

D-β-Hydroxybutyrate dehydrogenase is a lipid-requiring enzyme which is localized on the inner face of the mitochondrial inner membrane. The apoenzyme has been purified to homogeneity from beef heart; it is devoid of lipid and inactive. It can be functionally reconstituted with lecithin or phospholipid mixtures containing lecithin. The active form of the enzyme is the enzyme-phospholipid complex. Classical target analysis of radiation-inactivation data has now been used to determine the molecular size of the enzyme both in the native membrane (submitochondrial vesicles) and in the reconstituted enzyme inserted into phospholipid vesicles containing lecithin. For both forms of the enzyme, we find the same molecular size, ~110,000 daltons. This size is consistent with a tetramer. Radiation results in fragmentation of the polypeptide and the destruction of the polypeptide correlates with loss of enzymic function. A similar size is obtained when purified D-β-hydroxybutyrate dehydrogenase is inserted into a nonactivating mixture of phospholipid (i.e. in the absence of lecithin). We conclude that: 1) the native enzyme in submitochondrial vesicles and the purified active enzyme in phospholipid vesicles are the same size, approximating a tetramer; 2) radiation of D-β-hydroxybutyrate dehydrogenase results in loss of activity and fragmentation of the polypeptide; and 3) the role of lecithin in activation of D-β-hydroxybutyrate dehydrogenase is unrelated to determining oligomeric size of the enzyme since both active and nonactive forms exhibit the same structural size.

D-β-Hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD+ oxidoreductase, EC 1.1.1.30) is a lipid-requiring enzyme with a specific requirement of lecithin for enzymic activity. It has been found to be localized on the inner face of the mitochondrial inner membrane (McIntyre et al., 1978a). The enzyme has been purified from bovine heart mitochondria to homogeneity as the soluble, phospholipid-free apodehydrogenase which is inactive (Bock and Fleischer, 1974, 1975). When D-β-hydroxybutyrate apodehydrogenase is admixed with phospholipid vesicles, it appears to insert unidirectionally into the outer leaflet of the bilayer. When lecithin is present in the vesicles, an active enzyme-phospholipid complex is formed (Fleischer et al., 1974). The enzyme can also be activated with soluble lecithins, below the critical micellar concentration (Gazzotti et al., 1975). Analysis of the activation of the apodehydrogenase with soluble lecithins indicates that two lecithins per functional unit are required for activation (Cortese et al., 1982). The kinetic mechanism of D-β-hydroxybutyrate dehydrogenase is consistent with an ordered sequential reaction mechanism with kinetic parameters being similar for the enzyme in the native membrane and for the purified enzyme reactivated with MPL (Nielsen et al., 1973; Brenner et al.). Therefore, the isolation procedure does not appear to have significantly altered the enzyme and there does not appear to be substantial influence of other protein components of the mitochondrial membrane on enzymic catalysis (Nielsen et al., 1973).

The subunit size of D-β-hydroxybutyrate dehydrogenase is 31,000 daltons as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Bock and Fleischer, 1975). The hydrodynamic characteristics of both the inactive soluble apodehydrogenase and of the enzyme reactivated with short chain soluble lecithin (i.e. below the critical micellar concentration), indicated that the protein undergoes concentration-dependent self-association (McIntyre et al., 1978b). Active enzyme sedimentation indicates that the active form of the enzyme in the presence of soluble dioxanoyl lecithin is a dimer. These studies, together with NADH binding studies (1 mol of NADH binds per 2 mol of monomer, Gazzotti et al., 1974), indicated that the minimum functional size is a dimer of identical subunits. However, hydrodynamic techniques cannot be used to study either the size of the native enzyme in the mitochondrial membrane or the size of the purified enzyme inserted into natural phospholipid vesicles.

