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Sugar Transport by the Bacterial Phosphotransferase System

RADIOACTIVE AND ELECTRON PARAMAGNETIC RESONANCE LABELING OF THE *SALMONELLA TYPHIMURIUM* PHOSPHOCARRIER PROTEIN (HPr) AT THE NH_2-TERMINAL METHIONINE*

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Investigation of the complex interactions between the different proteins of the bacterial phosphotransferase system (PTS) would be greatly facilitated by the availability of specifically labeled proteins, such as the low molecular weight phosphocarrier HPr. *Salmonella typhimurium* HPr contains two methionine residues, one at the NH_2 terminus, and the other at amino acid residue 80. The method of Link and Stark (Link, T. P., and Stark, G. R. (1968) *J. Biol. Chem.* 243, 1082-1088) was used to alkylate HPr with [^{3}H]methyl iodide, and with the EPR probe, 3-[(2-bromoacetamido)methyl]-2,-2,5,5-tetramethyl-1-pyrrolidinyloxyl. The radiolabeled HPr was purified to apparent homogeneity, and was as active in phosphorylation assays *in vitro* as native HPr. The EPR-labeled HPr was isolated in better than 95% purity, contained no native HPr, and showed 80 ± 7% of the activity of the native protein. The specific activity of the radiolabeled protein indicated that it contained one [^{3}H]methyl group/mol of protein. Both proteins were treated with a *Staphylococcus aureus* protease, and the expected peptides were isolated by high pressure liquid chromatography. Amino acid (and ^{3}H) analyses showed that only the NH_2-terminal methionine had been derivatized in each case. EPR spectra were obtained from the labeled HPr at different temperatures and solvent viscosities. The motion of the nitroxide group in the derivative can best be described as a combination of internal flexibility of the label or of the NH_2 terminus of the protein superimposed on rotational motion of the protein as a whole. Since the EPR probe is close to the active site histidine (residue 15), it seems possible that the EPR-labeled HPr will give increased rotational correlation times in the presence of other PTS proteins, such as Enzyme I and III^{Glc}, despite the segmental flexibility, and preliminary experiments indicate this to be the case.

The accompanying reports (1, 2) describe the isolation, characterization, and primary amino acid sequence of the protein HPr[1] from *Salmonella typhimurium*. This protein acts as a phosphocarrier in the bacterial phosphotransferase system, and the physiological behavior of mutants defective in HPr suggests that it serves diverse functions *in vivo*.

Current studies in this laboratory are concerned not only with characterizing the individual components of the PTS, but also with their interactions *in vitro* and in natural membrane vesicles. For these experiments, it is advantageous to use HPr and other proteins specifically labeled in such a manner that we can examine properties of the proteins as they interact with one another before and during the phosphotransfer reactions. Ultimately, a complete understanding of the PTS will require study of the interactions of pairs of proteins, such as HPr and Enzyme I, or HPr and III^{Glc} (or II-A^{Man}), groups of three proteins (*e.g.* Enzyme I, HPr, and III^{Glc}, or HPr, III^{Glc}, and II-B^{Glc}), or of all four proteins plus and minus lipid and divalent cation. In addition, information on interactions between the corresponding phosphoproteins will be sought. Clearly, HPr is central to these studies since it lies between the initial and final steps in the phophotransfer pathway. For a variety of experiments, different types of probes covalently linked to HPr are required. For example, we have consistently noted that small quantities of HPr bind to membranes, quantities so small that accurate assay by the sugar phosphorylation method is very difficult. For this type of experiment, radiolabeled HPr would provide the necessary sensitivity and accuracy. Similarly, radiolabeled HPr would be very useful for determining binding constants to other PTS proteins by the gel filtration method (4). On the other hand, electron paramagnetic resonance-labeled HPr or the corresponding fluorescent-labeled protein (5) should provide much more detailed information concerning some of the interactions between HPr and other PTS components. While the fluorescent derivative will be used to study interactions between soluble components of the system, the EPR labeled material can be inserted into natural membrane vesicles (6) and should provide information on the state of HPr during sugar transport by the vesicles.

Two essential properties of the labeled proteins are (*a*) that they are active in the PTS, retaining the phosphocarrier function of the native protein; and (*b*) that the probes are located at specified amino acid residues, rather than distributed at random in the polypeptide chain, so that spectral changes can be correctly interpreted.

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[1] The abbreviations used are: HPr, histidine-containing phosphocarrier protein of the phosphotransferase system; PTS, phosphoenolpyruvate:glycose phosphotransferase system. EPR, electron paramagnetic spin resonance. The designations of the other components of the phosphotransferase system are described in Fig. 1 of Ref. 3. Unless otherwise stated, all sugars are of the D configuration and glycosides are pyranosides; amino acids are of the L configuration.

This paper describes the preparation of two derivatives of HPr, one containing a radiolabeled methyl group and the other an EPR probe. The methods of labeling were based on the procedures of Gundlach *et al.* (7) and Link and Stark (8). This technique consists of converting the thioether in methionine to a sulfonium ion by reaction with an alkyl halide, and it has been used to prepare spin-labeled cytochrome *c* (9). Since *S. typhimurium* HPr contains only 2 methionine residues, one located at the NH_2 terminus, and the other at residue 80 (84 total residues), it seemed possible that this procedure would give limited derivatization with retention of biological activity. In fact, the derivatives retained 80–100% of the biological activity of the native protein. The spin-labeled HPr derivative was used for EPR measurements and an analysis of these results is presented. An accompanying paper describes a similar fluorescent derivative of HPr (5).

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Methyl iodide was purchased from New England Nuclear; specific activities ranged from 50–100 μCi/μmol. The EPR probe, 3-[(2-bromoacetamido)methyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was obtained from the Syva Company (Palo Alto, CA). Homogeneous *S. typhimurium* HPr was prepared as described in the accompanying report (1).

All other commercial reagents and solvents were of the highest purity available. Glycerol, used for viscosity experiments, was Eastman Spectrograde quality.

General Methods for PTS Proteins—The described procedures (1, 3) were used for the measurement of protein, determination of HPr activity in the phosphotransferase assay, analytical polyacrylamide disc gel electrophoresis, fractionation on DEAE-cellulose, etc. All pH values of buffers including Tris were determined at room temperature and were not corrected for temperature shifts.

