Carbon-13 Nuclear Magnetic Resonance Studies of the Interaction of Lecithin with Purified D-β-Hydroxybutyrate Apodehydrogenase, a Lipid-Requiring Enzyme†

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ABSTRACT: The interaction of D-β-hydroxybutyrate apodehydrogenase (BDH), a lecithin-requiring enzyme purified from bovine heart mitochondria, with lecithin (PC) has been studied by ^{13}C NMR by using lecithin labeled either in the polar moiety, soybean [N-$^{13}CH_3$]PC, or in the hydrophobic moiety, [11-^{13}C]dioleoyl-PC. The lecithin was admixed together with phosphatidylethanolamine and diphosphatidylglycerol, the other two major mitochondrial phospholipids, and added as vesicles in aqueous buffer. With increasing BDH/PC ratio, the spin–lattice relaxation time (T_1) of the choline N-$^{13}CH_3$ signal (resonance at 54 ppm) decreased, whereas T_1 increased for the hydrophobic label, [11-^{13}C]dioleoyl-PC (resonance at 27 ppm). For relaxation by a dipole–dipole interaction mechanism, $1/T_1$ is proportional to τ_c, the rotational correlation time of the ^{13}C nucleus, so that changes in $1/T_1$ reflect changes in the rotational motion of the ^{13}C-labeled moiety. At the highest protein to lipid ratio studied, 4.8 mg of BDH added per mg of PC, $1/T_1$ of the N-$^{13}CH_3$ signal was enhanced 60% compared with that of phospholipid vesicles alone, whereas for the 11-^{13}C resonance $1/T_1$ was diminished by 33%. These studies show that, upon interaction of BDH with phospholipid vesicles containing lecithin, the rotational motion in the polar group of lecithin is constrained, whereas the motion in the hydrophobic region is increased. The increased motion of the hydrophobic moiety could result from disorder in the bilayer. Futher, the line shape of the 11-^{13}C lecithin resonance was broadened upon interaction with BDH. Such line broadening could result from chemical shift anisotropy or constrained lateral motion. The interaction of BDH with lecithin in phospholipid vesicles, as measured by ^{13}C NMR, is unique as compared with the two other systems previously studied.

Phospholipids are essential for the function of a number of membrane-bound enzymes (Fleischer & Fleischer, 1967; Rothfield & Romeo, 1971). For lipid-requiring enzymes, the phospholipid requirement may be specific or nonspecific. D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme which specifically requires lecithin for its function. The apodehydrogenase has been purified to homogeneity, devoid of phospholipid or detergents (Bock & Fleischer, 1975). The inactive apodehydrogenase is reactivated specifically by lecithin or phospholipid mixtures containing lecithin (Fleischer et al., 1974; Gazzotti et al., 1975). The formation of an enzyme–lecithin complex is a prerequisite for function and confers upon the enzyme the capability of binding the coenzyme, NADH (Gazzotti et al., 1974). Recent studies (Isaacson et al., 1979), using analogues of lecithin modified either in the hydrophobic region or in the polar moiety, have demonstrated the high structural specificity of the choline moiety of lecithin for activation of D-β-hydroxybutyrate apodehydrogenase. In the present study, ^{13}C NMR is used to measure the interaction of D-β-hydroxybutyrate apodehydrogenase with lecithin labeled either in the polar region, i.e., in the choline moiety (soybean [N-$^{13}CH_3$]lecithin), or in the fatty acyl moiety [bis([11-^{13}C]dioleoyl)lecithin]. A preliminary report has appeared (Fleischer et al., 1978).

Materials and Methods

Reagents. Chemicals were of reagent grade unless specified otherwise. Solutions were prepared in deionized water. DL-β-Hydroxybutyric acid (sodium salt), bovine plasma albumin (fraction V, powder), Tris [tris(hydroxymethyl)-aminomethane], and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO). A solution of bovine plasma albumin, used as the protein standard, was obtained from Armour Pharmaceutical Co. (Chicago, IL). NAD^+ was obtained from P-L Biochemicals, Inc. (Milwaukee, WI). LiBr, obtained from Matheson Coleman and Bell (Norwood, OH), was prepared as a 4 M stock solution which was purified by filtration sequentially through an activated-carbon column and a 0.22-μm membrane filter (Millipore Corporation, Bedford, MA).

Preparation of Phospholipids. Lipids from beef heart mitochondria were extracted from freshly prepared mitochondria as described previously (Fleischer et al., 1967). Mitochondrial lecithin, phosphatidylethanolamine, and diphosphatidylglycerol were purified from the total lipid extract by using silicic acid chromatography (Rouser et al., 1967; Isaacson et al., 1979). Soybean [N-$^{13}CH_3$]lecithin was prepared from soybean lecithin by demethylation, followed by methylation with [^{13}C]methyl iodide (Stoffel et al., 1971; Stoffel, 1974). [11-^{13}C]Dioleoyllecithin was prepared by acylation of glycerylphosphorylcholine with the acyl chloride of [11-^{13}C]oleic acid (Stoffel et al., 1972). Unlabeled dioleoyllecithin was prepared by acylation of glycerylphosphorylcholine by using the method of Warner & Benson (1977). Unlabeled soybean lecithin used in some of these studies was a gift of Dr. Hans Betzing (A. Natterman and Cie, GMBH, Köln, West Germany).

Preparation of D-β-Hydroxybutyrate Apodehydrogenase. The apodehydrogenase was purified from beef heart mitochondria as described by Bock & Fleischer (1974, 1975) and modified (Brenner, McIntyre, Latruffe, and Fleischer, unpublished experiments). The specific activity of the preparation used in these studies was 86 μmol of NAD^+ reduced per min per mg of protein, at 37 °C, when activated with mitochondrial phospholipid. This is equivalent to a specific activity of 35 μmol of NAD^+ reduced per min per mg of protein at 25 °C (Gazzotti et al., 1975).

Preparation of Phospholipid Vesicles in Aqueous Buffer (Liposomes). Mitochondrial phospholipid was microdispersed

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in 20 mM Tris-HCl[1] and 1 mM EDTA, pH 8.1, by dialysis from butanol–cholate (Fleischer & Klouwen, 1961). The other phospholipids were codispersed by sonication as follows. The various phospholipids were combined in the appropriate proportions in chloroform–methanol (2:1) and then evaporated to dryness. The dried lipid was resuspended in 20 mM Tris-HCl and 1 mM EDTA, pH 8.1, by vortexing and was microdispersed by sonication by using either a bath sonicator (Laboratory Supplies, Inc.) or a probe sonicator [Branson Sonifier B-12, with standard microtip (101–148–062) marketed by Heat Systems Ultrasonics, Inc., Plainview, L.I., NY] at 74–80 W in an atmosphere of nitrogen as described previously (Isaacson et al., 1979; Stoffel et al., 1974a). Phospholipid vesicles obtained by either method activated D-β-hydroxybutyrate apodehydrogenase to a similar extent. The lipid vesicles used in the ^{13}C NMR measurements were prepared by using the probe sonicator.

