Effect of Changes in the Phospholipid Composition on the Enzymatic Activity of D-β-Hydroxybutyrate Dehydrogenase in Rat Hepatocytes†

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ABSTRACT: The phospholipid composition of primary rat hepatocytes was manipulated by supplementing the medium with choline analogues. The unnatural analogue *l*-2-amino-1-butanol was incorporated into membrane phospholipids to the largest extent, whereas the natural choline analogues ethanolamine, *N*-methylethanolamine, and *N*,*N*-dimethylethanolamine were methylated to yield phosphatidylcholine. When cells were supplemented with [^{14}C]ethanolamine, greater than 25% of the total phosphatidylcholine contained radiolabel in the polar head group after 2 days of supplementation. The extent of phospholipid methylation was reduced by depriving the cells of serine and methionine. Under these conditions, *N*-methylethanolamine and *N*,*N*-dimethylethanolamine were incorporated into phospholipids and were not further metabolized to phosphatidylcholine. After 3 days of supplementation with *N*-methylethanolamine, the content of phosphatidylmethylethanolamine went from essentially 0 to 40% of the total phospholipids and surpassed the extent of incorporation of all other analogues. The formation of the new phospholipid species was primarily at the expense of phosphatidylcholine and phosphatidylethanolamine. D-β-Hydroxybutyrate dehydrogenase, which requires phosphatidylcholine for activity, was assayed in submitochondrial membranes isolated from supplemented cells. For cells supplemented with either *l*-2-amino-1-butanol or *N*-methylethanolamine, the K_m for NADH increased relative to choline-supplemented cells while the K_m for acetoacetate remained the same. For example, after 3 days of supplementation with *N*-methylethanolamine, the K_m for NADH was 3-fold higher than the value for the choline-supplemented control cells. The change in the K_m was due to the change in the lipid environment with no alteration in the enzyme itself. The results suggest that the phosphatidylcholine molecules necessary to activate the enzyme exchange with the other phospholipids in the membrane so that the K_m of the enzyme reflects the overall content of phosphatidylcholine as well as other properties of the membrane phospholipids.

The activity of many integral enzymes has been shown to be sensitive to the polar head group composition of membrane phospholipids (Gennis & Jonas, 1977). One such protein is D-β-hydroxybutyrate dehydrogenase (BDH),[1] which is found in mitochondria and catalyzes a reaction involved in the metabolism of ketone bodies: acetoacetate + NADH + H^+ ⇌ D-β-hydroxybutyrate + NAD^+. The lipid requirement for the enzymatic activity of BDH has been thoroughly examined (Fleischer et al., 1974b).

When the purified apoenzyme was examined in a reconstituted system, it was shown that the polar head groups of the phospholipids must contain choline to reactivate the apoenzyme (Isaacson et al., 1979; Grover et al., 1975). These studies were primarily directed at determining the effect of different phospholipids on the rate and efficiency of reconstitution.

When consideration was given to the specific kinetic properties of BDH, it was shown that the activity of BDH was sensitive to the surface charge of the phospholipids as well as the content of phosphatidylcholine (Clancy et al., 1981). For purified BDH in a reconstituted system, the K_m of the enzyme for NADH was shown to depend on the phosphatidylcholine content. This observation was consistent with the fact that BDH requires phosphatidylcholine for enzymatic activity (Sekuzu et al., 1961) and uses this phospholipid as a necessary cofactor for the binding of NADH (Gazzotti et al., 1974). Further studies were necessary such as manipulating the phospholipid composition of intact cells (Glaser et al., 1974) in order to evaluate how the phospholipid polar head group composition alters the enzyme kinetics of BDH in a biological membrane under more native conditions. This goal was initially frustrated by the fact that a number of cultured animal cells that were examined did not contain significant amounts of BDH. Of all the tissues examined in a rat, the largest amount of BDH was found in the liver (Lehninger et al., 1960), and a partially purified enzyme from this source was shown to require phosphatidylcholine for activity (Goetterer, 1967). Therefore, for this and other reasons, primary cultures of rat hepatocytes were selected for this study.