In the present work, we have used the radiation-inactivation technique (Pollard, 1953) to measure the molecular size of D-β-hydroxybutyrate dehydrogenase in the membrane (submitochondrial vesicles) as well as for the purified enzyme reconstituted in phospholipid vesicles. Further, we have compared the size of the reactivated enzyme with that of the enzyme inserted into a nonactivating mixture of phospholipids, i.e. in the absence of lecithin.1

1 The abbreviations used are MPL, the mixture of mitochondrial phospholipids extracted from beef heart mitochondria (Fleischer et al., 1967). Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

2 S. Brenner, J. O. McIntyre, N. Latruffe, and S. Fleischer, unpublished studies.

A preliminary report of portions of this work has been presented (J. O. McIntyre, P. Churchill, A. Maurer, C. Y. Jung, and S. Fleischer [1982] Target Molecular Weight of D-β-Hydroxybutyrate Dehydrogenase, 12th International Congress of Biochemistry, p. 365. Abstracts, Perth, Australia).
Target Molecular Weight of $\beta$-Hydroxybutyrate Dehydrogenase

EXPERIMENTAL PROCEDURES

Sucrose, density gradient grade, was obtained from Schwartz/Mann. glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from Escherichia coli was from Sigma. DL-$\beta$-hydroxybutyrate (sodium salt) was obtained from Sigma. Bovine plasma albumin solution, from Armour, was used as the protein standard. NAD$^+$ and dithiothreitol were obtained from Chemical Dynamics Corp. (South Plainfield, NJ). NAD$^+$ was also obtained from P-L Biochemicals, and Hepes from Calbiochem. All other chemicals were reagent grade. All solutions were prepared in deionized water.

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, the assay was carried out as described by Ross and Schatz (1973) using isoacetyl to carboxymethyl the dithiothreitol which would otherwise interfere with the assay for protein. Phosphorus was measured using a modification (Rouser and Fleischer, 1967) of the method of Chen et al. (1956).

$\beta$-Hydroxybutyrate dehydrogenase activity was measured spectrophotometrically as the rate of reduction of NAD$^+$ with $\beta$-hydroxybutyrate (sodium salt) as substrate as described previously (Bock and Fleischer, 1975). The activity was measured using the complex assay method in which a complex of the apodehydrogenase with MPL was preformed by incubation of the enzyme with the lipid vesicles as described below. An aliquot of the complex was added to the cuvette which was preincubated to 37 °C and contained 1 ml (final volume) of assay medium (10 mM potassium phosphate (pH 7.3), 0.5 mM EDTA, 0.4 mg/ml of bovine serum albumin, 0.3 mM dithiothreitol and 5 mM NAD$^+$). After 1 min incubation at room temperature, the cuvette was started by adding substrate $\beta$-hydroxybutyrate, to a final concentration of 20 mM. The initial rate of NADH production was measured at 340 nm and the $\beta$-hydroxybutyrate dehydrogenase specific activity of the sample was calculated taking the extinction coefficient of NADH to be 6.22 mM$^{-1}$ cm$^{-1}$. When submitochondrial vesicles were assayed, the method was modified. The sample (30 µl of 2 mg/ml) was added to the cuvette containing assay medium without substrate and then a 5-µl aliquot of antimycin A (0.1 mg/ml of ethanol) was added to block reoxidation of NADH by electron transport. After 2 min of incubation, the enzyme reaction was initiated by the addition of 0.1 ml of 0.2 M $\beta$-hydroxybutyrate. Glucose-6-phosphate dehydrogenase was assayed at 32 °C in 50 mM Tris-HCl (pH 7.8 at 25 °C), 3 mM MgCl$_2$, 3 mM NAD$^+$, and 3.3 mM glucose-6-phosphate (Olive and Levy, 1971).

Preparation of submitochondrial vesicles—$\beta$-Hydroxybutyrate dehydrogenase, and phospholipid vesicles—The heavy fraction of bovine heart mitochondria was prepared on a large scale (Blair, 1967) and were stored frozen at -80 °C. Submitochondrial vesicles were prepared from these mitochondria by nitrogen compression and shear, using the Parr bomb, as described by Fleischer et al. (1974a).

Mitochondrial lipids were prepared by extraction of bovine heart mitochondria with chloroform/methanol (2:1) and then back extracted to remove non-lipid materials (Folch as described in Fleischer et al., 1967). Mitochondrial phospholipids were prepared from the total lipid extract by separation of the neutral lipid using silicic acid chromatography (Rouser et al., 1971). Liposomes of MPL were prepared by dialysis of a butanol/cholate solution of MPL versus 20 mM Tris-HCl, 1 mM EDTA, pH 8.1, as described previously (Fleischer and Fleischer, 1967).