Preparation of D-β-Hydroxybutyrate Apodehydrogenase–Liposome Complexes for ^{13}C NMR Measurements. Ten milligrams of ^{13}C-labeled lecithin (either [11-^{13}C]dioleoyl- or soybean [*N*-$^{13}CH_3$]lecithin) was codispersed together with mitochondrial phosphatidylethanolamine and diphosphatidylglycerol (phosphorus ratio of 1.0:0.8:0.2) in 2.5 mL of 0.1 M LiBr, 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, and 8 mM dithiothreitol by sonication (Branson probe sonifier, see above) for 15 min under N_2. An aliquot of the ^{13}C-labeled lecithin, 2.5 mg in 0.63 mL as codispersion, was diluted with buffer to 11 mL, and 4 mL of D_2O was added. Purified stock D-β-hydroxybutyrate apodehydrogenase, 12 mg in 5 mL of 0.4 M LiBr, 1 mM Hepes (pH 7.0), and 5 mM dithiothreitol, was added to the phospholipid dispersion, and the mixture incubated for 15 min at room temperature (~25 °C) to form the active enzyme–lecithin complex (Isaacson et al., 1979). At this ratio of lecithin to apodehydrogenase, approximately half-maximal activation is obtained; cf. Figure 1 and Tables I and II. The sample was then concentrated to between 2.5 and 3.0 mL, under N_2 at 4 °C, by ultrafiltration by using a PM-10 membrane filter (Amicon Corp., Lexington, MA). Following measurement of D-β-hydroxybutyrate dehydrogenase activity, the ^{13}C NMR spectrum and spin–lattice relaxation time (T_1) were obtained at 6 °C (see below). This first sample contained 12 mg of D-β-hydroxybuturate apodehydrogenase and 2.5 mg of ^{13}C-labeled lecithin (as codispersion) in 0.1 M LiBr, 15 mM Tris-HCl (pH 8.1), 1 mM EDTA, 5 mM dithiothreitol, and 20% (v/v) D_2O. Following the ^{13}C NMR measurements, D-β-hydroxybutyrate dehydrogenase activity was measured and then an additional 2.5 mg of the ^{13}C-labeled lecithin codispersion in 11 mL of buffer (0.1 M LiBr, 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, and 8 mM dithiothreitol) and 4 mL of D_2O were added to the enzyme–phospholipid complex (~25°C). With this amount of lecithin, maximal activation was obtained. The sample was then concentrated to 2.5–3.0 mL as before. This second sample contained 12 mg of D-β-hydroxybutyrate apodehydrogenase and 5.0 mg of ^{13}C-labeled lecithin as codispersion with phosphatidylethanolamine and diphosphatidylglycerol. D-β-Hydroxybutyrate dehydrogenase activity was measured and the spin–lattice relaxation time (6 °C) was obtained from ^{13}C NMR measurements. After these measurements, a third addition of ^{13}C-labeled lecithin codispersion, 5 mg of lecithin, was made to the enzyme–phospholipid complex to obtain 12 mg of D-β-hydroxybutyrate apodehydrogenase and 10 mg of ^{13}C-labeled lecithin. The sample was again concentrated to 2.5–3.0 mL, activity was assayed, and T_1 was measured at both 6 and 20 °C. NADH was then added to a final concentration of 0.5 mM, and T_1 was measured at both 6 and 20 °C. D-β-Hydroxybutyrate dehydrogenase activity was measured after each operation. The activation obtained at each lecithin to D-β-hydroxybutyrate dehydrogenase ratio was similar to that obtained in the activation studies (cf. Figure 1), and activity of the complexes usually remained constant while the ^{13}C NMR measurements were made. However, there was loss in activity (~70%) following the measurement of T_1 (at 20 °C) for the complex containing 10 mg of ^{13}C-labeled lecithin. Activity was partially restored (to ~60% of original) by incubation of the complex with additional dithiothreitol (8 mM). There was no detectable change in T_1 with this reversible inactivation of D-β-hydroxybutyrate dehydrogenase.

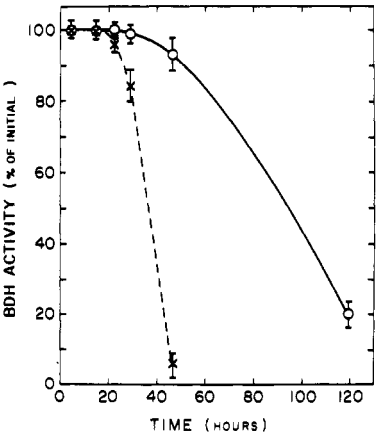

FIGURE 1: Stability of D-β-hydroxybutyrate apodehydrogenase (BDH) complexed with lipid vesicles. BDH was activated with lecithin (PC; either beef heart mitochondrial, dioleoyl, or soybean) as mixed dispersions with beef heart mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2) or with mitochondrial phospholipid by incubating the BDH (0.1 mg/mL) with the lipid vesicles (30 mol of lecithin per mol of BDH) as described in Figure 2. After 15-min preincubation at 25 °C to obtain optimum activation of the apoenzyme, the samples were either stored at 25 (×) or at 0 °C (○), and an aliquot (5, 10, or 20 μL) was removed at the times indicated for measurement of BDH activity at 37 °C as described in Figure 2. Activity is expressed as the percentage of the initial specific activity (between 80 and 90 μmol of NAD^+ reduced per min per mg of BDH; cf. Figure 2). The stability of BDH complexed with different phospholipid dispersions was not significantly different, and the average of the four samples is shown for clarity. The diminished activity at 46 (25 °C) or at 120 h (0 °C) was partially reversed (to ~70% of the initial activity) by incubation (at 25 °C) for 15 min with additional dithiothreitol (5 mM). The loss of activity is thus mainly referable to the oxidation of dithiothreitol. Stability can be improved by careful maintenance of an anaerobic atmosphere; e.g., a sample stored under N_2 at 4 °C showed no measurable loss of activity during 72 h and had a half-life of ~400 h (Bock and Fleischer, unpublished experiments).

^{13}C NMR Spectroscopy. Proton-noise-decoupled ^{13}C NMR spectra were obtained at 22.63 MHz with a Bruker WH-90 pulse spectrometer operating in the Fourier transform mode. An internal deuterium field frequency lock was used. The free induction decay signals were obtained following a 20-μs pulse by using a 180° – t – 90° pulse sequence (Freeman & Hill, 1970; Vold et al., 1968) to obtain the spin–lattice relaxation time (T_1), where t is the delay time between the 180° and 90° pulses. The T_1 value was determined by the inversion recovery technique according to $M_0 - M_z = 2M_0 \exp(t/T_1)$, where M_0 is the equilibrium amplitude of the fully relaxed spectrum (i.e., the free induction decay for $t = \infty$) and M_z is the amplitude of a partially relaxed spectrum (amplitude in arbitrary units). Between 6 and 10 h was required to obtain the T_1 mea-

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride.

surements for each sample. Sample tubes of 10-mm outer diameter were used. The upper part was constricted to about 1-mm inner diameter so that the small liquid surface in the capillary minimized the liquid–vapor interchange, which might affect the T_1 value due to contributions from rapidly relaxing nuclei in the vapor phase (Aksnes et al., 1968). All samples were flushed thoroughly with purified argon gas. The temperature (either 6 or 20 °C) was controlled by a Bruker ST 100/700 variable temperature controller with an accuracy of better than ±1 °C as checked by inserting a thermistor into the open tubes under decoupling conditions.

Assays. Protein was measured by the procedure of Lowry et al. (1951) with bovine plasma albumin as protein standard. When dithiothreitol was present in the sample, iodoacetate was used to carboxymethylate the dithiothreitol which would otherwise interfere with the assay for protein (Ross & Schatz, 1973). Phosphorus was measured by using either a modification (Rouser & Fleischer, 1964) of Chen et al. (1956) or by the method of Bartlett (1959).

D-β-Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically as the rate of reduction of NAD^+ with D-β-hydroxybutyrate as substrate (Bock & Fleischer, 1975). The complex assay was used in which an enzyme–phospholipid complex was preformed for the optimum reactivation time (15 min) at room temperature (~25 °C) with approximately 10 μg of the apodehydrogenase and variable amounts of phospholipid microdispersion in 0.1 mL containing 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, and 5 mM dithiothreitol. The enzymic activity of the preformed complex was measured by the addition of a small aliquot (5 or 10 μL) of the complex to a 1-mL cuvette which contained the standard assay medium (10 mM potassium phosphate (pH 7.35), 0.5 mM EDTA, 0.4 mg/mL bovine plasma albumin, 1.27% (v/v) ethanol, 0.3 mM dithiothreitol, 2 mM NAD^+, and 20 mM DL-β-hydroxybutyrate), preincubated to the desired temperature (usually 37 °C). The enzymic activity of the enzyme–phospholipid complexes prepared for the ^{13}C NMR measurements was measured in a similar manner, except that the sample was more concentrated (3 mg/mL), a smaller aliquot (1–3 μL) of the complex was used and activity was measured in a 3-mL cuvette at room temperature (~25 °C).