Preparation of Electron Paramagnetic Resonance Samples—EPR samples of spin-labeled HPr in solutions of varying glycerol concentration were prepared by mixing weighed amounts of a spin-labeled HPr solution (1.75 mg/ml in 0.05 M potassium phosphate, pH 6.5, density 1.01 ± 0.007 g/cm^3) with weighed amounts of previously prepared dilutions of glycerol in buffer. The original dilutions of glycerol in buffer were also prepared by weight. The viscosities of glycerol-buffer mixtures were measured at 30 °C and were found to agree with the literature values predicted for the measured weight percentages (10) to within 0.3%. Viscosities of the solutions at other temperatures were estimated by extrapolation from literature tables (11). In some experiments, sucrose was used to vary the viscosity. The concentrations of sucrose solutions were determined by refractive index measurements and viscosities were taken from tables (11).

EPR Spectral Measurements—Solutions of spin-labeled HPr in buffer-glycerol or -sucrose mixtures were contained in 50-μl glass disposable pipettes (Corning), sealed at one end, for EPR spectral mesurements. The EPR spectra were recorded on a Varian E-12 spectrometer which was equipped with a variable temperature unit. The sample capillaries were positioned in the center of the temperature-regulating Dewar with a holder designed by R. D. Kornberg (see Ref. 12). Temperature was monitored with a copper-constantan thermocouple which was placed in the sample holder just above the portion of the sample being measured. The gradient of temperature between the usual position of the thermocouple and the measured part of the sample was found to be negligible. Spectrometer settings for EPR measurements were: scan width, 100 G for the complete spectrum and 20 G for measurement of the width of the center line; microwave power, 20 microwatts; modulation amplitude, 0.5–0.8 G, adjusted so that no spectral distortions were created; scan time, 8–16 s; and time constant, 0.25–0.5 s.

Spectral Simulations and Estimates of Correlation Times—Expected correlation times were calculated assuming the validity of the Stokes-Einstein relation (Equation 1). In using Equation 1, the partial specific volume of HPr was

$$\tau_c = \frac{4\pi r^3 \eta}{3kT} \quad (1)$$

taken as 0.742 cm^3/g; τ_c, rotational correlation time; r, the radius of the molecules (assuming a sphere); η, viscosity; k, Boltzmann constant; T, temperature.

Simulations of EPR spectra were carried out on an IBM3033 computer using a published program (13) provided by Professor J. H. Freed of Cornell University. Simulations for spectra exhibiting relatively rapid motion were also carried out on a DEC-10 using a variation of the above program. The input parameters for the program are the principal values of the anisotropic magnetic interaction tensors, T_2 and two rotational diffusion rates about orthogonal axes.

RESULTS

Synthesis of Radioactive HPr—[^{3}H]Methyl iodide was distilled to the bottom of a break-seal ampule by warming the upper portion while the bottom was maintained in a mixture of dry ice and methyl Cellosolve. The break-seal was ruptured, and a solution containing 6.0 mg of homogeneous HPr in 1.6 ml of 0.025 M potassium citrate, 0.05 M phosphate buffer, pH 5.0, was added so that it washed down the sides of the ampule while the latter was kept in the dry ice bath. The top of the ampule was then sealed, and the frozen solution was permitted to thaw. The reaction mixture was shaken in the dark for 24 h at room temperature. In the reaction described here, the specific activity of the labeled methyl iodide was stated to be 79 mCi/mmol by the manufacturer; 8.9 mg were used. The reaction was stopped by adding 3 ml of 0.01 M Tris-HCl buffer, pH 7.5, in ice, shaking for 10 min, and dialyzing at 4 °C for 24 h against 18 liters (3 × 6 liters each) of the Tris buffer, at which time no radioactivity could be detected in the dialysate. The dialysis bag had a nominal cut-off of 3500 daltons (A. H. Thomas, 3787-H45). The retained solution containing approximately 10^7 cpm was applied to a 10-ml DE-23 column, previously equilibrated with 0.01 M Tris-HCl buffer, pH 7.5. The column was washed with 25 ml of a solution containing 0.005 M KCl in the Tris buffer at 4 °C. All remaining steps were conducted at this temperature. The column was eluted with a linear gradient ranging from 0.005 to 0.05 M KCl in the Tris buffer; total volume, 800 ml. Fractions were collected and assayed as indicated in Fig. 1*A*. Two protein peaks were detected, one in the expected position for HPr, which contained no radioactivity, and a peak which eluted at a lower salt concentration, contained protein and radioactivity, and exhibited HPr phosphotransfer activity in the phosphotransferase assay.

The pooled fractions of the labeled peak were dialyzed against distilled water, lyophilized, and dissolved in the minimum volume of distilled water. In some preparations at this stage, the final product was turbid and lemon-colored. Centrifugation removed the precipitate and most of the color. The final solution contained 0.82 mg of protein/ml, the yield was about 1.03 mg, and its specific activity was 5×10^7 cpm/μmol, which agreed with the calculated value for the labeled methyl iodide (7×10^7).

The purity of the protein was assessed by polyacrylamide disc gel electrophoresis and is shown in Fig. 2. Its rate of migration with respect to the tracking dye was 0.47 under conditions where native HPr migrated at 0.53. Mixtures of the two proteins were readily resolved. In gels that were overloaded with the labeled protein, two very faint protein contaminants were just visible, both migrating more rapidly than either the main band or native HPr. These may be derivatives of HPr-1 and HPr-2 (14).

Synthesis of Spin-labeled HPr—A 40-mg sample of the spin-label reagent, 3-[(2-bromoacetamido)methyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, was dissolved in 3.2 ml of 0.025 M potassium citrate, 0.05 M phosphate buffer, pH 5.0, and added to 12 mg of HPr (lyophilized from a solution in water). The reaction mixture was incubated in the dark at 37 °C for 24 h, and the reaction was stopped by adding 3 ml of 0.01 M Tris-HCl buffer (pH 7.5, at room temperature) in the cold, followed by shaking in the cold for 10 min. The turbid mixture was transferred to a dialysis bag with a 3500 molecular weight