Single molecular species of phospholipids (dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, and 1-palmitoyl-2-oleoyl-phosphatidylpropan-1,3-diol) were synthesized according to the methods of Eibl (1978, 1980, 1981a, and 1981b). A vesicle preparation of a ternary mixture of these three phospholipids, at a $\alpha$-oleiithic/diethanolamine/dioleoylphosphatidylpropan-1,3-diol (5:4:1 ratio by phosphorus), was prepared as described previously (Eibl et al., 1982). This mixture of lipids, since it does not contain cholesterol, or choline-containing species of phospholipid, does not activate the apodehydrogenase (Fleischer et al., 1974a; Isaacson et al., 1979, Churchill et al., 1979).

$\beta$-Hydroxybutyrate dehydrogenase was purified from beef heart mitochondria essentially as described by Bock and Fleischer (1974, 1975) and stored in a liquid nitrogen refrigerator at -1 to -2 mg of protein/ml of 0.4 M LiBr, 5 mM Hepes, 5 mM dithiothreitol (pH 7.0). Mitochondrial phospholipids were prepared from bovine heart mitochondria according to the methods of Fleischer et al. (1974). Mitochondrial phospholipids were prepared from bovine heart mitochondria by chloroform/methanol (2:1) and then back extracted to remove non-lipid materials (Folch as described in Fleischer et al., 1967). Mitochondrial phospholipids were prepared from the total lipid extract by separation of the neutral lipid using silicic acid chromatography (Rouser et al., 1971). Liposomes of MPL were prepared by dialysis of a butanol/cholate solution of MPL versus 20 mM Tris-HCl, 1 mM EDTA, 5 mM dithiothreitol, and 5 mM NAD$^+$ with between 16 and 100 µg of enzyme/ml and various amounts of MPL, as indicated. Enzymatic activity was measured following 1 h preincubation at 25 °C to obtain optimal activity, after which time activity remained constant (Fleischer et al., 1979). Since the purified enzyme preparations used in these studies ranged between 115 and 135 µmol of NAD$^+$ reduced/min/mg of protein, at 37 °C, when optimally activated with MPL. The enzyme, added to phospholipids in aqueous buffer, appears to insert unidirectionally into the outer face of the phospholipid vesicles (Churchill et al., 1981).

Preparation of Samples for Radiation—The effect of irradiation on the native enzyme in the mitochondrial inner membrane was determined using submitochondrial vesicles. In intact mitochondria, the mitochondrial inner membrane presents a permeability barrier for NADH to reach the enzyme, which is localized on the inner face, thus complicating the measurement of enzymic activity (McIntyre et al., 1979a). For this reason, submitochondrial vesicles were used since enzymic activity is readily measured in such inside-out vesicles which have their matrix face exposed to the medium (Fleischer et al., 1974a). Submitochondrial vesicles were diluted to 2 mg of protein/ml in 0.25 M sucrose, 5 mM Hepes (pH 7.3), 1 mM EDTA, and 5 mM dithiothreitol. $\beta$-Hydroxybutyrate dehydrogenase (0.96 mg) was activated with MPL vesicles (58 or 174 µg of P) which had previously been centrifuged at 100,000 x g for 60 min and filtered through a 0.22 µm Tuffyn filter (Gelman Filter, Millipore Corp., Bedford, MA) in a total volume of 0.6 ml of 0.25 M sucrose, 5 mM Hepes, 1 mM EDTA, and 5 mM dithiothreitol. After incubation for 1 h at 25 °C, the active enzyme-phospholipid complex was then diluted with the same buffer to 24 ml. Formation of an inactive enzyme-phospholipid complex was achieved in like manner except that, instead of MPL, a nonactivating ternary mixture of phospholipids was used (see above). This mixture of lipids without lecithin did not activate the enzyme but forms a nonactive complex (Gazzotti et al., 1975). Glucose-6-phosphate dehydrogenase, used as an internal standard was diluted into 0.3 M sucrose, 0.1 M KCl, and 5 mM Hepes (pH 7.0) to a concentration of 5 units/ml. For each preparation, 2-ml aliquots were placed out to a depth of 1.14 mm in open aluminum trays (surface area -17.6 cm$^2$). The trays were each frozen by mounting them level over an insulated channel which was subsequently filled with liquid nitrogen. Freezing time was approximately 10-15 s per tray. The trays were shipped frozen in liquid nitrogen to Van de Graaff generator, producing a high-energy beam of 1.5 MeV electrons. Radiations were measured using the transmittance change of blue cellophane calibrated against a chemical dosimeter (Frack and Hite, 1966). The dose for each sample was controlled by varying the number of passes through the electron beam from zero to 16 with a measured dose of between 0.55 and 6.05 Mrad/pass. The radiation chamber was chilled with a stream of liquid nitrogen and the sample temperature (-40 to -50 °C) was monitored as described previously (Sacconi et al., 1981). Samples of either submitochondrial vesicles or enzyme-MPL or enzyme-nonactivating phospholipid complexes or glucose-6-phosphate dehydrogenase standard, were irradiated in pairs, so that reliable comparisons could be made between different sample preparations. After radiation, the samples were stored in a liquid nitrogen refrigerator for 24 h. The samples were then thawed and enzymic activities were promptly measured. Within experimental error, the samples were quantitatively recovered from the plates after thawing, as estimated by measurement of absorbance at maximum absorbance of each enzymic reaction. Thus, loss of activity after radiation was not due to loss of sample. Storage of the samples in the liquid nitrogen refrigerator for up to 4 days did not affect the results. Measured enzymic activities were stable for samples stored up to 9 h after thawing. Identical samples could be obtained after quick-freezing and storage of the samples in liquid nitrogen for up to 1 month. Control samples either simply frozen and thawed using the same protocol or manipulated through the Van de Graaff generator with the radiation beam turned "off" (irradiated), showed no loss of activity compared with the initial activity prior to freezing.