Results

The interaction of purified D-β-hydroxybutyrate apodehydrogenase with lecithin, to form an active enzyme–phospholipid complex, has been studied by using ^{13}C NMR. The T_1 values of ^{13}C in specific positions of lecithin were measured in the absence and presence of D-β-hydroxybutyrate apodehydrogenase. The measurement of T_1 times requires pulsing of the sample for many hours in order to obtain sufficient signal to noise ratio so that it was vital for these studies to establish conditions at which the enzyme–phospholipid complex would remain stable. The enzymic activity of D-β-hydroxybutyrate apodehydrogenase, complexed with phospholipid vesicles and in the presence of 5 mM dithiothreitol, does not measurably decrease for 20-h incubation at either 0 or 25 °C (Figure 1). After 20-h incubation, activity decreases rapidly under the conditions of this experiment with 50% activity remaining after 37- and 94-h incubation at 25 and 0 °C, respectively. When the activity is diminished to about 20% of initial activity, the lost activity can be substantially restored (to ~70% of the initial value) by incubation of the enzyme–phospholipid complex for 15 min with additional 5 mM dithiothreitol. The inactivation, subsequent to 20-h incubation, observed at either 0 or 25 °C (Figure 1) seems referable mainly to oxidation of the dithiothreitol. Stability can be improved by careful maintenance of an anaerobic atmosphere; e.g., a sample stored under N_2 at 4 °C showed no measurable loss of activity for 72 h and was 50% active after 400 h. The ^{13}C NMR measurements required between 6 and 10 h to obtain the T_1 for each sample so that, with each addition of phospholipid, fresh dithiothreitol (5 mM) was added to the sample to minimize loss of activity arising from limiting dithiothreitol-reducing equivalents.

Optimal activation of D-β-hydroxybutyrate apodehydrogenase is obtained with mitochondrial phospholipid or with mitochondrial lecithin codispersed with mitochondrial phosphatidylethanolamine and diphosphatidylglycerol (lecithin–phosphatidylethanolamine–diphosphatidylglycerol in a phosphorus ratio of 1.0:0.8:0.2) (Isaacson et al., 1979). Therefore, in these studies, lecithin was added codispersed with phosphatidylethanolamine and diphosphatidylglycerol in the form of phospholipid vesicles. A typical activation curve is shown (Figure 2A). The activation of the apoenzyme was measured by using a constant amount of apodehydrogenase (10 μg) and titrating with increasing amounts of each phospholipid vesicle preparation, measuring the enzymic activity of a small aliquot of the preformed complex. The characteristics of the activation by phospholipid vesicles containing either beef heart mitochondrial lecithin or soybean lecithin or dioleoyllecithin are similar. The activation is dependent on the ratio of lecithin to apodehydrogenase and is proportional to the amount of lecithin added up to about 10 mol of lecithin per mol of apodehydrogenase subunit. Optimal specific activity is obtained when the ratio reaches or exceeds 30. The activation data is replotted as a function of the increasing ratio of apodehydrogenase to lecithin (Figure 2B). With low ratios of apodehydrogenase to lecithin (up to 1.3 mg of apodehydrogenase per mg of lecithin), all of the apoenzyme is activated. With increasing ratio of apodehydrogenase to lecithin, the amount of activated apoenzyme increases to a saturating value (2.78 mg/mg of lecithin). This experiment demonstrates that D-β-hydroxybutyrate apodehydrogenase interacts optimally with either dioleoyllecithin or soybean lecithin; both lecithins give optimal values for specific activity of the enzyme (in excess lecithin) and for the maximal amount of apodehydrogenase activated (in excess apodehydrogenase).

The interaction of D-β-hydroxybutyrate apodehydrogenase with the polar region of the lecithin molecule was studied by measuring the ^{13}C NMR spin–lattice relaxation time of the ^{13}C nucleus in soybean [N-$^{13}CH_3$]lecithin as a function of varying the ratio of the apodehydrogenase to the ^{13}C-labeled lecithin. In addition to phospholipid alone, three samples were prepared by the sequential addition of phospholipid to the apodehydrogenase to obtain samples with the apodehydrogenase to ^{13}C-labeled lecithin ratios indicated by the arrows in Figure 2B. For each sample, a series of partially relaxed spectra was obtained by varying the delay time (t) between the 180° and 90° pulses in the 180° – t – 90° pulse sequence (cf. Materials and Methods). The ^{13}C NMR spectrum of soybean [N-$^{13}CH_3$]lecithin (as codispersion with beef heart mitochondrial phosphatidylethanolamine and diphosphatidylglycerol) exhibits a sharp resonance at 54.1 ppm relative to tetramethylsilane (Figure 3A). T_1 was calculated from the amplitude of the signal at 54.1 ppm with different delay times in the pulse sequence (cf. Materials and Methods) and is 182 ± 19 ms at 6 °C. T_1 increased with increasing temperature (cf. Table I) as expected for increased thermal motion (Stoffel et al., 1974a). When D-β-hydroxybutyrate apodehydrogenase is inserted into the phospholipid vesicles, the

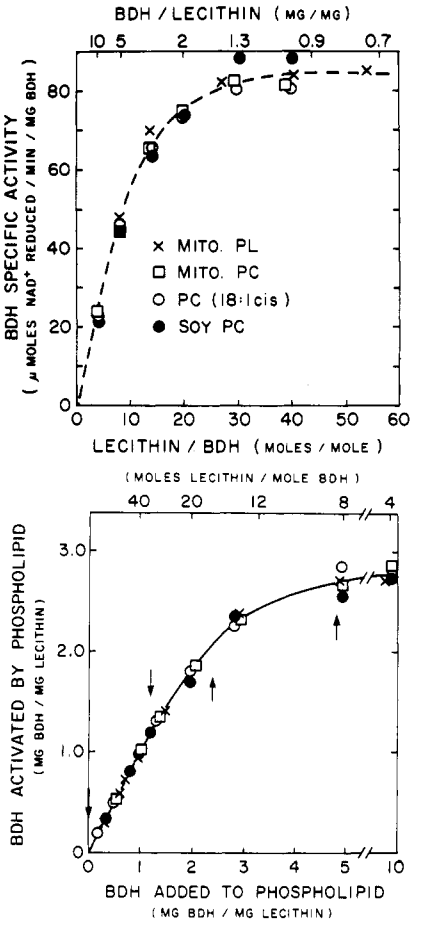

FIGURE 2: Activation of D-β-hydroxybutyrate apodehydrogenase (BDH) with different lecithins. A complex was preformed by admixing, in 0.1 mL of 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, and 5 mM dithiothreitol, the apoenzyme (10 μg) with variable amounts of lecithin (PC), either as beef heart mitochondrial phospholipid (×) or as codispersions of PC [mitochondrial (□), dioleoyl (○), or soybean (●)] together with beef heart mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) in a PC/PE/DPG phosphorus ratio of 1.0:8:0.2. Mitochondrial phospholipid is composed of PC, PE, DPG, and minor components (mainly phosphatidylinositol), which constitute 40, 37, 20, and 3%, respectively, of the total phosphorus (Fleischer et al., 1967). After preincubation for 15 min at room temperature (~25 °C), the enzymic activity of each complex was measured by the addition of a 5- or 10-μL aliquot to the cuvette which contained the standard enzymic assay medium [10 mM potassium phosphate (pH 7.35), 0.5 mM EDTA, 0.4 mg/mL bovine plasma albumin, 1.25% (v/v) ethanol, 0.3 mM dithiothreitol, 2 mM NAD^+, and 20 mM DL-β-hydroxybutyrate] preincubated to 37 °C. (A) Activation of the apodehydrogenase as a function of increasing phospholipid concentration. The BDH specific activity (μmol of NAD^+ reduced per min per mg of BDH) was calculated from the initial rate of reduction of NAD^+ measured at 340 nm. The activation of BDH by the four phospholipid vesicle preparations is the same within experimental error. The maximum specific activity obtained varies between 80 and 90 μmol of NAD^+ reduced per min per mg of BDH at 37 °C; this is equivalent to a specific activity of between 32 and 36 at 25 °C. (B) Activation of the apodehydrogenase as a function of increasing ratio of BDH to phospholipid. The data presented is a replot of that in Figure 2A. The amount of BDH activated was calculated from the specific activity obtained at each ratio of BDH to phospholipid and the maximal specific activity obtained with a saturating amount of the particular phospholipid vesicle preparation (cf. Figure 2A). The saturating amount of BDH activated, expressed relative to lecithin rather than to total phospholipid, is similar (2.78 mg of BDH per mg of lecithin) for the four phospholipid vesicle preparations; this is equivalent to 1.34 mg of BDH per mg of total phospholipid for the lecithins codispersed with PE and DPG and 1.25 mg of BDH per mg of mitochondrial phospholipid. The arrows mark the BDH/lecithin ratios of the four complexes prepared for ^{13}C NMR measurements by using either soybean [N-$^{13}CH_3$]lecithin (cf. Figure 4 and Table I) or [11-^{13}C]dioleoyllecithin (cf. Figure 6 and Table II).