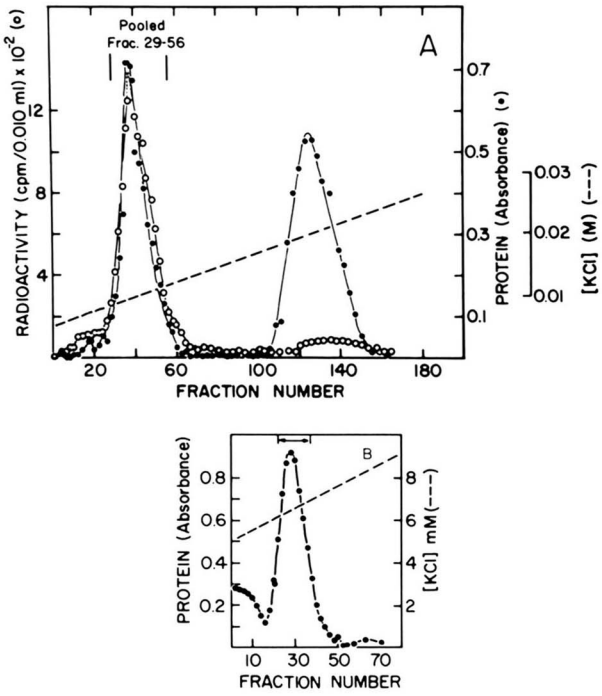


FIG. 1. **Fractionation of HPr derivatives.** The dialyzed reaction mixtures were transferred to DE-23 columns, and eluted with KCl in Tris buffer as described in the text. *A*, purification of [^{3}H]methyl-labeled HPr. Fractions, 2.1 ml each, were collected at a flow rate of 0.4 ml/min. Aliquots were assayed for protein (absorbance at 215 nm (●)), radioactivity (○), and activity in the PTS *in vitro* assay (1). The curves for the latter were coincident with the two protein peaks (data not shown). Fractions 29 through 56 were pooled for isolation of the active protein. The second protein peak (Fractions 105 through 155) was eluted at the expected position for HPr, and contained negligible quantities of radioactivity. *B*, purification of spin-labeled HPr. Fractions, 2.5 ml each, were collected at a flow rate of 0.6 ml/min. Only the early portion of the chromatogram is shown. Native HPr was eluted much later in the gradient, in its expected position, as in *A*. Fractions 22 through 37 were combined for further purification and analysis.

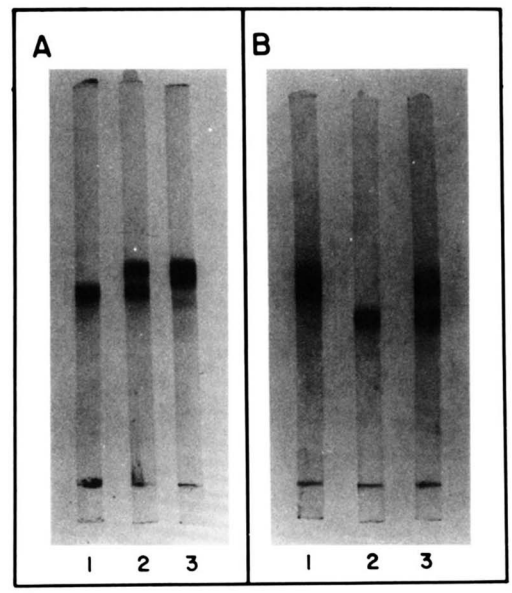


FIG. 2. **Polyacrylamide disc gel electrophoresis of derivatized HPr proteins.** The method of Ornstein (16) and Davis (17) was used except for slight modification of the stacking-gel buffer. The resolving gels contained 7.5% acrylamide, 0.25% bisacrylamide, and Tris-HCl buffer, pH 8.9. The stacking gels (0.25 ml) contained 0.6% acrylamide and 0.6% bisacrylamide in Tris-phosphate buffer, pH 7.5. Samples were applied to the gels in final volumes of 0.14 ml containing 0.02 ml of glycerol and 0.05 ml of the Tris-phosphate buffer, pH 7.5 (0.18 M phosphoric acid, 0.235 M Tris, titrated to pH 7.5). *A*, [^{3}H] methyl-labeled HPr: *Lane 1*, 17.5 μg of native HPr; *Lane 2*, 15 μg of native HPr plus 13 μg of [^{3}H]HPr; *Lane 3*, 35 μg of [^{3}H]HPr. *B*, EPR-labeled HPr: *Lane 1*, 35 μg of EPR-labeled HPr; *Lane 2*, 15 μg of native HPr; *Lane 3*, 17.5 μg of EPR-labeled HPr plus 15 μg of native HPr.

cut-off (A. H. Thomas, 3787-H45), together with 9 ml of 0.01 M Tris-HCl buffer, pH 7.5, and dialyzed in the dark at 0–4 °C for 12 h against a total of 12 liters of the Tris buffer (2 × 6 liters). All remaining steps were performed at 0–4 °C.

The dialyzed solution was centrifuged to remove precipitate. Analysis of typical reaction mixtures at this point by polyacrylamide disc gel electrophoresis showed four protein bands, a major band which migrated at the same rate as native HPr, another band present at the same or higher concentration (as judged by the intensity of Coomassie blue staining), which migrated somewhat more slowly, and trace quantities of two more slowly moving bands. Based on our previous experience with mutant forms of HPr (1), the major band which migrated more slowly than native HPr was presumed to be the desired spin-labeled protein, since the spin-labeled protein should have contained an additional positive charge due to the sulfonium group. The mixture was transferred to a 16-ml column of DE-23 (1) and washed with 50 ml of 0.01 M Tris-HCl buffer, pH 7.5, containing 0.005 M KCl. The column was then eluted with a linear shallow KCl gradient in the Tris buffer (total volume, 1600 ml, from 0.005 to 0.024 M KCl). Native HPr is eluted at a higher salt concentration (Fig. 1*A*). Fig. 1*B* shows the column profile at low concentrations of KCl from 0.005 to 0.009 M. Some unidentified protein was eluted in the early fractions, followed by a protein peak which was eluted between 0.0055 and 0.0074 M KCl. The latter showed HPr activity in the phosphotransferase assay, and comprised about 40% of the total protein eluted from the column. The peak fractions were combined as indicated in Fig. 1*B*, and dialyzed against 0.005 M potassium phosphate buffer, pH 6.5, in the dark as described above.

While native or ^{3}H-labeled HPr can be lyophilized in the phosphate buffer without loss of activity, spin-labeled HPr cannot. The solution was therefore concentrated by immersing the dialysis bag in dry Sephadex G-25 beads, and by repeatedly replacing the moist beads at the side of the dialysis bag. In this way, the pooled fractions were concentrated from about 40 ml to less than 1 ml with retention of phosphocarrier activity in the PTS assay.