Data Presentation and Calculations—The data were analyzed with a single target, single hit model of radiation inactivation (Pollard, 1953). Plots of the logarithm of the percentage of surviving enzymic
activity were linear over one order of magnitude with correlation coefficients >0.98 (>0.96 for PAGE data). The data were fitted by least-squares with the value for the control sample weighted as twice the other values since the error in this point is smaller. Apparent molecular weights were calculated using the formula of Kepner and Macey (1968), i.e. molecular weight = \(6.4 \times 10^7/D_{50}\) (rads), where \(D_{50}\) is the dose of absorbed radiation required to reduce the enzyme activity or PAGE band intensity to 37% of the original. The data were fitted by the same as in the membrane of submitochondrial vesicles.

P-hydroxybutyrate dehydrogenase therefore appears to be the irradiated enzyme-MPL complexes served to activate freshly added D-P-hydroxybutyrate apodehydrogenase similarly to the nonirradiated sample (Fig. 2). Two parameters of the activation can be evaluated by titrating the apodehydrogenase with phospholipid vesicles or membranes (Gazzotti et al., 1975; McIntyre et al., 1979). Maximal specific activity, obtained in excess phospholipid, was the same with the irradiated and nonirradiated enzyme-MPL samples as with MPL alone. Efficiency of activation, the amount of lipid required to obtain half-maximal activation, is an index of the accessible lipid in the membrane (McIntyre et al., 1979). For this parameter, a higher number denotes poorer efficiency. The values obtained for activation of newly titrated apodehydrogenase by control and two irradiated samples were the same (23 mol of phospholipid/mol of added apodehydrogenase). This was somewhat less efficient than that obtained with MPL alone (19 mol of phospholipid/mol of added apodehydrogenase, Fig. 2). This lower efficiency of activation by the enzyme-MPL complexes versus MPL merely reflects that the enzyme, in the liposome, diminishes in part the amount of accessible phospholipid. If the loss in activity of the enzyme-MPL complexes after irradiation treatment were due to either the generation of inhibitors or phospholipid destruction, a decrease in the maximal specific activity and/or efficiency of activation would be obtained with freshly added apodehydrogenase. Neither of these effects is observed, suggesting that a process other than lipid damage or generation of inhibitors, results in loss in enzymic activity. Further, the loss of activity could be directly correlated with destruction of the enzyme.

**Correlation of Loss of Activity with Polypeptide Fragmentation**—The amount of enzyme in the purified enzyme-phospholipid complex, after exposure to various doses of radiation up to 8.8 megard, was measured by densitometry of PAGE and enzyme-MPL complexes, which had previously been irradiated, were prepared for PAGE as follows: Each sample (75 µl of 40 µg of D-β-hydroxybutyrate dehydrogenase/ml) was diluted with 25 µl of sample buffer (2 % Tris-HCl, pH 6.8, 8.0% sodium dodecyl sulfate, 2% (v/v) β-mercaptoethanol, 22% (v/v) glycerol and 0.22 mg bromophenol blue/ml) and incubated for 30 min at room temperature; 15 µl (450 mg enzyme) of each sample was applied to the gel. Sample denaturation appeared to be complete after this treatment since similar results were obtained with samples denatured by boiling in sample buffer, although there was greater error introduced by variations in the sample volume after boiling. Electrophoresis (1.5 milliwatts/gel) was carried out using a Hoefer slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) with cooling. Electrophoresis was terminated when the tracking dye (bromophenol blue) reached the end of the gel (1-2 h).