N-$^{13}CH_3$ resonance of soybean lecithin remains sharp and at 54.1 ppm (Figure 3B,C). (The ratio of signal to noise is poorer than with phospholipid alone due to technical limitations on the amount and concentration of sample available for this measurement and the minimal signal accumulation time used.) T_1 was calculated to be 114 ± 7 ms at 6 °C for the highest ratio of apodehydrogenase to phospholipid (4.8 mg/mg of lecithin) and is thus significantly diminished compared with the T_1 of N-$^{13}CH_3$ of soybean lecithin in phospholipid vesicles in the absence of D-β-hydroxybutyrate apodehydrogenase. The motion of the ^{13}C nucleus in soybean [N-$^{13}CH_3$]lecithin is therefore constrained by insertion of the apodehydrogenase into the phospholipid vesicles containing this lecithin.[2] At the highest protein to lipid ratio used (4.8 mg of apodehydrogenase per mg of lecithin), the apoenzyme is in excess; that is to say, nearly all of the lecithin which can be titrated has interacted with the apoenzyme. Under these conditions approximately 60% of the apodehydrogenase has been activated (cf. Figure 2B). T_1 of [N-$^{13}CH_3$]lecithin (114 ± 7 ms at 6 °C) therefore approaches the T_1 which would be obtained at saturating apodehydrogenase. Upon subsequent additions of phospholipid vesicles, containing soybean [N-$^{13}CH_3$]lecithin, to form enzyme–phospholipid complexes of decreasing enzyme to phospholipid ratio, T_1 increases (Table I). There is a linear correlation between the decrease in T_1 and the increasing ratio of D-β-hydroxybutyrate apodehydrogenase to lecithin (Figure 4). A similar decrease is observed for T_1 measured at 20 °C. The measured T_1 of N-$^{13}CH_3$ in soybean lecithin therefore reflects an average of T_1 for lecithin bound to the apodehydrogenase and T_1 for unbound lecithin, i.e., in phospholipid vesicles in the absence of added apodehydrogenase. T_1 for N-$^{13}CH_3$ in soybean lecithin for the enzyme–liposome complex was not significantly altered by the addition of 0.5 mM NADH (Table I).

A similar series of measurements was made by using [11-^{13}C]dioleoyllecithin to study the interaction of D-β-hydroxybutyrate apodehydrogenase with a portion of the hydrophobic region of the lecithin molecule. Series of partially relaxed ^{13}C NMR spectra of [11-^{13}C]dioleoyllecithin, codispersed with mitochondrial phosphatidylethanolamine and diphosphatidylglycerol, in the absence and after the addition of D-β-hydroxybutyrate apodehydrogenase are shown in Figures 5A and 5B, respectively. The resonance is at 27 ppm relative to tetramethylsilane. T_1 was calculated to be 211 ± 12 ms at 20 °C; at 6 °C, T_1 is diminished to 113 ± 16 ms (cf. Table II). In the presence of D-β-hydroxybutyrate apodehydrogenase, T_1 measured at 6 °C increases linearly with the ratio of apodehydrogenase added to the phospholipid vesicles (Figure 6). At the highest apodehydrogenase to phospholipid ratio possible in these studies, 4.8 mg of apodehydrogenase per mg of lecithin, T_1 is 168 ± 23 ms. The molecular motion in the region of the ^{13}C nucleus of [11-^{13}C]dioleoyllecithin therefore appears to increase when the apodehydrogenase is inserted into the phospholipid vesicles. Addition of NADH to the apodehydrogenase–phospholipid complex does not significantly alter the T_1 of the ^{13}C-labeled lecithin at either 6 or 20 °C (Table II).

The shape of the resonance line of the ^{13}C nucleus in [11-^{13}C]dioleoyllecithin in the presence of D-β-hydroxybutyrate apodehydrogenase is altered; the base of the resonance line is broadened after complex formation with the apode-

[2] For relaxation by a dipole–dipole interaction mechanism, T_1 is proportional to $1/\tau_c$, where τ_c is the rotational correlation time of the ^{13}C nucleus. Thus, changes in T_1 reflect changes in the rotational motion of the ^{13}C-labeled moiety.

Table I: Carbon-13 NMR Measurement of Spin-Lattice Relaxation Times (T_1) for the Interaction of D-β-Hydroxybutyrate Apodehydrogenase (BDH) with Soybean [N-$^{13}CH_3$]Lecithin (PC)[a]

amount of BDH (mg)	amount of lecithin (mg)	BDH/PC (mg/mg)	PC/BDH (mol/mol)	BDH sp act. at ~25 °C[b]	spin-lattice relaxation time: temp of measurement (°C)	spin-lattice relaxation time: T_1 (ms)
			A. Phospholipid Vesicles			
0	10.0				6	182 ± 19
					20	289 ± 9
			B. BDH + Phospholipid Complexes			
12	2.5	4.8	8.2	17	6	114 ± 7
12	5.0	2.4	16.4	36	6	152 ± 12
12	10.0	1.2	32.8	31	6	166 ± 4
					20	256 ± 7
			C. Complex (BDH + Phospholipid) + NADH (0.5 mM)			
12	10.0	1.2	32.8		6	178 ± 19
					20	228 ± 8

[a] ^{13}C NMR spectra and spin-lattice relaxation times (T_1) were obtained as described in Figure 3. The complexes with varying ratios of BDH to ^{13}C-labeled lecithin were prepared as described in Figure 4. [b] The BDH specific activity (μmol of NAD^+ reduced/min per mg of BDH) for each BDH-phospholipid complex was measured as described in Figure 2 except that 3-mL curvettes were used and the assay temperature was ~25 °C. Specific activities of 17, 36, and 31 at 25 °C are equivalent to 42, 89, and 78, respectively, at 37 °C. The specific activities obtained (corrected for temperature) are in good agreement with those obtained for activation of BDH with phospholipid vesicles containing unlabeled lecithin (cf. Figure 2A). The diminished activity for the complex with 1.2 mg of BDH per mg of PC compared with that of 2.4 mg of BDH per mg of PC probably represents reversible inactivation arising from extensive handling of the sample since the samples with decreasing ratios of BDH to PC were prepared by sequential addition of PC to the initial sample (4.8 mg of BDH per mg of PC).

Table II: Carbon-13 NMR Measurement of Spin-Lattice Relaxation Times (T_1) for the Interaction of D-β-Hydroxybutyrate Apodehydrogenase (BDH) with [11-^{13}C]Dioleoyllecithin (PC)[a]

amount of BDH (mg)	amount of lecithin (mg)	BDH/PC (mg/mg)	PC/BDH (mol/mol)	BDH sp act. at ~25 °C[b]	spin-lattice relaxation time: temp of measurement (°C)	spin-lattice relaxation time: T_1 (ms)
			A. Phospholipid Vesicles			
0	10.0	0			6	113 ± 16
					20	211 ± 12
			B. BDH + Phospholipid Complexes			
12	2.5	4.8	8.2	16	6	168 ± 23
12	5.0	2.4	16.4	36	6	145 ± 21
12	10.0	1.2	32.8	33	6	126 ± 12
					20	216 ± 9
			C. Complex (BDH + Phospholipid) + NADH (0.5 mM)			
12	10.0	1.2	32.8		6	105 ± 25
					20	179 ± 6

[a] ^{13}C NMR spectra and spin-lattice relaxation times (T_1) were obtained as described in Figure 5. The complexes with varying ratios of BDH to ^{13}C-labeled lecithin were prepared as described in Figure 4, except that [11-^{13}C]dioleoyllecithin was used instead of soybean [N-$^{13}CH_3$]lecithin (cf. Figure 6). [b] The BDH specific activity (μmol of NAD^+ reduced per min per mg of BDH) for each BDH-phospholipid complex was measured as described in Figure 2 except that 3-mL cuvettes were used and the assay temperature was ~25 °C. Specific activities of 16, 36, and 33 at 25 °C are equivalent to 40, 89, and 82, respectively, at 37 °C. The specific activities obtained (corrected for temperature) are in good agreement with those obtained for the activation of BDH with phospholipid vesicles containing unlabeled lecithin (cf. Figure 2A).

hydrogenase (Figure 5B), although the sharp resonance is still observed in this sample. The T_1 relaxation times of the broad and sharp components are similar. Actually, the lipid vesicles containing the [11-^{13}C]dioleoyllecithin admixed with phosphatidylethanolamine and diphosphatidylglycerol exhibit some line broadening in the absence of the apodehydrogenase (Figure 5A). However, the incorporation of D-β-hydroxybutyrate apodehydrogenase into the liposomes results in increased line broadening and a decrease in amplitude of the sharp component. In the limit, when the amount of apodehydrogenase added is 4.8 mg/mg of lecithin, the sharp signal is completely lost and only the broad signal is observed (not shown).