A number of recent attempts have been undertaken to manipulate the phospholipid polar head group composition of rat liver. In one approach, the unnatural base analogues *N*-isopropylethanolamine and *N*-methyl-*N*-isopropylamine were injected intraperitoneally into rats (Lee et al., 1975; Moore et al., 1978). These analogues were incorporated to the extent of 9% and 19% of the total phospholipid of rat liver, respectively. Another approach involved addition of choline analogues to the medium of a suspension of primary hepatocytes. Once again, an unnatural base, *N*,*N*-diethylethanolamine, was incorporated to the largest extent and comprised approximately 20% of the total phospholipid (Akesson, 1977a). Efforts to supplement the natural bases ethanolamine, *N*-methylethanolamine, and *N*,*N*-dimethylethanolamine were not as successful primarily due to the fact that all these analogues were methylated to form phosphatidylcholine by the methylation pathway that exists in rat liver (Sundler & Akesson,

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[1] Abbreviations: BDH, D-β-hydroxybutyrate dehydrogenase; CL, cardiolipin; PBA, phosphatidylbutanolamine; PC, phosphatidylcholine; PDEEA, phosphatidyldiethylethanolamine; PDMEA, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMEA, phosphatidylmethylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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1975; Akesson, 1977b). There was an enhanced accumulation of ethanolamine reported when suspension hepatocytes were sustained in medium containing ethanolamine and lacking methionine (Akesson, 1978).

The results of this study show that the removal of methionine and serine from the supplementation medium can have a large impact on the incorporation of choline analogues in the phospholipid. The natural analogue *N*-methylethanolamine was now incorporated into membrane phospholipids to the largest extent. When BDH was assayed in these membranes, the enzymatic activity was significantly influenced by the polar head group composition of the phospholipids.

Materials and Methods

Isolation and Culture of Hepatocytes. Hank's modified balanced salts without calcium was made as described by Patterson (1979). Dulbecco's modified Eagle's medium without choline, arginine, serine, glutamine, and methionine was made as described by Leffert et al. (1979). Porcine insulin (26 units/mg) was a generous gift of William Bromer of Eli Lilly and Co.

Predigestion buffer was made by adding insulin (2 μg/mL) and hydrocortisone (2 μg/mL) to the Hank's modified balanced salts without calcium. The digestion buffer consisted of 150 mg of type I collagenase (catalog CO-130) from Sigma Chemical Co. in 200 mL of predigestion buffer. The plating medium contained choline (4 μg/mL), ornithine (0.5 mM), serine (0.4 mM), glutamine (4.0 mM), methionine (0.2 mM), insulin (10 μg/mL), hydrocortisone (10 μg/mL), and 20% newborn calf serum (Irvine Scientific) in the Dulbecco's modified Eagle's medium described above.

Hepatocytes were isolated from female Sprague Dawley rats (100–200 g) by using the procedure of Davis et al. (1979). The cells were suspended in plating medium at a concentration of 10^6 cells/mL, and 9 mL was added per 100-mm tissue culture dish (Corning).

After 24 h, the medium was aspirated from the dishes, and the attached cells were washed with the Dulbecco's modified Eagle's medium described above and then placed in supplementation medium.

Supplementation medium was made by adding ornithine (0.4 mM), glutamine (4.0 mM), insulin (10 μg/mL), and hydrocortisone (10 μg/mL) to the above-mentioned Dulbecco's modified Eagle's medium. Choline or choline analogues were added at 40 μg/mL. Serine and methionine were added at 0.4 and 0.2 mM, respectively. *N,N*-Dimethylethanolamine, *N*-methylethanolamine, and ethanolamine were obtained from Eastman Kodak. *l*-2-Amino-1-butanol and *N,N*-diethylethanolamine were obtained from Aldrich. [1,2-^{14}C]-Ethanolamine hydrochloride was obtained from New England Nuclear.