The enzymic activities of the initial samples before freezing were 130 and 8,000 reduced/min/mg of protein at 37°C for BDH-MPL and SMV, respectively. "Sham" irradiated controls (electron beam turned off) retained full activity. The slope of each inactivation profile was determined by least-squares fit of the data. The molecular weight (M, 6.6 Mrad) and 114,000 8,000 Daltons for the enzyme in SMV and purified BDH-MPL, respectively. Theoretical inactivation lines (solid) are shown for either monomer (a), dimer (b), or tetramer (c) of BDH with target sizes of 30, 60, or 120 kilodaltons, respectively. Experimental data from a number of experiments are averaged in Table I.

**RESULTS**

**Effect of Radiation on Enzymic Activity**—Inactivation of D-β-hydroxybutyrate dehydrogenase, in both submitochondrial vesicles and the purified enzyme reconstituted into MVL vesicles, occurred as a simple exponential function of radiation dose (Fig. 1). The lines drawn in Fig. 1 were determined by least-squares fit of the weighted data (cf. "Experimental Procedures") to the theoretical exponential decay of activity with dose. From the slope of these lines, \(D_{37}\) values were determined from which target sizes were calculated (Fig. 1). From several such studies, average values for molecular sizes of 106,000 ± 8,000 and 119,000 ± 8,000 were determined for D-β-hydroxybutyrate dehydrogenase activity in submitochondrial vesicles and enzyme-MPL complex, respectively (Table I). These values are the same within experimental error and were found not to be significantly different using Student's t test (p < 0.05). The oligomeric structure of purified and reactivated D-β-hydroxybutyrate dehydrogenase therefore appears to be the same as in the membrane of submitochondrial vesicles.

Loss in enzymic activity by irradiation might have resulted from the destruction of the phospholipid, which is essential for function, or from the generation of potent inhibitors of the enzyme. This does not appear to be the case. The lipid of the irradiated enzyme-MPL complexes served to activate freshly added D-β-hydroxybutyrate apodehydrogenase similarly to the nonirradiated sample (Fig. 2). Two parameters of the activation can be evaluated by titrating the apodehydrogenase activity or PAGE band intensity to 37% of the original.
newly inserted BDH (120 ± 5 μmol of min/mg of NAD+ reduced/min/mg of BDH) was obtained with each of the samples. The efficiency of activation (amount of lipid which gives half-maximal activation, see text) was the same (23 μg of P/mg of BDH) for the three BDH-MPL complexes which had received different dosages of irradiation. MPL, to which BDH had not previously been added, had somewhat better efficiency of activation (19 μg of P/mg of BDH). The reactivation characteristics of freshly introduced BDH by the lipid in BDH-MPL complexes was, within experimental error, independent of the irradiation exposure.

in sodium dodecyl sulfate using silver staining (Fig. 3). There was a progressive decrease in silver stain intensity of the enzyme band with increasing radiation dose (Fig. 3, A and B). The gels with decreased enzyme band intensity generally showed some lower molecular weight material as a broadened smear, which stained only faintly and variably at this level of loading. The fragments could be observed routinely at higher protein loading either by the silver stain or with Coomassie brilliant blue (not shown). Irradiation did not give rise to higher molecular weight material in either the stacking or running gel or at the interface between the two. Therefore, we conclude that irradiation of D-β-hydroxybutyrate dehydrogenase results in polypeptide fragmentation with production of a distribution of lower molecular weight peptides. Calibration of the enzyme band intensity versus the amount of applied enzyme is linear in the concentration range from 45 to 450 pg (Fig. 3C). The plot of the logarithm of the integrated peak area of the PAGE band as a function of radiation dose for the enzyme-MPL complex gave a straight line (Fig. 4). Target size analysis of radiation-induced fragmentation of the polypeptide, from two separate experiments, yields a structural size of about 104,000 daltons, the same within experimental error as that obtained by enzymic activity measurements of these same samples (Fig. 4 and Table I). Thus, irradiation leads to concomitant loss of enzymic activity and fragmentation of the protein.
The size of the inactive enzyme, inserted into phospholipid vesicles devoid of lecithin was also measured. The decrease in intensity of the enzyme band on polyacrylamide gels with increasing radiation dose was similar to that of the active enzyme-MPL complex (Fig. 4). Target size analysis yielded a molecular size of approximately 110,000 daltons. Thus, the nonactive enzyme in phospholipid has a size comparable to that of the active enzyme-MPL complex and as well as the enzyme in the membrane of submitochondrial vesicles (cf. Table I).