Discussion

The interaction of lecithin with purified D-β-hydroxybutyrate apodehydrogenase, a lipid-requiring enzyme, has been studied by ^{13}C NMR by using lecithin with a ^{13}C label in the polar (choline) moiety (soybean [N-$^{13}CH_3$]lecithin) and in the hydrophobic fatty acyl chain [bis([11-^{13}C]dioleoyl)lecithin]. This study was achieved by purification of sufficient quantities of D-β-hydroxybutyrate apodehydrogenase to homogeneity and devoid of phospholipid. Addition of phospholipid-containing lecithin to the apodehydrogenase results in formation of an active enzyme–phospholipid complex, making possible the study of the motion of the lecithin molecule in lipid vesicles in the absence and presence of the apodehydrogenase. Even using 12 mg of the purified apodehydrogenase, we were limited in sensitivity for the ^{13}C NMR measurements, and it was necessary to make sequential additions of phospholipid to the apodehydrogenase to optimize use of the enzyme. Although the enzyme–phospholipid complex is relatively stable (cf. Figure 1), the requirement of sequential additions limited the length of time during which the ^{13}C NMR measurements could

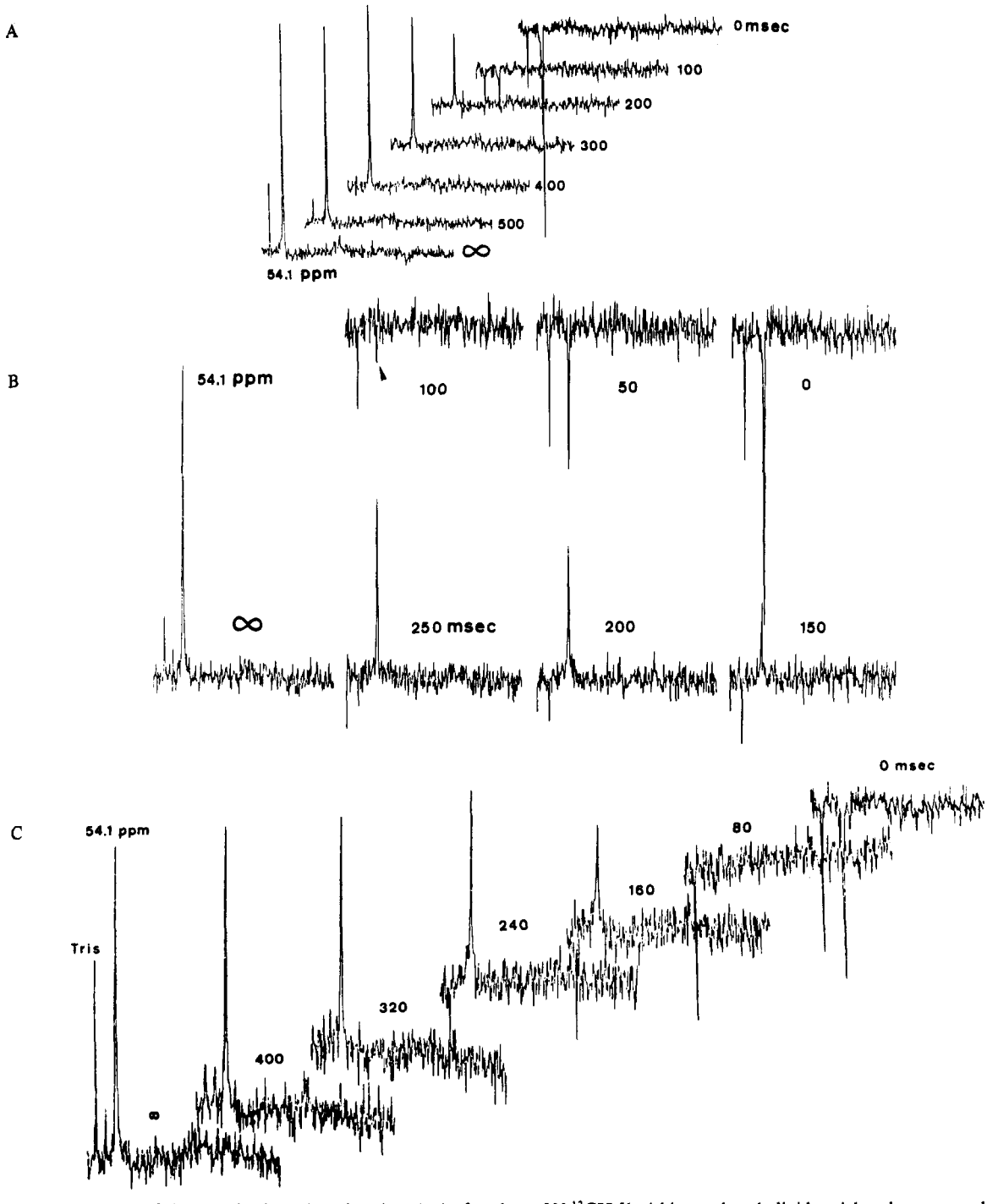

FIGURE 3: Measurement of the spin–lattice relaxation time (T_1) of soybean [N-$^{13}CH_3$]lecithin as phospholipid vesicles alone or combined with D-β-hydroxybutyrate apodehydrogenase. Soybean [N-$^{13}CH_3$]lecithin (PC) was codispersed with mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) at a PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2 (cf. Materials and Methods), and the solution was adjusted to final concentrations of buffer [0.1 M LiBr, 20 mM Tris-HCl (pH 8.1), 1 mM EDTA, 5 mM dithiothreitol, and 20% (v/v) D_2O] (NMR assay buffer). The ^{13}C NMR spectra were obtained at 22.63 MHz with a Bruker WH-90 pulse spectrometer operating in the Fourier transform mode. A 180° – t – 90° pulse sequence was employed where t is the delay time between the 180° and 90° pulses. Operating temperature was 6 ± 1 °C. A series of partially relaxed spectra is shown in each of Figure 3A–C in which the delay time (t) is indicated to the right of each tracing. The spin–lattice relaxation time (T_1) was computed from the slope of the plot of ln ($M_0 - M_z$) vs. t since $M_0 - M_z = 2M_0 \exp(t/T_1)$, where M_0 is the equilibrium amplitude of the fully relaxed spectrum (i.e., the free induction decay for $t = \infty$) and M_z is the amplitude of a partially relaxed spectrum (amplitude in arbitrary units). The N-$^{13}CH_3$ resonance of soybean lecithin is at 54.1 ppm relative to tetramethylsilane. (A) Phospholipid vesicles (PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2) at 10 mg of [N-$^{13}CH_3$]lecithin in ~3 mL of NMR assay buffer. Number of scans = 2000. The T_1 time (182 ± 19 ms) was independent of the concentration of phospholipid vesicles in the range 2–10 mg of lecithin/mL. (B) Phospholipid vesicles (10 mg of [N-$^{13}CH_3$]lecithin) complexed with 12 mg of purified D-β-hydroxybutyrate apodehydrogenase in approximately 3 mL of NMR assay buffer. Number of scans = 4000. The T_1 time, calculated from the amplitude of the 54.1-ppm signal at different delay times t, as indicated, was 166 ± 4 ms. (C) The same as Figure 3B except with 2.5 mg of [N-$^{13}CH_3$]lecithin as phospholipid vesicles. Number of scans = 16 000; T_1 = 114 ± 7 ms.