Cell Harvest and Membrane Preparation. To harvest the cells, the medium was aspirated from the culture dishes, and the cells were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2). The cells were scraped in phosphate-buffered saline at 4 °C and centrifuged for 10 min at 1000*g*.

A modified procedure of Fleischer et al. (1974a) was used to isolate submitochondrial membranes which employed a French press (American Instrument Co.) instead of a Parr bomb. Isolation of submitochondrial membranes from intact hepatocytes was accomplished by gently resuspending the 1000*g* pellet of cells into 4 mL of 5 mM Tris, pH 7.4, 0.25 M sucrose, and 1.0 mM EDTA at a concentration of 10^7 cells/mL. This suspension of cells was subjected to 20 000 psi in a French press. Submitochondrial membranes were then isolated by differential centrifugation as previously described by Fleischer et al. (1974a). The yield of BDH in submitochondrial membranes was the same for all supplementation conditions. Studies on the K_m of BDH were carried out in submitochondrial membranes rather than whole mitochondria to ensure the accessibility of the substrates to BDH since the enzyme is located on the inner side of the inner mitochondrial membrane (McIntyre et al., 1978).

Lipid Analysis. For the extraction of whole cell lipids from primary rat hepatocytes, the cells were washed with phosphate-buffered saline at 37 °C and scraped in phosphate-buffered saline at 4 °C. Isolated membranes were also diluted in phosphate-buffered saline, and the lipid was extracted by using the method of Bligh & Dyer (1959) as described by Ames (1968). Neutral lipids were separated from phospholipids as described previously (Ferguson et al., 1975).

The polar head group composition of the phospholipids was determined by two-dimensional thin-layer chromatography on silica gel G (250 μm; Analtech, Inc.) as described previously (Glaser et al., 1974). The plates were briefly exposed to iodine vapor in order to visualize the phospholipids. After the iodine stain disappeared, the spots were scraped into 13 × 75 mm test tubes, the phospholipids were hydrolyzed, and the phosphate was quantitated according to the procedure of Kates (1972). Before an absorbance reading was taken, the tubes were centrifuged at 1000 rpm for 5 min to clarify the solution.

Phospholipids were isolated by using a Unisil column (Clarkson Chemical Co.), and fatty acid methyl esters were prepared by the $NaOH–CH_3OH$ method as described previously (Ferguson et al., 1975). The methyl esters were analyzed by gas–liquid chromatography as described previously (Gilmore et al., 1979). A temperature program was used with 10 min of chromatography at 160 °C followed by a 2 °C/min gradient until a temperature of 190 °C was reached, and then the column was held at 190 °C for an additional 20 min.

Enzyme Assays. The standard BDH assay mixture contained 2 mM dithioerythritol, 0.5 mg/mL bovine serum albumin (fatty acid free), 25 mM sodium chloride, 2.5 μg/mL antimycin A, 0.2 mM NADH, and 5 mM sodium phosphate, pH 7.0. Each mixture of the above components was preincubated with BDH-containing membranes for 10 min at 37 °C before the reaction was started by the addition of 2.0 mM acetoacetate. The decrease in absorbance at 340 nM was converted to units of activity by using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH.

Protein was determined according to the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Lactate dehydrogenase was assayed at 27 °C in a mixture containing 0.16 mM NADH and 20 mM Tris, pH 7.4. The reaction was initiated with the addition of 5 mM pyruvate, and the reaction was monitored at 340 nm.

BDH Reconstitution. BDH was solubilized free of phospholipids and purified to homogeneity according to the method of Boch & Fleischer (1974). When BDH was stored at protein concentrations less than 1 mg/mL, ethylene glycol was added to the purified apoenzyme to 50% (v/v). The enzyme was stored at −20 °C. BDH was reconstituted with phospholipids (Clancy et al., 1981) and assayed as described above.