The concentrated solution was dialyzed in the dark against fresh phosphate buffer, centrifuged to remove any precipitate, and the final solution (0.78 ml) was stored at −20 °C. This solution contained 1.7 mg of protein (as assayed by the microbiuret procedure (15)).

Polyacrylamide gel electrophoresis was used to determine the purity of the product described above (Fig. 2). When the gels were overloaded with protein, faint traces of contaminants were observed. Judging from a series of such gels with different concentrations of the sample, we estimate that the labeled protein was greater than 95% pure, and that it did not contain native HPr. It migrated at an R_F of 0.45 relative to the tracking dye, under conditions where native HPr migrated at 0.51. After mixing, the two proteins were readily resolved by electrophoresis.

Activity of Radiolabeled and Spin-labeled HPr in Phosphotransferase Systems—A requisite for the labeled HPr molecules was that they be active as phosphocarriers in the PTS. [^{3}H]Methyl- and EPR-labeled HPr samples were therefore tested for activity in the *in vitro* sugar phosphorylating

assay with methyl α-glucoside as the acceptor sugar, limiting amounts of Enzyme I (1, 3), and membranes from SB2950 (a deletion strain lacking Enzyme I and HPr). The assay was conducted as described (1), with four concentrations of the spin-labeled HPr and various concentrations of native HPr. The results showed that the EPR-labeled protein was 80 ± 7% as active in this assay as the native protein. Similar measurements were made with six different concentrations of radiolabeled HPr, which was found to be fully as active as the native protein (100 ± 10%).

Linkage of [^{3}H]Methyl and EPR Probe in Derivatized HPr Proteins—In a series of preliminary experiments, attempts were made to determine whether the substituting groups (radioactive and EPR) were linked to the NH_2-terminal methionine residue by treating the HPr derivatives and native HPr with four different preparations of leucine aminopeptidase, followed by analysis of the released amino acids as a function of time of incubation. These experiments were unsuccessful, primarily because all of the enzyme samples contained other proteases that gave cleavages elsewhere in the protein chain. Therefore, a different approach was followed.

The successful method involved cleavage of HPr by a protease from *Staphylococcus aureus*, which hydrolyzes proteins at the carboxyl side of glutamic acid residues. When applied to HPr, seven peptides were obtained, separable by ion exchange chromatography (2). The peptides are designated S-1 through S-7, respectively.

The method described in the accompanying paper (2) was modified to accommodate the small quantities of derivatized HPr available for analysis. Each incubation mixture contained the following components in final volumes of 60 μl: 200 μg of native or derivatized HPr, 3 μg of the *S. aureus* protease, and 3 μmol of NH_4HCO_3. Hydrolysis was allowed to proceed for 18 h at 37 °C. Control mixtures contained the protease but no HPr. After incubation, the sample was frozen, lyophilized, and dissolved in 0.5 ml of 0.05 M pyridyl acetate, pH 2.75. Each sample was then transferred to a high pressure liquid chromatography column 150 mm long with a 3-mm inner diameter, containing Benson BP-B5 7–10 μm resin (Benson Co., Reno, NE) first equilibrated with the same buffer. The peptides were eluted at 60 p.s.i. with a concave gradient of pyridyl acetate, ranging from 0.1 M, pH 3, to 1.2 M, pH 5; 100 fractions, 0.4 ml each, were collected. For detection of peptides, the fractions were dried in a vacuum desiccator containing a centrifuge which served to concentrate the samples to the bottom of the tubes; traces of pyridyl acetate were removed by twice adding 300 μl of water and drying. Each of the samples was dissolved in 100 μl of water, and 25-μl aliquots of each fraction were chromatographed on Silica Gel G plates, with chloroform:methanol:NH_4OH (2:2:1, v/v/v) as solvent. The plates were then dried at 100 °C for 30 min, after which peptides were visualized by spraying the plates with 5% triethylamine in dry acetone, drying for 2 min at room temperature, and then spraying with 0.05% fluorescamine in dry acetone. Fluorescent bands corresponding to peptides were visible under UV irradiation.

The remaining aliquots of appropriate fractions were pooled, dried in hydrolysis tubes under nitrogen, and hydrolyzed with 250 μl of constant boiling HCl for 24 h at 110 °C. The hydrolysates were dried and the amino acids were analyzed on a Durrum automated amino acid analyzer. Peptides were identified by comparing their compositions with those of peptides isolated from HPr after treatment with the *S. aureus* protease. As described below, there was some nonspecific cleavage of certain peptides; for example, S-4 was sometimes hydrolyzed to S-4a and S-4b. However, these were clearly distinguishable from the known peptides (see Fig. 3).

The NH_2-terminal methionine residue was found in S-1 and the second methionine, residue 80, was contained in S-6 (2). These two peptides were very clearly resolved since S-1 was eluted first from the column, and S-6 was eluted last (Fig. 3*A*). It was important to be able to identify these two peptides so that an unequivocal assignment of the derivatized methionine residue could be made. We assumed that formation of sulfonium derivatives of one (or both) of the methionine residues would increase the positive charge of the respective peptide, resulting in a significant change in the position of peptide S-1 or S-6 (or both) as it was eluted from the column. The peptides obtained by cleavage of [^{3}H]methyl and spin-labeled HPr with *S. aureus* protease, were therefore fractionated according to this protocol. Fig. 3*B* shows the results of cleavage of the [^{3}H]methyl-labeled HPr. Peptides S-2 through S-7 were found in their expected positions. Peptide S-1, however, was not found in its usual position, but a new peptide located between S-4 and S-6 was observed. The fractions comprising this peak all contained radioactivity; indeed these were the only radioactive fractions obtained. Analysis of the amino acids in the pooled fractions gave phenylalanine, glutamic acid (or glutamine), and a trace of methionine, consistent with the composition of modified S-1. In this experiment, the total recovery of radioactivity in the peptide was about 74%; that is, of the 4.2×10^6 dpm in the initial labeled HPr subjected to proteol-