DISCUSSION

D-β-Hydroxybutyrate dehydrogenase is one of the most extensively studied lipid-requiring enzymes. This is the first study in which the radiation-inactivation technique has been used to compare the target size of a purified and reconstituted membrane enzyme with that of the enzyme in the native membrane. Within experimental error, the size of D-β-hydroxybutyrate dehydrogenase, either native or reconstituted, is approximated a tetramer. Inactivation analysis has also been used to evaluate the influence of lecithin on the size of this lipid-requiring enzyme. We find the same oligomeric size for the enzyme in activating mixtures of phospholipid containing lecithin as with a nonactivating phospholipid mixture. Therefore, the lecithin does not activate the enzyme by way of regulating oligomeric size. We find that inactivation of enzyme activity upon irradiation is not attended by destruction of the phospholipid which is required for enzyme function, but arises from fragmentation of the polypeptide. Further freeze-fracture electron microscopy studies indicate that loss of D-β-hydroxybutyrate dehydrogenase activity by irradiation of submitochondrial vesicles is not attended by gross morphological changes in membrane structure.5

The size of D-β-hydroxybutyrate dehydrogenase obtained by classical target theory analysis of radiation inactivation data is approximately 110,000 daltons both in submitochondrial vesicles and for the purified enzyme reactivated by insertion into vesicles of MPL. Therefore, the oligomeric structure of D-β-hydroxybutyrate dehydrogenase does not appear to be altered following isolation, purification, and reactivation with MPL. This is consistent with the unaltered kinetic parameters of this preparation, reactivated with MPL, compared with the native enzyme (Nielsen et al., 1973). The subunit size by PAGE in sodium dodecyl sulfate is 31,000 daltons (Bock and Fleischer, 1975), but appears slightly smaller (28,500 daltons) by equilibrium sedimentation under denaturing conditions.5 The radiation inactivation data analyzed by the single target, single hit model of radiation inactivation (Pollard, 1953). Apparent molecular weights were calculated using the formula of Kepner and Macey (1968), i.e.

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M = 4.4 \times 10^{3}/D_{m}\ (\text{rads}),
\]

where \(D_{m}\) is the dose of absorbed radiation required to reduce the enzymatic activity to 37% of the original. The errors given are the standard deviation of the slope of the linear least-square fit of the data (Fig. 4). Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides was incorporated in some samples as a standard and was found to have a target size of 90,000 ± 5,000 daltons in the presence or absence of membranes. This size is 87% of the size (103,700 daltons) of this enzyme measured by sedimentation equilibrium at 20 °C (Olive and Levy, 1971).

5 A. Maurer and S. Fleischer, unpublished studies.
studied at low concentration of enzyme (30 μg of protein/ml or lower). The phospholipid-free apodehydrogenase in aqueous buffer was studied by conventional sedimentation analysis at higher protein concentrations (60–1200 μg of protein/ml) and was found to undergo concentration-dependent self-association. Under these conditions, the enzyme associates to form a heterogeneous population of components, some much larger than a tetramer (McIntyre et al., 1979b). Therefore, the presence of phospholipid serves to limit self-association to a tetramer. Active enzyme sedimentation studies of the enzyme, activated with soluble lecithins, show a dimeric functional unit (McIntyre et al., 1978b). The molecular size of the enzyme, activated with soluble lecithin, could not be confirmed by the radiation-inactivation method due to the following technical limitations: (a) the lipid-free enzyme and the complex with soluble lecithin both exhibit concentration-dependence of molecular size (McIntyre et al., 1978b); (b) the specific activity of the enzyme activated with such lecithins is concentration-dependent (McIntyre et al., 1978b); (c) soluble lecithins exhibit film-flow properties; and (d) there is a temperature-dependent change in critical micellar concentration of the short chain soluble lecithins. Although a tetramer appears to be the native oligomeric structure, other oligomeric species may have enzymic activity under different conditions, such as in the presence of soluble lecithins.