be performed at each enzyme to phospholipid ratio. For the sample with the highest apodehydrogenase to lecithin ratio (4.8 mg of apodehydrogenase per mg of lecithin), the signal to noise was suboptimal (cf. Figure 3C), yet the spectra were sufficient to obtain an adequate measurement of T_1. However, the limitations of the amount of sample and pulsing time limit an

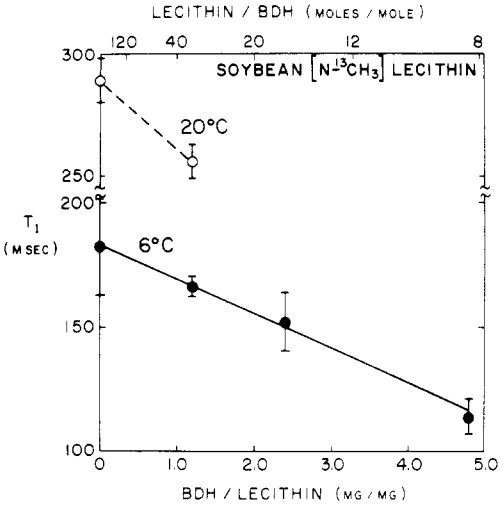

FIGURE 4: The variation in the measured spin–lattice relaxation time (T_1) of soybean [N-$^{13}CH_3$]lecithin as a function of varying the ratio of D-β-hydroxybutyrate apodehydrogenase (BDH) to lecithin (PC). Soybean [^{13}N-CH_3]lecithin was codispersed with beef heart mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2) as described under Materials and Methods. The T_1 time at 6 (●) and at 20 °C (○) of the [N-$^{13}CH_3$]lecithin in the absence of BDH was measured by using 10 mg of [N-$^{13}CH_3$]lecithin as microdispersion in ~3 mL of NMR assay buffer (cf. Figure 3A). A complex of BDH (12 mg) with 2.5 mg of soybean [N-$^{13}CH_3$]lecithin, as codispersion with PE and DPG (PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2), was prepared (cf. Materials and Methods) (BDH/lecithin ratio 4.8 mg of BDH per mg of lecithin), and the T_1 of the [N-$^{13}CH_3$]lecithin was measured at 6 °C (cf. Figure 3C). Subsequent additions of soybean [N-$^{13}CH_3$]lecithin (as codispersion with PE and DPG) were made and incubated (cf. Materials and Methods) to obtain BDH–phospholipid complexes with the BDH/lecithin ratios indicated (2.4 and 1.2 mg of BDH per mg of lecithin). With each addition of phospholipid, the NMR assay buffer (containing fresh dithiothreitol) was replaced by dilution of the sample to 20 mL and reconcentration (Amicon ultrafiltration) to ~3 mL to help insure sufficient reducing equivalents. An anaerobic atmosphere (N_2 or argon) was maintained wherever possible. For each sample, T_1 was measured at 6 °C (●) and, for the final BDH–phospholipid complex (1.2 mg of BDH/mg of lecithin), also at 20 °C (○). BDH specific activity was measured before and after each T_1 measurement for each BDH–phospholipid complex as described in Figure 2 except that 3-mL cuvettes were used and the assay temperature was ~25 °C. The BDH specific activities (at ~25°C) were 17, 36, and 31 μmol of NAD^+ reduced per min per mg of BDH (equivalent to 42, 90, and 78, respectively, at 37 °C) for the BDH–phospholipid complexes of 4.8, 2.4, and 1.2 mg of BDH per mg of lecithin, respectively (cf. Table I).

accurate line shape analysis or nuclear Overhauser enhancement (NOE) measurement to establish the nature of the relaxation mechanism of the ^{13}C nucleus in lecithin in the presence of D-β-hydroxybutyrate apodehydrogenase. (Accurate NOE measurements require a minimal noise level and can therefore only be carried out with larger amounts of material.)

For relaxation by a dipole–dipole interaction mechanism, T_1 is proportional to $1/\tau_c$, where τ_c is the rotational correlation time of the ^{13}C nucleus. Although we were unable in these studies to establish by NOE measurements (due to the limitations mentioned above) whether the relaxation mechanism is exclusively dipole–dipole or whether the narrowing limits are fulfilled (NOE > 1.5), other ^{13}C NMR and NOE measurements of the motion of the ^{13}C nuclei in soybean [N-$^{13}CH_3$]lecithin and [11-^{13}C]dioleoyllecithin indicate that the relaxation mechanism is indeed exclusively dipole–dipole and that the molecules do not rotate isotropically (Stoffel, unpublished experiments). The analysis of the molecular motion is not yet resolved, although $1/T_1$ appears proportional to τ_c. Therefore, $1/T_1$ is an expression of motion, and a comparison of the molecular motion exhibited by a particular ^{13}C nucleus, under different conditions, can be obtained by comparison of $1/T_1$ values. Neither the isotropic tumbling nor the translational movement of the whole lipid vesicle influences the T_1 values of [11-^{13}C]dioleoyllecithin since T_1 is not altered by increasing viscosity (Stoffel et al., 1974a). Changes in T_1 values therefore reflect changes in the rotational mobility of the labeled carbon segment.

The interaction of D-β-hydroxybutyrate apodehydrogenase with phospholipid vesicles containing ^{13}C-labeled lecithin alters the motion of the lecithin molecule as measured by the change in $1/T_1$ (cf. Table III). For soybean [N-$^{13}CH_3$]lecithin, $1/T_1$ increased by 60% for the apodehydrogenase–phospholipid complex (4.8 mg of apodehydrogenase per mg of lecithin) compared with phospholipid vesicles alone. This indicates a decreased motion of the ^{13}C nucleus in the choline moiety bound to the enzyme, reflecting the high specificity of lecithin for activation of D-β-hydroxybutyrate dehydrogenase (Fleischer et al., 1974; Gazzotti et al., 1975; Isaacson et al., 1979). This is the first confirmation by using a biophysical technique of the specific interaction of this lecithin-requiring enzyme with the choline moiety of lecithin and is the only case known to us in which the polar head group of a phospholipid is constrained by interaction with protein. The average mobility of the polar head group in the bilayer increases with the subsequent addition of phospholipid to the initial apodehydrogenase–phospholipid complex (Figure 4), implying that T_1 for the constrained phospholipid (at 4.8 mg of apodehydrogenase per mg of lecithin) represents an average for both bound and free lecithin. We observe no significant line broadening of the N-$^{13}CH_3$ label in the presence of excess D-β-hydroxybutyrate apodehydrogenase.

The effect of D-β-hydroxybutyrate apodehydrogenase on the T_1 time of [11-^{13}C]dioleoyllecithin is also without precedence. In contrast to the results obtained with [N-$^{13}CH_3$]lecithin, the $1/T_1$ decreased by 33% in the presence of excess apodehydrogenase. The longer T_1 relaxation time compared with that of [11-^{13}C]lecithin in the phospholipid bilayer suggests an increased motion arising from the insertion of the apodehydrogenase. It is noteworthy that the efficiency of activation of the apodehydrogenase by lecithins with fatty acyl moieties of varying chain length is diminished with decreasing chain length (Gazzotti et al., 1975). The hydrophobic region of lecithin is clearly essential for activation and is required for the effective interaction of lecithin with D-β-hydroxybutyrate apodehydrogenase (Isaacson et al., 1979).