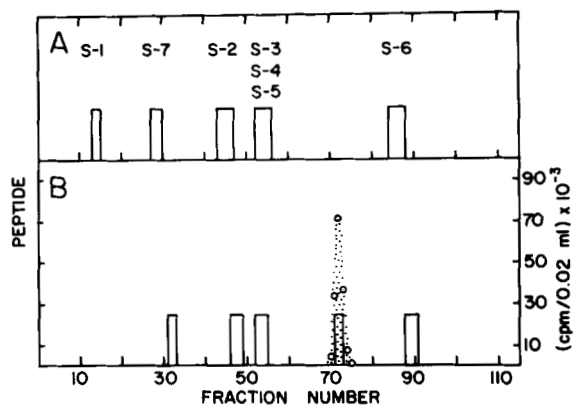


FIG. 3. **Separation of peptides after hydrolysis of HPr and [^{3}H]methyl HPr with *S. aureus* protease.** The HPr samples were digested with the protease, transferred to the high pressure liquid chromatography column, eluted with a pyridyl acetate gradient at a flow rate of 0.180 ml/min, and each fraction was analyzed for peptide by thin layer chromatography as described in the text. The fractions containing peptide were pooled as indicated, hydrolyzed, and analyzed for amino acids. *A*, native HPr. The *bars* show the location of pooled fractions. Identification of each peptide was based on its amino acid composition, compared to the compositions of peptides formed from HPr by the *S. aureus* protease (2). The molar ratios of the peptides isolated in the present experiments agreed with those previously reported. The yield of the more stable amino acids, which represented the yield of peptide in the pooled fractions, ranged between 50–80% of the expected values (based on the quantity of HPr used for the protease treatment). *B*, ^{3}H-labeled HPr. The labeled HPr was subjected to hydrolysis and fractionation as described above. Except for S-1, the other known peptides were eluted in the same sequence, but some were displaced to the right by two to five fractions. S-1 was not detected, but a new peptide appeared coincident with the radioactive peak shown. These were the only fractions that exhibited radioactivity. The pooled radioactive peak gave the following amino acid analysis (molar ratio): Glx, Phe, Met (2.9:1.0:0.36). *S*-Methyl methionine is only partially converted to methionine on vigorous acid hydrolysis and the sulfonium derivative is normally eluted from the amino acid analyzer in the ammonia peak (8). Thus, the radioactive fractions are concluded to be derivatized S-1. In this experiment, S-6 was obtained in higher yield (about 60%) than S-6 from native HPr (about 50%).

ysis, 3.09×10^6 dpm were found in the peptide. After the peptides had been eluted, we were unable to detect any additional radioactivity by sequentially washing the column with 2 M pyridine, 8 M pyridine, and 0.5 M NaOH.

From these results, and the fact that the labeled HPr contained about 1 mol of labeled methyl group (based on specific activity, see above), we concluded that only the NH_2-terminal methionyl residue had been modified by the methyl iodide treatment. Similar conclusions were drawn from analysis of the spin-labeled HPr, which gave essentially the same results as the radiolabeled HPr. Fluorescent HPr, reported in the next paper (5), also gave similar results, although in this case the label is much less stable, and some degradation occurred during peptide cleavage and fractionation of the peptides.

Experimental EPR Spectra

Paramagnetic resonance spectra were recorded for HPr in buffer plus 0 to 50% sucrose, or plus 0 to 21% glycerol (w/w), solutions. The temperatures of measurement were varied between 0 and 40 °C. Assuming that HPr undergoes simple Brownian diffusion in these solutions, its rotational correlation time, τ_c, should be proportional to the viscosity divided by the temperature (°K), η/T. The experimental values of η/T used in this study were varied by almost an order of magnitude.

Fig. 4*a* shows representative EPR spectra for spin-labeled HPr at several values of rotational correlation time. Clearly the spectra are quite sensitive to changes in η/T, and thus to correlation time. However, the experimental EPR spectra show evidence of a much shorter correlation time (faster motion) for HPr than is expected for rotation of a hydrated protein sphere of its size. This can be seen by comparing the experimental spectra of Fig. 4*a* with the computer-simulated ones shown in Fig. 4*b*. (The spectral simulations are discussed below.)

Experimental EPR spectra resulting from molecules tumbling isotropically with correlation times shorter than 3×10^{-9} s can be evaluated semiquantitatively by using an approach described by Stone *et al.* (18). In this analysis, EPR spectral parameters are related to the rotational correlation time by Equation 2.

$$\tau_c \propto \left[\sqrt{\frac{h_0}{h_{+1}}} + \sqrt{\frac{h_0}{h_{-1}}} - 2 \right] \Delta\omega_0 \approx F \qquad (2)$$

Here, h_{+1}, h_0, and h_{-1} are the heights of the low-, mid-, and high-field lines of the EPR spectrum, respectively, and $\Delta\omega_0$ is the peak to peak width of the mid-field line. Referring again to Fig. 4*b*, it is clear that this formula can only be applied to those cases where the motion is fast enough that the spectra consist simply of three lines. For instance, h_{+1} and h_{-1} are meaningless parameters for the top spectrum of Fig. 4*b*. Since correlation time is proportional to the function F given in Equation 2, and also to η/T, a plot of log F *versus* log η/T is expected to be linear. Slight deviations from linearity may result in cases where the assumption of axial symmetry of elements of g and T (used to derive Equation 2) does not hold (18). Fig. 5 shows two comparisons of values of F from simulated and experimental EPR spectra. In Fig. 5*a*, the data are plotted to emphasize effects of glycerol concentration on the experimental parameters (each symbol refers to a particular glycerol concentration). In Fig. 5*b* the effects of temperature are emphasized by assigning a symbol to a particular temperature. Two points are evident from this figure. First, the difference in the intercepts on the F axis for the experimental and simulated data suggests that the motion of the spin-labeled portion of HPr is about 5 times faster than is the rate of rotation of the protein as a whole. Second, Fig. 5*b* shows clear evidence of a temperature-dependent, but viscosity-independent equilibrium in the labeled HPr because the F axis intercepts of the plots depend on the temperature of measurement. Precise values for experimental spectral parameters for the labeled HPr solutions at selected values of η/T are given in Table I.

Although the majority of the EPR data discussed in this report were obtained with glycerol-buffer solutions, some measurements were also made, as mentioned earlier, with sucrose-buffer solutions. No differences were found, for a particular η/T, between the EPR spectra of spin-labeled HPr in glycerol- and sucrose-containing solutions. Several data points (×) from the sucrose-buffer solutions are shown in Fig. 5 to support this conclusion.