Results

So that the effect of the phospholipid composition on the enzymatic activity of BDH could be studied, several cell lines were screened to determine whether they contained BDH. Cells that did not contain any significant amount of BDH were mouse LM cells, Chang liver cells, mouse liver NCTC clone 1469, Morris rat hepatoma MH_1C_1, and primary chicken

Table I: Phospholipid Composition of Primary Rat Hepatocytes Grown in Media Containing Serine, Methionine, and Different Analogues of Choline[a]

	phospholipid composition (% phosphate)									
supplement	PC	PE	PI/PS	cardio-lipin	sphingo-myelin	PMEA	PDMEA	PBA	PDEEA	PX
choline	56.8	18.5	13.7	4.8	6.1					
ethanolamine	52.5	25.0	12.0	4.7	4.7					
N-methylethanolamine	44.8	28.4	13.9	4.4	5.5	2.2	0.8			
N,N-dimethylethanolamine	52.7	18.3	16.0	6.1	6.9					
l-2-amino-1-butanol	40.0	11.9	15.5	4.0	3.2			18.0		7.4
N,N-diethylethanolamine	42.3	21.9	14.9	7.6	6.1				4.4	3.2

[a] Cells were grown for 3 days in media containing a 40 μg/mL sample of the indicated supplement in place of choline plus serine and methionine. Several minor phospholipids comprising less than 2% of the total phospholipids were not present in sufficient amounts for accurate determination and were not included in the analysis. The phospholipid PX is the putative N-methylated derivative of either PBA or PDEEA.

embryo fibroblasts. Because rat liver was known to contain large quantities of BDH (Lehninger et al., 1960), efforts were undertaken to establish a primary rat hepatocyte culture and manipulate the lipid composition of these cells.

Hepatocytes were isolated and plated on culture dishes basically as described by Davis et al. (1979). Initial attempts to incorporate analogues of choline into the phospholipids of primary rat hepatocytes employed techniques described previously (Glaser et al., 1974; Hale et al., 1977) and gave the results shown in Table I. An unnatural choline analogue, *l*-2-amino-1-butanol, was incorporated to the largest extent, and phosphatidylbutanolamine comprised 18% of the total phospholipids after 3 days in supplementation medium. Another novel phospholipid was formed in these cells that represented 7.4% of the total phospholipids. Although it was not positively identified, it probably was the N-methylated derivative of phosphatidylbutanolamine. The formation of the new phospholipids in these membranes was primarily at the expense of phosphatidylcholine and phosphatidylethanolamine. Supplementation with either ethanolamine, *N*-methylethanolamine, or *N,N*-dimethylethanolamine was largely unsuccessful in producing changes in the phospholipid composition. When these analogues are incorporated into phospholipids, they are substrates for the methylation pathway which converts phosphatidylethanolamine into phosphatidylcholine.

The methylation pathway is quite active in hepatocytes as shown in Figure 1. When hepatocytes were placed in medium that contained [^{14}C]ethanolamine, methionine, and serine, there was a rapid rate of incorporation of [^{14}C]ethanolamine into phosphatidylethanolamine, and by 24 h, approximately 20% of the total phospholipids was ^{14}C-labeled phosphatidylethanolamine. Since the total amount of labeled and unlabeled phosphatidylethanolamine in the cells was less than 25% of the total phospholipids, greater than 80% of the phosphatidylethanolamine was labeled. These primary hepatocytes do not divide in culture, and consequently, the data show that the phospholipids must undergo rapid turnover in order to incorporate the label. There was also a rapid rate of incorporation of [^{14}C]ethanolamine into phosphatidylcholine that was linear with time. At 48 h, greater than 25% of the total phosphatidylcholine in the cell contained [^{14}C]ethanolamine that had been incorporated into phospholipid and converted to phosphatidylcholine. Therefore, a significant amount of the phosphatidylcholine was derived from the methylation pathway. One objective of this study was to reduce the phosphatidylcholine content as much as possible in order to observe the effect on the activity of BDH. To reduce the extent of phospholipid methylation and lower the level of phosphatidylcholine, serine and methionine were omitted from the