The apparent molecular size of purified enzyme-MPL complex was not significantly different at two different lipid/protein ratios used in this study. The MPL vesicles, prepared as described here, have an average diameter of 650 Å by quasielastic light scattering measurements so that there are approximately 530 or 175 enzyme monomers/phospholipid vesicle for 60 to 180 mol of phospholipid/mol of enzyme monomer, respectively. Thus, the apparent molecular size of the δ-β-hydroxybutyrate dehydrogenase in MPL vesicles is not influenced by the number of polypeptides per phospholipid vesicle within the range of this study.

The loss of enzymic activity upon irradiation of the purified δ-β-hydroxybutyrate dehydrogenase-MPL complex appears to result from radiation-induced fragmentation of the 31,000-dalton band (Bock and Fleischer, 1975) observed by PAGE in sodium dodecyl sulfate. Fragmentation of the polypeptide exhibits the same radiation dose dependency as that of the enzyme in activating the enzyme is not related to modulating the oligomeric size. The difference in oligomeric size of the enzyme reactivated with soluble lecithins (see above) as compared with the enzyme inserted into phospholipid vesicles is an important one. It points out that enzymic activity is more critically dependent on the presence of lecithin rather than whether the enzyme exists as a dimer or tetramer.

The temperature of irradiation influences the apparent target size of some enzymes (Kempner and Schlegel, 1979; Schlegel et al., 1979). In studies of the (H' + K')-ATPase, a 20% higher molecular size was obtained for the enzyme as a lyophilized film at 20 °C as compared with the apparent target size in frozen suspensions at −50 °C (Saccomani et al., 1981). The temperature for radiation used in the study reported here (−50 °C) and that reported by Saccomani et al., (1981) in the frozen state were the same. In our studies, glucose-6-phosphate dehydrogenase, has been used as an internal standard, to correct for temperature and other variables. The target size for the internal standard, irradiated under conditions similar to those used for δ-β-hydroxybutyrate dehydrogenase, was 90,000 ± 5,000 (cf. Table I) or 13% lower than the size as measured by sedimentation equilibrium at 20 °C (Olive and Levy, 1971). This correction factor is, within experimental error, the same as the temperature correction factor obtained by Saccomani et al. (1981). Thus, the ~110,000-dalton target size that we obtain for δ-β-hydroxybutyrate dehydrogenase appears to be underestimated by 10–20% due to the conditions for the low temperature of irradiation. When a temperature correction factor of 13% is applied to our results with δ-β-hydroxybutyrate dehydrogenase, a target size of ~124,000 daltons is obtained, which is close to a tetramer.

The usefulness of target size analysis lies in the ability to determine the functional molecular weight of a protein regardless of the state of purification as long as the size is unaltered by sample preparation. In the study reported here, we have obtained the size of δ-β-hydroxybutyrate dehydrogenase both in the membrane and as the purified enzyme-phospholipid complex. The functional sizes of a number of transport proteins have previously been reported. The (Na' + K')-ATPases of human erythrocyte ghosts, guinea pig kidney microsomes, and plasma membrane preparations have been shown to have sizes of 250,000 daltons (Kempner and Macey, 1968). The calcium pump protein of sarcoplasmic

*E. Chang, J. O. McIntyre, and S. Fleischer, unpublished studies.
Target Molecular Weight of \( \text{D-} \text{\beta}-\text{Hydroxybutyrate Dehydrogenase} \)