Computer Simulation of Solution EPR Spectra

The effects of molecular motion on the solution EPR spectra of the labeled HPr were simulated with a computer program (13) which allowed for two motional models: (i) isotropic rotational diffusion; or (ii) anisotropic diffusion characterized by two rotational correlation times. In agreement with data of

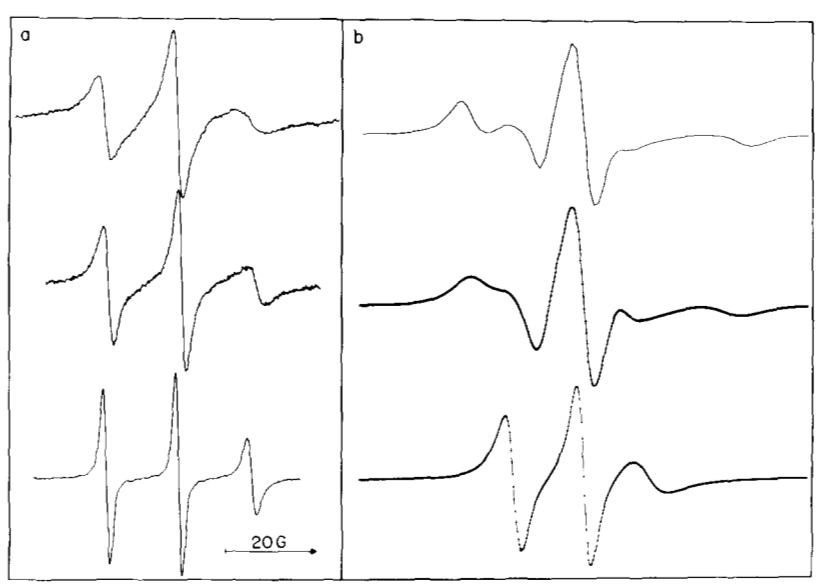


FIG. 4. **Experimental spectra (*a*) of spin-labeled HPr in solutions of several different viscosities are compared with the computer-simulated spectra (*b*) for isotropic Brownian rotation of a hydrated sphere the size of HPr.** The temperatures (percentages of glycerol, w/w) for the experimental spectra are, from *top* to *bottom*, 0 °C (21.1%), 10 °C (16.8%), and 30 °C (0%). The parameters for the simulated spectra are for Z, Y, and X components of T and g, 36.0, 5.85, and 5.85 G and 2.0028, 2.0072, and 2.0094, respectively. The correlation time, τ_c, is an input parameter for the simulations. The τ_c value was converted to η/T for this plot by using Equation 1 and the partial specific protein volume as described under "Experimental Procedures." Correlation times used for the simulated spectra were, from *top* to *bottom*, 10.4, 6.1, and 2.1 ns.

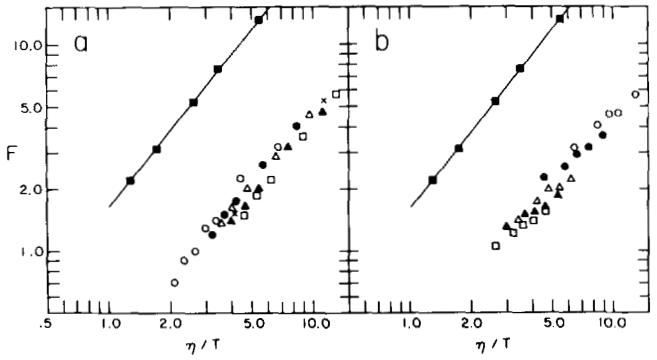


FIG. 5. **Values of the function, *F*, given by Equation 2, are plotted *versus* η/T for computer-simulated EPR spectra and for the experimental spectra of spin-labeled HPr at various temperatures and viscosities.** The simulated data are given by ■. *Experimental points* are plotted to show, in *a*, the dependence of experimental values of *F* on glycerol concentration and, in *b*, the dependence on temperature. In *a*, the per cent by weight of glycerol in buffer is designated by ○ (0%), ● (8.2%), △ (12.6%), ▲ (16.8%), and □ (21.1%). Two points for sucrose solutions are also shown (×). In *b*, the temperature is designated by ○ (0 °C), ● (10 °C), △ (20 °C), ▲ (25 °C), and □ (30 °C). Two of the points shown in *a* for 0% glycerol at 35 and 40 °C are not shown in *b* because no measurements were made at these temperatures at other viscosities. Correlation times for the simulated spectra are, from high to low values of *F*, 4.4, 2.7, 2.1, 1.4, and 1.0 ns in both *a* and *b*.

TABLE I

EPR spectral data and correlation times for EPR-labeled HPr in glycerol or sucrose solutions

The following abbreviations are used: *T*, Kelvin temperature; η, viscosity in Pascal-seconds (1 millipoise = 10^{-4} Pascal-seconds); w_0, peak to peak width of the derivative of the middle line ($m_I = 0$) of the EPR spectrum; h_{+1}, h_0, and h_{-1} are the peak to peak heights of the low-, mid-, and high-field lines of the EPR spectrum; $\tau_c^{S\text{-}E}$ is the rotational correlation time predicted by the Stokes-Einstein relation (Equation 1).

Glycerol or sucrose	*T*	η	w_0	h_0/h_{+1}	h_0/h_{-1}	$\tau_c^{S\text{-}E}$
%	*K*	*Pascal-seconds*	*G*			*ns*
Glycerol						
0	303	0.0007999	1.50	1.160	2.667	2.10
16.8	303	0.001236	1.55	1.218	3.204	3.25
16.8	293	0.001604	1.85	1.344	3.826	4.36
16.8	283	0.002170	2.25	1.512	4.741	6.11
16.8	273	0.00310	2.50	1.852	6.25	9.06
21.1	273	0.00357	2.70	2.000	7.300	10.4
Sucrose						
15.5	303	0.001258	1.65	1.254	3.358	3.35
15.5	293	0.001623	1.85	1.350	4.051	4.38
15.5	283	0.002178	2.20	1.638	4.914	6.14
15.5	273	0.003066	2.65	2.000	6.741	8.93

Fig. 5, the experimental spectra were reasonably approximated by simulations assuming isotropic diffusion with a correlation time 5 times faster than expected for HPr at a particular viscosity and temperature. A variety of anisotropic diffusion models were tested, in which the rate of diffusion about an axis parallel to the nitroxide *z* axis was slower than the rate about an axis in the *x-y* molecular plane. None of the anisotropic diffusion models gave substantially better fits of simulated and experimental spectra than did the isotropic motion model.