In addition to the increased T_1 time of [11-^{13}C]dioleoyllecithin by the formation of a complex with D-β-hydroxybutyrate apodehydrogenase, the line shape of the 11-^{13}C resonance is broadened (cf. Figure 5B). In the presence of excess apodehydrogenase (4.8 mg/mg of lecithin), the resonance is very broad and the sharp signal is not observed. Upon further addition of lecithin to obtain the complex with 2.4 mg of apodehydrogenase per mg of lecithin, the sharp spike reappears although the base remains broad. The line broadening may arise either from a heterogeneity of microenvironments with resulting chemical shift anisotropy or from immobilization giving rise to enhanced spin–spin relaxation (i.e., the spin–spin relaxation time, T_2, is reduced and the signal is "lifetime broadened" according to the uncertainty principle (Knowles et al., 1976). It is significant that no line broadening was observed previously when ^{13}C-labeled phospholipid was bound to either high-density apolipoprotein (Stoffel et al., 1974b,c) or the calcium pump protein of sarcoplasmic reticulum (Stoffel

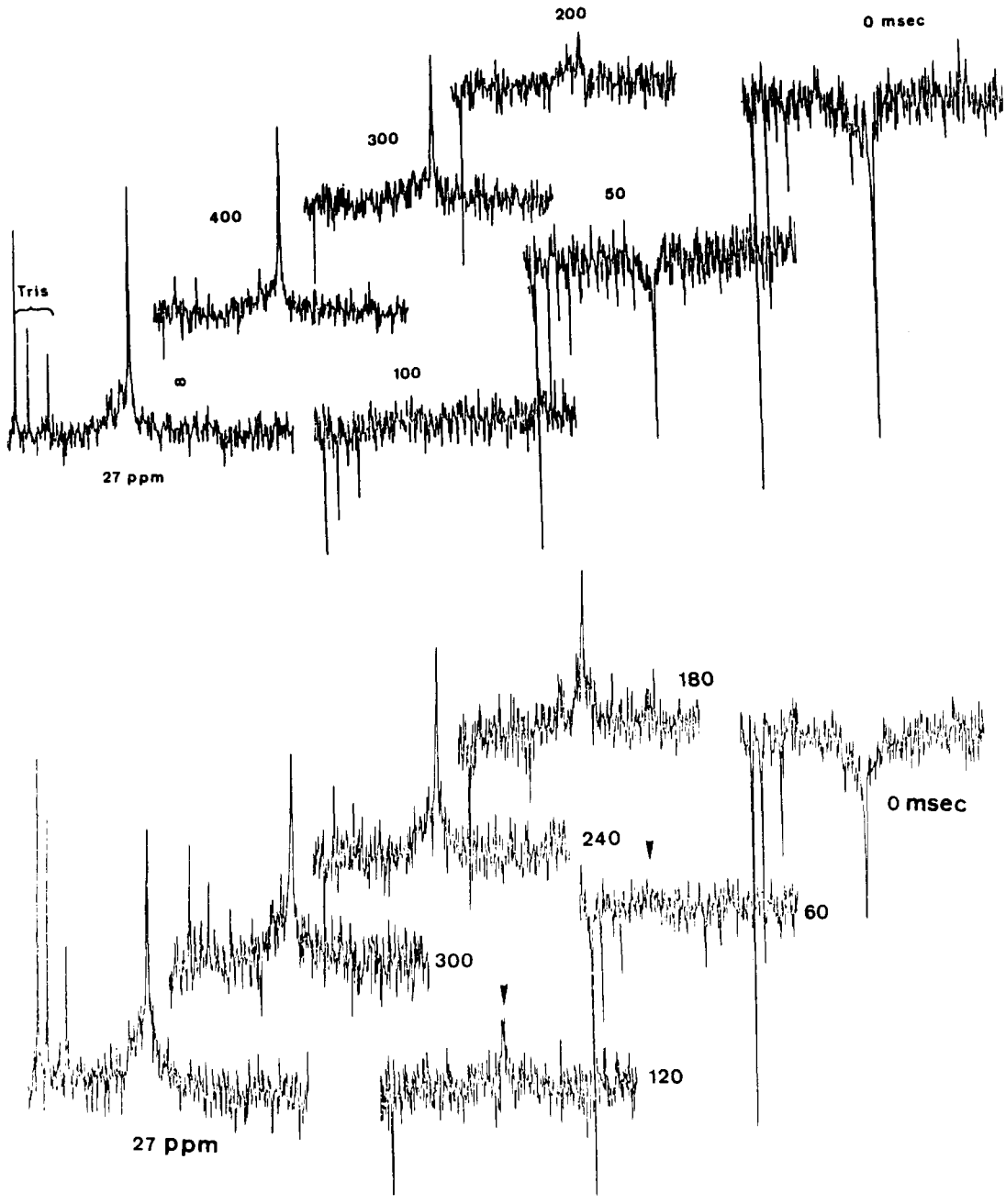

FIGURE 5: Measurement of the spin–lattice relaxation time (T_1) of [11-^{13}C]dioleoyllecithin as phospholipid vesicles alone or complexed with D-β-hydroxybutyrate apodehydrogenase. Samples were prepared and partially relaxed ^{13}C NMR spectra obtained as described in Figure 3 except that [11-^{13}C]dioleoyllecithin was used in place of soybean [N-$^{13}CH_3$] lecithin. The resonance of 11-^{13}C of [11-^{13}C]dioleoyllecithin is at 27 ppm relative to tetramethylsilane. (A) Partially relaxed spectra of 10 mg of [11-^{13}C]dioleoyllecithin as codispersion with mitochondrial phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) (PC/PE/DPG phosphorus ratio of 1.0:0.8:0.2) at 20 °C in ~3 mL of NMR assay buffer (cf. Figure 3). Number of scans = 6000. T_1, computed from the slope of ln ($M_0 - M_z$) vs. t (cf. Figure 3), was 211 ± 12 ms. (B) Partially relaxed spectra of 10 mg of [11-^{13}C]dioleoyllecithin as codispersion (cf. Figure 5A) complexed with 12 mg of D-β-hydroxybutyrate apodehydrogenase (BDH) in the presence of 0.5 mM NADH in ~3 mL of NMR assay buffer at 6 °C. Number of scans = 10000. T_1 was calculated to be 105 ± 25 ms. It should be noted that the base of the resonance line of the 11-^{13}C of the dioleoyllecithin is broadened after complex formation with the apodehydrogenase. The peak heights of the spike and the broad base were found to be proportional at the different delay times so that the T_1 times for both components of the resonance are similar. In the limit, the sample with the most D-β-hydroxybutyrate apodehydrogenase (4.8 mg of BDH per mg of lecithin) has lost the sharp spike in the 27-ppm resonance, and only the broad base of the resonance can be observed (not shown).

et al., 1977), although there was a significant decrease in T_1 (see below). Whether the line broadening, observed in [11-^{13}C]dioleoyllecithin upon addition of the apodehydrogenase, arises from immobilization of the alkane chains (constrained lateral diffusion and decreased T_2) or from chemical shift anisotropy, the ^{13}C-labeled phospholipid must be localized in two or more distinct environments. The broadened portion of the ^{13}C resonance of [11-^{13}C]dioleoyllecithin could represent "boundary" or "annulus" phospholipid as suggested by Jost et al. (1973) and Metcalfe & Warren (1977), respectively, or it could reflect a gradient of "constrained" motion extending between the protein and the free lipid bilayer.

When the line shape satisfies a Lorentzian line-shape function, the spin–spin relaxation time, T_2, can be estimated from the line width at half-height, ΔV, by using the relationship $\Delta V = 1/(\pi T_2)$. This relationship is not affected by proton-noise decoupling (W. Stoffel, H. Utsumi, and B. D.