Eibl, H. (1989) Chem. Phys. Lipids 26, 405-429
Eibl, H. (1981a) Chem. Phys. Lipids 28, 1-5
Eibl, H., and Kovatchev, S. (1981b) Methods Enzymol. 72, 632-639
Eibl, H., Churchhill, P., McIntyre, J. O., and Fleischer, S. (1982) Biochem. Int. 4, 551-558
Fleischer, S., Rousset, G., Fleischer, B., Casu, A., and Kritchevsky, G. (1987) J. Lipid Res. 8, 170-189
Fleischer, S., Bock, H.-G., and Gazzotti, P. (1974) in Membrane Proteins in Transport and Phosphorylation (Klingenberg, M., and Azone, G. F., eds) pp. 125-136, North-Holland Publishing Co., Amsterdam
Fleischer, S., Meissner, G., Smigel, M., and Wood, R. (1974b) Methods Enzymol. 31, 292-299
Fleischer, S., and Fleischer, B. (1967) Methods Enzymol. 10, 406-433
Frck, H., and Hart, E. J. (1966) in Radiation Dosimetry (Attix, F. H., and Roesch, W. C., eds) Vol. 11, pp. 167-239, Academic Press, New York
Gazzotti, P., Bock, H.-G., and Fleischer, S. (1975) J. Biol. Chem. 250, 5782-5790
Haa, J. S., Cha, C., and Haas, H. N. (1980) J. Biol. Chem. 255, 351-364
Kepner, G. R., and Macey, R. I. (1968) Biochim. Biophys. Acta 163, 180-203
Kemper, E. S., and Schlegel, W. (1979) Anal. Biochem. 92, 2-10
Lämml, U. K. (1970) Nature (Lond.) 227, 680-685
Lehninger, A. L., Sadth, H. D., and Wise, J. B. (1960) J. Biol. Chem. 235, 2450-2455
Lo, M. M. S., Barnard, E. A., and Dolly, J. O. (1982) Biochemistry 21, 2210-2217
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
Merrill, C., Goldmann, D., and van Keuren, M. L. (1983) Methods Enzymol. 96, in press
McIntyre, J. O., Bock, H.-G., and Fleischer, S. (1978a) Biochim. Biophys. Acta 515, 255-267
McIntyre, J. O., Holladay, L. A., Smigel, M., Puett, D., and Fleischer, S. (1978b) Biochemistry 17, 4169-4177
Mintyre, J. O., Wang, C., and Fleischer, S. (1979) J. Biol. Chem. 254, 5199-5207
Nielson, N. C., and Fleischer, S. (1979) Science 166, 1017-1019
Nielson, N. C., Zahler, W. L., and Fleischer, S. (1973) J. Biol. Chem. 248, 2556-2562
Olive, C., and Levy, H. R. (1971) J. Biol. Chem. 246, 2043-2046
Parkinson, D., and Callingham, B. A. (1982) Radiat. Res. 90, 252-259
Pollard, E. C. (1959) Adv. Biol. Med. Phys. 3, 153-189
Ross, E., and Schatz, G. (1973) Anal. Biochem. 54, 304-306
Rousset, G., and Fleischer, S. (1987) Methods Enzymol. 10, 385-406
Rousset, G., Kritchevsky, G., and Yamamoto, A. (1967) in Lipid Chromatographic Analysis (Marinetti, G. V., ed) Vol 1, pp. 99-162, Marcel Dekker, New York
Saccomani, G., Sach, G., Cuppoletti, J., and Jung, C. Y. (1981) J. Biol. Chem. 256, 7727-7729
Schlegel, W., Kemppner, E. S., and Rodbell, M. (1979) J. Biol. Chem. 254, 5168-5176
Stevens, B. R., Harms, P., Kemppner, E. S., and Wright, E. M. (1982) Fed. Proc. 41, 1264
Thompson, D. A., Suárez-Villafañe, M., and Ferguson-Miller, S. (1992) Biophys. J. 37, 285-292
Vegh, K., Spiegler, P., Chamberlain, C., and Moomaert, W. F. H. M. (1968) Biochim. Biophys. Acta 163, 266-268

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REFERENCES
Blair, P. V. (1967) Methods Enzymol. 10, 78-81
Bock H.-G., and Fleischer, S. (1974) Methods Enzymol. 31, 374-381
Bock, H.-G., and Fleischer, S. (1975) J. Biol. Chem. 250, 5774-5783
Chen, P. S., Toribara, T., and Warren, H. (1956) Anal. Chem. 28, 1756-1758
Cortese, J. O., Vidal, J. C., Churchhill, P., McIntyre, J. O., and Fleischer, S. (1982) Biochemistry 21, 3896-3906
Cuppoletti, J., Jung, C. Y., and Green, F. A. (1981) J. Biol. Chem. 256, 1305-1306
Eibl, H., and Nicksch, A. (1978) Chem. Phys. Lipids 22, 1-8

1 L. Hymel, A. Maurer, C. J. Berensky, C. Y. Jung, and S. Fleischer, in preparation.