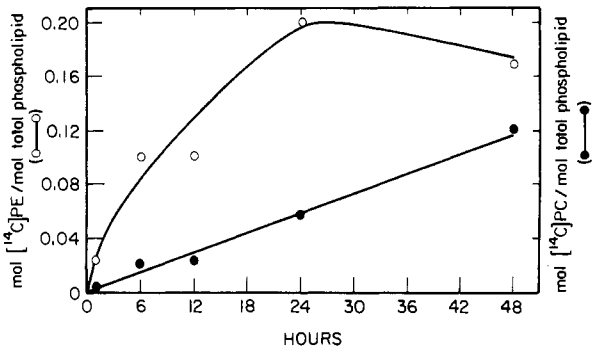

FIGURE 1: Incorporation of [^{14}C]ethanolamine into phosphatidylcholine and phosphatidylethanolamine of primary rat hepatocytes. After 1 day in culture, the hepatocytes were placed in medium containing [^{14}C]ethanolamine at 40 μg/mL (specific activity = 1.5 × 10^3 cpm/nmol) plus serine and methionine. The ordinate shows the moles of [^{14}C]ethanolamine incorporated into either phosphatidylcholine (●) or phosphatidylethanolamine (○) divided by the total moles of cellular phospholipid.

culture medium. When hepatocytes were placed in medium containing [^{14}C]ethanolamine without serine and methionine, the label was rapidly incorporated into phosphatidylethanolamine. After 24 h, greater than 80% of the phosphatidylethanolamine was labeled. However, after 48 h of supplementation, only 6% of the phosphatidylcholine contained the ^{14}C label, indicating that the rate of methylation was markedly reduced.

Supplementation of hepatocyte cultures with different analogues of choline was reexamined in a medium lacking serine and methionine. The data in Table II show that these conditions had a large effect on the final phospholipid composition when cells were supplemented with *N*-methylethanolamine or *N,N*-dimethylethanolamine, whereas the incorporation of the unnatural analogues was unaffected. Also, the decrease in the phosphatidylcholine content for all supplementation conditions was larger than that for cells grown in the presence of serine and methionine (Table I). Without serine and methionine, *N*-methylethanolamine was incorporated to the largest extent and comprised approximately 40% of the total phospholipids. For *N*-methylethanolamine-supplemented cells, the phosphatidylethanolamine content also fell to very low levels. The absence of serine and methionine or the presence of the analogues had no apparent effect on the viability of the cells. There were some small changes in the composition of the minor phospholipid species, but these did not exceed the error in the determination. Under these conditions, the content of the putative N-methylated derivative of phosphatidylbutanolamine was also reduced when the cells were supplemented with *l*-2-amino-1-butanol.

Table II: Phospholipid Composition of Primary Rat Hepatocytes Grown in Media Containing Different Analogues of Choline and without Serine and Methionine[a]

supplement	phospholipid composition (% phosphate)									
	PC	PE	PI/PS	cardio-lipin	sphingo-myelin	PMEA	PDMEA	PBA	PDEEA	PX
choline	50.3	26.8	13.1	5.2	4.6					
ethanolamine	48.1	29.6	12.5	4.9	4.9					
N-methylethanolamine	25.8	6.7	13.4	3.1	2.6	40.2	8.2			
N,N-dimethylethanolamine	46.2	15.1	11.8	4.2	5.2	4.2	13.3			
l-2-amino-1-butanol	33.6	19.7	16.8	4.3	3.8			20.9		1.0
N,N-diethylethanolamine	41.3	27.2	12.7	4.6	4.2				9.9	

[a] Same conditions as the experiment in Table I except the media did not contain serine or methionine.