Table III: Changes in T_1 Relaxation Times in [^{13}C]Lecithin after Formation of Lipid–Protein Complex[a]

lipid–protein complex	temp (°C)	T_1 relaxation time (ms) lipid vesicles[b]	+apo-BDH	% ΔT_1[c]	% $\Delta(1/T_1)$[c]	motion[c]
apo-BDH with						
PC (18:2) [N-$^{13}CH_3$]	6	182	114	−37	+60	slower
PC (18:2) [N-$^{13}CH_3$]	20	289				
PC (18:1) [11-^{13}C]	6	113	168[d]	+49	−33	faster
PC (18:1) [11-^{13}C]	20	211				
			+apo-HDL			
apo-HDL[e] with						
PC (18:0, 18:2) [N-$^{13}CH_3$]	37	430	485	+13	−11	faster
PC (18:0, 18:2) [14-^{13}C]	37	705	440	−38	+60	slower
			+CPP			
SR (Ca^{2+} pump protein)[f] with						
PC (18:2) [N-$^{13}CH_3$]	37	510	480	−6	+6	ca. no change
PC (18:0, 18:2) [14-^{13}C]	37	640	380	−40	+68	slower
PC (18:2) [14-^{13}C]	37	780	620	−21	+26	slower

[a] ^{13}C NMR spectra and T_1 times were obtained as described under Materials and Methods. The data obtained at 37 °C in the absence and presence of high-density apolipoprotein (apo-HDL) were reported previously (Stoffel et al., 1974b). The data for sarcoplasmic reticulum (SR) calcium pump protein (CPP) are taken from Stoffel et al. (1977). The other data are taken from Tables I and II. [b] For T_1 measurements, at 20 and 6 °C in the absence or presence of the apodehydrogenase (apo-BDH), the lipid vesicles contained lecithin, phosphatidylethanolamine, and diphosphatidylglycerol (phosphorus ratio of 1.0:0.8:0.2), the major components of beef heart mitochondrial phospholipid. For measurement of T_1 of ^{13}C-labeled lecithin in the absence and presence of apo-HDL at 37 °C, the lipid vesicles contained lecithin, sphingomyelin, cholesterol, and cholesterol esters in the proportions present in native HDL. T_1 of ^{13}C-labeled lecithin is not markedly altered by the composition of the lipid vesicles (Stoffel et al., 1974b). [c] % $\Delta T_1 = [T_{1(P\text{-}L)} - T_{1(L)}]100/T_{1(L)}$, where $T_{1(P\text{-}L)}$ and $T_{1(L)}$ refer to the T_1 of the protein–phospholipid complex and phospholipid vesicles, respectively. % $\Delta(1/T_1) = [1/T_{1(P\text{-}L)} - 1/T_{1(L)}]100/[1/T_{1(L)}]$. Since $1/T_1$ is proportional to τ_c (see text), an increase in % $\Delta(1/T_1)$ reflects an increase in τ_c or slower motion. [d] Exhibits line broadening suggesting constrained lipid (see text). [e] Stoffel et al. (1974b). [f] Stoffel et al. (1977).

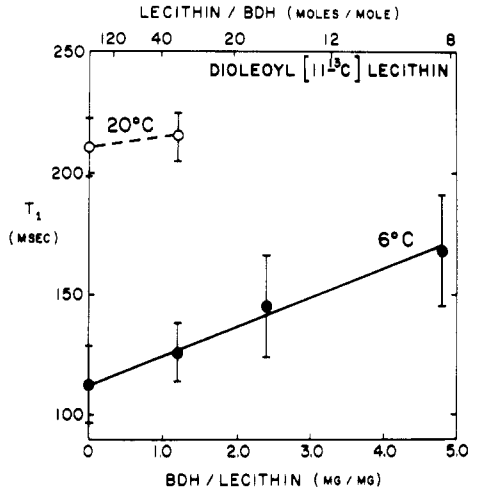

FIGURE 6: The variation in the measured spin–lattice relaxation time (T_1) of [11-^{13}C]dioleoyllecithin as a function of varying the ratio of D-β-hydroxybutyrate apodehydrogenase (BDH) to lecithin (PC). The samples were prepared as described in Figure 4 except that [11-^{13}C]dioleoyllecithin was used instead of soybean [N-$^{13}CH_3$]lecithin. The T_1 for each sample was measured as described in Figure 5. The BDH specific activities at ~25 °C (measured as described in Figure 4) were 16, 36, and 33 μmol of NAD^+ reduced per min per mg of BDH (equivalent to 40, 89, and 82 at 37 °C) for the BDH–phospholipid complexes containing 4.8, 2.4, and 1.2 mg of BDH per mg of lecithin, respectively (cf. Table II).

Tunggal, unpublished experiments). We estimate T_2 to be approximately 2–5 ms for the broadened component and 15–25 ms for the narrow component of the 11-^{13}C resonance in [11-^{13}C]dioleoyllecithin. The estimation of T_2 by Lorentzian line-shape analysis is complicated by the presence of narrow lines which may add disproportionately to the width at half-height determination. Further, anisotropic and restricted motions of component lecithin molecules, in addition to possible chemical shifts, may contribute to a broadening of the line shape so that the T_2 values are considered only to be estimates. The apparent T_2, obtained from the line shape, and the spin-lattice relaxation time (T_1) have been correlated with the lateral motion of the molecule and the rotational motion of the ^{13}C nucleus, respectively (Stoffel et al., 1974a, 1977). Thus, although we are unable to accurately measure T_2, the lateral diffusion rate of the bound phospholipid can be estimated to be 5 times slower than that of the free phospholipid, according to the extent of line broadening. By contrast, T_1 of [11-^{13}C]dioleoyllecithin is increased when D-β-hydroxybutyrate apodehydrogenase is added, indicating an increased rotational motion of the ^{13}C nucleus. Further, the semilogarithmic plots to obtain T_1 are not biphasic, so that the T_1 value reflects an average for the ^{13}C nuclei in the different environments. This means that the internal mobility of the labeled carbon segment depends only on the amount of phospholipid present in the complex.

Two other phospholipid–protein complexes, namely high-density lipoprotein (Stoffel et al., 1974b,c) and sarcoplasmic reticulum (Stoffel et al., 1977), have been studied previously by ^{13}C NMR. The results obtained for the three systems are compared in Table III. No significant change in $1/T_1$ of [N-$^{13}CH_3$]lecithin was observed in sarcoplasmic reticulum compared with that of the phospholipid vesicles alone. For high-density lipoprotein, there was a small decrease in $1/T_1$ (−11%) after formation of the phospholipid–lipoprotein complex, probably due to the increasing curvature of the particle surface. For [14-^{13}C]lecithin, $1/T_1$ was increased significantly in the protein–phospholipid complex as compared with the lipid vesicles by both apolipoprotein (+60%) and calcium pump protein (+68 and +26%, depending on the species of lecithin) (cf. Table III). Thus, for these two systems, the motion in the hydrophobic moiety of the lecithin was slowed down upon interaction with the protein. No line broadening of the ^{13}C resonance (14-^{13}C or N-$^{13}CH_3$) was observed upon the interaction of ^{13}C-labeled lecithin with either high-density apolipoprotein or sarcoplasmic reticulum. The ^{13}C NMR results obtained here for D-β-hydroxybutyrate apodehydrogenase–phospholipid complexes are unique for both polar and hydrophobic regions of the lecithin molecule. Upon

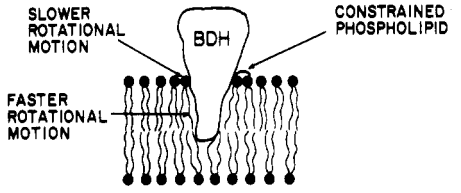

FIGURE 7: A diagrammatic representation of D-β-hydroxybutyrate dehydrogenase (BDH) inlaid in a phospholipid bilayer. BDH is shown in an *inlaid* orientation (McIntyre et al., 1978). The insertion of BDH into phospholipid vesicles results in slower rotational motion of the polar moiety of [*N*-$^{13}CH_3$]lecithin (cf. Table I and Figure 4) and faster rotational motion of the ^{13}C-labeled segment of the fatty acyl chain of the [11-^{13}C]lecithin (cf. Table II and Figure 6) as compared with the motion in the phospholipid vesicles alone. Constrained lateral motion of lecithin in the bilayer is suggested from the line broadening of the ^{13}C resonance of [11-^{13}C]lecithin.

addition of the apodehydrogenase to phospholipid vesicles containing ^{13}C-labeled lecithin, we find (1) a significant increase in $1/T_1$ (+60%) for [*N*-$^{13}CH_3$]-labeled lecithin, indicating reduced motion of the polar moiety; (2) a significant decrease in $1/T_1$ (−33%) for [11-^{13}C]-labeled lecithin, reflecting increased mobility of the labeled carbon segment of the hydrophobic moiety; and (3) line broadening of the 11-^{13}C resonance which suggests constrained lateral motion of the phospholipid or a heterogeneous microenvironment of the ^{13}C nucleus with resulting chemical shift anisotropy. A diagrammatic representation of D-β-hydroxybutyrate dehydrogenase inlaid in a phospholipid bilayer is shown in Figure 7. Comparison of the results obtained by ^{13}C NMR studies of three protein–phospholipid complexes demonstrates that the nature of the interaction of protein with phospholipid is characteristic for each specific protein.

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