) 5.1%; (7) 0%. *S. faecium:* (1) 16.5%; (2) 8.5%; (3) 20%; (4) 54.5%; (5) 0%; (6) 0%; (7) 0%. *S. faecium*-MR: (1) 1%; (2) 5.7%; (3) 6.9%; (4) 81.3%; (5) 5.1%; (6) 0%; (7) 0%. The derivative with four glutamates predominates in all the strains examined.

The folates are a family of closely related chemical structures whose number may exceed 140 members (Baugh and Krumdieck, 1971). Of this number perhaps 30 structures have been identified by one criterion or another (Shiota, 1970). In recent years, the availability of the poly-γ-glutamates of folic acid by chemical synthesis (Krumdieck and Baugh, 1969; Meienhofer and Jacobs, 1970) and increasing evidence of a functional coenzymatic role for them (Large and Quayle, 1963; Guest and Jones, 1960; Whitfield and Weissbach, 1968; Burton *et al.*, 1969) have focused considerable attention upon these naturally occurring peptides. Numerous reports have appeared in the recent literature assigning structures to the poly-γ-glutamates of folic acid from a variety of biological specimens (Buehring *et al.*, 1974; Shin *et al.*, 1972a,b; Osborne-White and Smith, 1973; Corrocher *et al.*, 1972). The majority of these reports describe techniques of Sephadex gel filtration and/or DEAE-cellulose chromatography, and differential microbiological assays before and after deconjugation with a variety of crude pteroylglutamyl-γ-polyglutamyl hydrolases to identify and assign structures to the various folates. At least two investigators have employed more direct methods (Houlihan and Scott, 1972; Curthoys *et al.*, 1972). In this laboratory we have repeatedly observed that the folates behave abnormally on Sephadex gels, with chain lengths below the tri- eluting much later than one would predict based on molecular weight, apparently due to adsorptive properties conferred by the pteridine. In addition, their unusual behavior decreases as the polyglutamyl chain is lengthened in the fully oxidized folates. It also appears unlikely that details of structure such as the state of reduction and nature of single-carbon substituents could be assigned unequivocally by this technique since the molecular weight changes are minimal and the changes in adsorptive properties due to reduction and substitution of single-carbon units on the pteridine are not predictable. Similarly, the use of DEAE-cellulose to identify the natural folates has not been feasible in this laboratory. For example, cirtovorum factor (N^5-formyl-5,6,7,8-tetrahydrofolic acid) is eluted along with pteroylglutamyl-(glutamyl)$_3$-glutamic acid (Pte-G_5)[1] from columns of DEAE-cellulose under a variety of elution conditions. Similar unexpected elution characteristics have also been noted to exist between the fully and partially reduced pteroyl polyglutamates (Kisliuk *et al.*, 1974). These workers showed that adding five glutamyl residues to tetrahydrofolic acid does not alter its affinity for DEAE-cellulose as much as does converting it to dihydrofolic acid.

It appeared that this multiplicity of problems could be greatly simplified if the heterogeneity of this large group of

† From the Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Alabama 36688. *Received June 24, 1974.* Principal support for these investigations was received from the American Cancer Society Grant CI-86-O and National Institutes of Health Grant 1-RO1-CA-16048.

[1] Abbreviations used are: Pte-G_5, pteroylglutamyl-(glutamyl)$_3$-glutamic acid; pABG$_n$, *p*-aminobenzoyl polyglutamate.