Table III: Effect of Methionine on the Incorporation of Choline and Choline Analogues into the Phospholipids of Primary Rat Hepatocytes[a]

supplement	methionine concn (mM)	phospholipid composition (% phosphate)				
		PC	PE	other	PMEA	PDMEA
choline	0	57.4	18.8	23.8		
choline	0.1	56.8	18.5	24.6		
choline	0.5	59.2	15.9	24.9		
N-methylethanolamine	0	30.0	8.7	20.7	34.0	6.8
N-methylethanolamine	0.1	33.3	9.1	23.2	26.0	8.2
N-methylethanolamine	0.5	44.8	28.4	23.8	2.2	0.8
N,N-dimethylethanolamine	0	47.0	14.0	22.2	0.6	16.0
N,N-dimethylethanolamine	0.1	49.6	25.8	24.6		

[a] The conditions were the same as those for the experiment in Table I. The media also contained 0.4 mM serine.

When choline or choline analogues were used to supplement the medium in the presence of different concentrations of methionine, phosphatidylmethylethanolamine or phosphatidyldimethylethanolamine was completely methylated to phosphatidylcholine as shown in Table III. The addition of 0.1 mM methionine to the medium led to the complete methylation of phosphatidyldimethylethanolamine but not phosphatidylmethylethanolamine. However, 0.5 mM methionine fully methylated phosphatidylmethylethanolamine. The addition of methionine along with *N*-methylethanolamine or *N,N*-dimethylethanolamine also caused a large increase in the content of phosphatidylethanolamine. The reason for this is not clear.

The fatty acid compositions of the phospholipids for cells under different supplementation conditions as described in Table II were analyzed and were found to have no significant differences in composition (data not shown).

When hepatocytes were supplemented with an analogue of choline, the analogue was rapidly taken up by the cells and incorporated into phospholipids. For example, *N*-methylethanolamine was incorporated to an extent of 20% of the total phospholipids during the first 6 h of supplementation as shown in Figure 2B. After 6 h of supplementation, a slower rate of incorporation ensued. Phosphatidylmethylethanolamine increased in content primarily at the expense of phosphatidylcholine and phosphatidylethanolamine. When the enzymatic activity of BDH was assayed in submitochondrial membranes, the K_m for NADH remained unaffected in cells supplemented with choline (Figure 2A), while the K_m increased in cells supplemented with *N*-methylethanolamine (Figure 2B). The K_m for NADH of the enzyme did not change appreciably during the first 24 h of supplementation despite the fact that there was a large increase in the content of phosphatidylmethylethanolamine in the phospholipids. After 3 days of supplementation with *N*-methylethanolamine, the K_m of BDH increased 3-fold.

The phospholipid compositions of submitochondrial membranes isolated from rat liver hepatocytes grown for 3 days

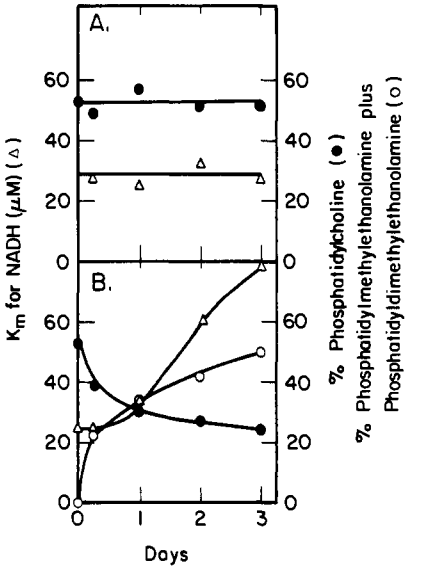

FIGURE 2: K_m for NADH of BDH (△) in submitochondrial membranes and the percent of phosphatidylcholine (●) or the percent of phosphatidylmethylethanolamine plus phosphatidyldimethylethanolamine (○) in cells supplemented with either choline (A) or *N*-methylethanolamine (B). Hepatocytes were supplemented as described in Table I except the medium did not contain serine or methionine.

in medium containing different analogues of choline are given in Table IV. The phospholipid compositions of submitochondrial membranes were similar to those obtained with whole cells. *N*-Methylethanolamine and *l*-2-amino-1-butanol were incorporated to 36% and 18% of the total phospholipids, respectively.