DISCUSSION

For reasons given in the introductory section, the availability of HPr derivatized with different probes will greatly facilitate studies aimed at understanding how individual proteins of the PTS interact with one another, with membrane components, and whether, for example, they attach and detach from the membrane components during sugar transport in vesicles. An essential requirement for a satisfactory derivative is that it should retain its ability to participate in the phosphotransfer reactions of the PTS. For instance, HPr is easily dansylated, and extensive studies led to conditions where an average of 1 mol of dansyl group was introduced/mol of protein. However, these derivatives were not studied further because they showed no phosphotransfer activity.

Successful modification of HPr was achieved by treating the protein with alkyl halides at acid pH, a method developed for alkylating methionyl residues to give the corresponding sulfonium derivatives (7, 8). Since HPr contains only 2 methionine residues (2), only a limited number of derivatives can be formed in each reaction mixture. In this report, radiolabeled methyl iodide and the EPR probe, 3-[(2-bromoacetamido)methyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, were used to alkylate the protein, and in an accompanying report (5) a corresponding fluorescent probe was used.

The positive charge introduced by the sulfonium group is sufficient to permit separation of the derivatives from native HPr on gel electrophoresis, and also permitted separation and purification of the derivatives by ion exchange chromatography. The radiolabeled protein exhibited the expected specific activity if one [^{3}H]methyl group had been introduced/mol of protein, and appeared homogeneous by gel electrophoresis with possible trace contamination by proteins migrating like HPr-1 and HPr-2 (14). The EPR-labeled derivative was somewhat less stable (although it can be stored in the frozen state for many months), and, for example, could not be concentrated in phosphate buffer by lyophilizing. We estimate the final product to be more than 95% pure.

Both preparations were active in the phosphotransfer reactions of the PTS. The *in vitro* methyl α-glucoside phosphorylating assay depends on interaction between HPr and two proteins of the PTS, Enzyme I and III^{Glc}. With the use of limiting amounts of Enzyme I, the radiolabeled HPr derivative was 100 ± 10% as active as native HPr, while the EPR derivative was 80 ± 7% as active.

The positions of the labels in the HPr derivative were determined by subjection of the proteins to digestion with an *S. aureus* protease, fractionation of the resulting peptides, and amino acid analyses of the latter. The analyses showed that each of the labels was located exclusively on the NH_2-terminal methionine, and was not found at any other position. Since the alkylation reactions were conducted under conditions where the alkylating agent was present in very large excess, for 24 h at 27–37 °C, the lack of reactivity of Met-80 suggests that it is not readily available to the aqueous environment which is in accord with the predicted secondary structure of HPr (2), where this residue is in the terminal helix.

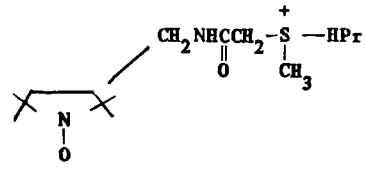
Electron paramagnetic resonance spectra of spin-labeled macromolecules are sensitive to motions with correlation times from 10^{-10} to 10^{-4} s, if spectra obtained under both saturating and nonsaturating conditions are considered (19). Since this range of correlation times is exhibited by molecules in the molecular weight range from about 100 to greater than 10^7 when they tumble in aqueous solution, EPR offers an opportunity to investigate associations of components of a multiprotein complex, such as the PTS. To maximize changes in rotational motion, the smallest component of the system is the preferred one to label, and HPr, the smallest of the PTS proteins, was therefore selected for this purpose. Viscosity changes as small as 20% gave detectable changes in the EPR spectra of the labeled HPr. However, as indicated below, the motion is not simple.

The experimental data shown in Fig. 5 are reasonably fit by a straight line that is nearly parallel to the one obtained from simulated spectra. However, if lines are drawn through the points of Fig. 5 that correspond to constant temperatures, slightly different intercepts on the F axis are obtained for different temperatures (Fig. 5*b*). This is similar to the situation that was observed for spin-labeled hemoglobin (20) where again spectral motion parameters are linear to η/T for viscosity variations at a *single* temperature, but separate plots were obtained at *different* temperatures. The data for spin-labeled hemoglobin suggested the presence of a viscosity-independent thermal equilibrium in the protein. The data for the labeled HPr shown in Fig. 5*b*, taken at individual temperatures, suggest that there may be a viscosity-independent contribution to the motion. Additional evidence for motions that are independent of rotation of HPr is provided by the observed changes in the spectra of frozen HPr solutions at various temperatures. The outer extrema of the spectra are separated by 36.0 G at −25 °C and by 37.0 G at −196 °C.

Although a variety of computer simulations of EPR spectra were tested, discrepancies between experimental and simulated spectra for EPR-labeled HPr remained. The fit could be improved slightly by varying the spectral input parameters from the values determined for the frozen solution, but these variations are entirely empirical and do not conform to a well defined motional model. Thus, at the present time, the motion of the nitroxide in the derivative can best be described as some internal flexibility of the protein or label superimposed on rotational motion of the whole protein. While the spectra clearly respond to changes in the rotation of the whole protein, the other contributions to the motions of spin-labeled HPr are not well defined.

In future experiments, EPR evidence for binding of spin-labeled HPr to other components of the PTS will be sought. Such binding should be accompanied by a change in rotational correlation time of HPr. A similar approach has been used to quantitate the binding of spin-labeled peptides to neurophysin (21); changes in correlation time were evident from the EPR spectra of the peptides in the presence of varying amounts of neurophysin. However, based on the work presented here, there may be two types of spectral change if the labeled HPr forms a complex with other PTS components. There may be a change in rotational correlation time and/or a change in the conformation of the spin-labeled HPr. For instance, we thought it possible that phosphorylation of HPr might result in a conformational change which would be detected as a change in the freedom of motion of the probe. We performed preliminary experiments of this type using an excess of phosphoenolpyruvate and a catalytic amount of Enzyme I and found that the EPR spectra of HPr under these phosphorylating conditions were identical to those of the unphosphorylated protein, and, thus, that there was no detectable conformational change in this case.

In addition to information about motion, the EPR spectra of spin labels convey information about the polarity of the label environment. The trace of the elements of the hyperfine tensor for nitroxide EPR spectra is considerably smaller for hydrocarbon environments than for aqueous solutions. The separation (approximately the trace) of lines in the spectrum of rapidly tumbling HPr is 15.9–16.0 G. This separation for the spin label alkylating reagent *per se* in water is 16.0 G, 16.2 G for the label in 9 M aqueous lithium chloride, and 14.2 G for the label in benzene. Thus, it is clear that the label in the protein is in a polar environment. Polarity of environment is also reflected in observed parameters for frozen, or otherwise immobilized, protein samples. The largest separation of the outer spectral extrema yet observed for a protein is for a spin label hapten bound by anti-nitroxide antibodies, 39.5 G (22). Here, the large splitting was attributed to the presence of a positive charge, such as guanidinium, near the nitroxide. The value of this parameter for spin-labeled HPr frozen at −196 °C (37.0 G) is similar to that of precipitated carbonmonoxyhemoglobin at the same teperature, 37.5 G (21), in which case, hydrogen bonding to the nitroxide was thought to influence this spectral parameter. In contrast, this parameter for crystals containing nonpolar nitroxides is 31–32 G (23). The high value of the outer separation in spectra of spin-labeled HPr in frozen solution suggests that a positive charge may be near the nitroxide. The positive sulfonium group at the site of attachment of the probe to HPr may be close enough to the N—O bond to influence this spectral feature,



An accompanying manuscript describes studies of HPr by time-resolved fluorescence (5). Two times were found to fit the decay curve better than one. One of these times (15 ns in buffered water at 20 °C) is considerably longer than expected if HPr is spherical. One explanation of this long time is that the molecule is highly asymmetric. In comparing the spin-label and fluorescence data, the shape of the continuous wave type of EPR spectra we report will be dominated by the faster motional component when several motions are present. EPR spectral simulations have also been made assuming two correlation times. As long as the fast component corresponds to rotation about the nitroxide x and y axes, a slow component about an axis through the nitroxide z axis has little effect on the spectra even when the slow rate is half that expected for the rotational motion of a hydrated sphere the size of HPr. In addition, the absolute values of diffusion rates obtained by EPR and fluorescence techniques cannot be compared directly, because the observed rates in both techniques depend on the orientation of the probe relative to the principal diffusion axes that describe the motion of the protein molecule. In one sense, a better estimate of overall rotational motion of a protein can be obtained from a protein labeled at several different sites than from the uniquely labeled derivatives described here, because then there is random orientation of the probe axes relative to protein motion axes. Of course, randomly labeled enzymes or phosphocarrier proteins are unacceptable derivatives when the activity of the labeled protein is of interest, as it is here.

As a final point in this discussion, we note that the EPR probe, at the NH_2 terminus of HPr, is located close to the active site histidine, residue 15. Thus, it is reasonable to expect that interactions between HPr and different protein species of the PTS, such as Enzyme I and III^{Glc}, may be reflected by the behavior of the EPR probe, regardless of the fact that it exhibits a considerable degree of flexibility when measurements are made with the HPr derivative alone. In fact, preliminary experiments with stoichiometric quantities of Enzyme I indicate binding between the two proteins, and similar results were obtained with III^{Glc} and the EPR-labeled HPr (24).

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REFERENCES

1. Beneski, D. A., Nakazawa, A., Weigel, N., Hartman, P. E., and Roseman, S. (1982) *J. Biol. Chem.* **257,** 14492-14498
2. Weigel, N., Powers, D. A., and Roseman, S. (1982) *J. Biol. Chem.* **257,** 14499-14509
3. Weigel, N., Waygood, E. B., Kukuruzinska, M. A., Nakazawa, A., and Roseman, S. (1982) *J. Biol. Chem.* **257,** 14461-14469
4. Turner, B. W., Pettigrew, D. W., and Ackers, G. K. (1981) *Methods Enzymol.* **76,** 596-628
5. Hildenbrand, K., Brand, L., and Roseman, S. (1982) *J. Biol. Chem.* **257,** 14518-14525
6. Beneski, D. A., Misko, T. P., and Roseman, S. (1982) *J. Biol. Chem.* **257,** 14565-14575
7. Gundlach, H. G., Stein, W. H., and Moore, S. (1959) *J. Biol. Chem.* **234,** 1754-1760
8. Link, T. P., and Stark, G. R. (1968) *J. Biol. Chem.* **243,** 1082-1088
9. Azzi, A., Tamburro, A. M., Farnia, G., and Gobbi, E. (1972) *Biochim. Biophys. Acta* **256,** 619-624
10. Miner, C. S. (ed) (1953) *Glycerol*, Reinhold Publishing Corporation, New York
11. *Handbook of Biochemistry*, CRC Press, Cleveland, Ohio
12. Gaffney, B. J., and McNamee, C. (1974) *Methods Enzymol.* **32B,** p. 195
13. Freed, J. H. (1976) in *Spin Labeling, Theory and Applications*, (Berliner, L. J., ed) Vol. 1, pp. 53-132, Academic Press, New York
14. Anderson, B., Weigel, N., Kundig, W., and Roseman, S. (1971) *J. Biol. Chem.* **246,** 7023-7033
15. Layne, E. (1957) *Methods Enzymol.* **3,** 447-454
16. Ornstein, L. (1964) *Ann. N. Y. Acad. Sci.* **121,** 321-349
17. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404-427
18. Stone, T. J., Buckman, T., Nordio, P. L., and McConnell, H. M. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **54,** 1010-1017
19. Berliner, L. J. (ed) (1976, 1979) *Spin Labeling, Theory and Applications*, Vols. 1 and 2, Academic Press, New York
20. Johnson, M. E. (1978) *Biochemistry* **17,** 1223-1228; (1979) *FEBS Lett.* **97,** 363-366
21. Lundt, S. L., and Breslow, E. (1976) *J. Phys. Chem.* **80,** 1123-1126
22. Humphries, G. M. K., and McConnell, H. M. (1976) *Biophys. J.* **16,** 275-277
23. Lajzerowicz-Bonneteau, J. (1976) in *Spin Labeling, Theory and Applications*, (Berliner, L. J., ed) Vol. 1, pp. 239-249, Academic Press, New York
24. Gaffney, B. J., and Cheung, A. (1981) *Abstracts of the Seventh International Biophysics Congress and the Third Pan-American Biochemistry Congress, Mexico City, Aug 23-29*