For BDH assayed in submitochondrial membranes isolated from cells supplemented with *l*-2-amino-1-butanol, the K_m for NADH increased, although the increase was smaller than that observed with membranes from *N*-methylethanolamine-supplemented cells (Table V). The K_m for acetoacetate was unaffected by the alterations in the phospholipid composition.

Table IV: Phospholipid Composition of Submitochondrial Membranes Isolated from Primary Rat Hepatocytes Grown in Media Containing Different Analogues of Choline without Serine and Methionine

	phospholipid composition (% phosphate)								
supplement	PC	PE	PI/PS	cardio-lipin	sphingo-myelin	PMEA	PDMEA	PBA	PX
choline	50.2	22.6	10.0	7.4	5.6				
N-methylethanolamine	25.6	7.7	14.9	4.2	4.2	36.2	7.0		
l-2-amino-1-butanol	38.0	17.0	18.0	1.8	4.6			18.0	2.0

[a] Same conditions as the experiment in Table I except the media did not contain serine or methionine.

Table V: Effect of Different Supplementation Conditions on the Enzymatic Activity of BDH in Submitochondrial Membranes Isolated from Primary Rat Hepatocytes[a]

substrate	supplement	K_m for NADH (μM)	V_{max} (μmol min^{-1} mg^{-1})
NADH	choline	27	0.11
NADH	*N*-methylethanolamine	75	0.07
NADH	*l*-2-amino-1-butanol	46	0.077
acetoacetate	choline	430	0.12
acetoacetate	*N*-methylethanolamine	390	0.064
acetoacetate	*l*-2-amino-1-butanol	360	0.075

[a] Hepatocytes were supplemented as described in Table I except the media did not contain serine or methionine.

Table VI: Apparent K_m for NADH of BDH in a Reconstituted System Containing either the Pure Enzyme in Phospholipids Isolated from Supplemented Mitochondria or the Enzyme Isolated from Supplemented Mitochondria and the Same Phospholipids[a]

variable	supplement	K_m for NADH (μM)
	Experiment 1[b]	
enzyme	choline	24
	N-methylethanolamine	18
	Experiment 2[c]	
phospholipid	choline	32
	N-methylethanolamine	97

[a] In both experiments, hepatocytes were grown for 3 days in media containing a 40 μg/mL sample of the indicated supplement minus serine and methionine. [b] In experiment 1, mitochondrial membranes were isolated from cells supplemented with either choline or *N*-methylethanolamine. BDH was solubilized by phospholipase A_2 digestion of the respective membranes (Boch & Fleischer, 1974) and reconstitution with the same preparation of phospholipid vesicles comprised of beef heart phosphatidylcholine and egg phosphatidic acid (0.8/0.2). [c] In experiment 2, the same pure enzyme preparation was reconstituted with phospholipids isolated from submitochondrial membranes derived from cells supplemented with either choline or *N*-methylethanolamine.

The maximal velocity of BDH showed a small decrease for cells supplemented with *N*-methylethanolamine or *l*-2-amino-1-butanol relative to the choline membranes.

It is possible that a change in the structure of BDH occurred during the time when the cells were being supplemented and that this change might have led to the observed alteration in the K_m for NADH. For examination of this point, mitochondrial membranes were isolated from cells supplemented with either *N*-methylethanolamine or choline. BDH was solubilized from the two different membranes and reconstituted into identical phospholipid vesicles. BDH was greater than 90% solubilized, and the reconstituted enzyme from either choline- or *N*-methylethanolamine-supplemented mitochondria had a similar K_m for NADH, suggesting that there was no change in the enzyme itself (Table VI, experiment 1).

The phospholipids of the submitochondrial membranes were isolated from cells supplemented with either *N*-methylethanolamine or choline in order to show that the K_m of BDH directly reflected its lipid environment. The same preparation of purified BDH was now reconstituted into unilamellar vesicles of the two different phospholipids. The purified apoenzyme reconstituted with the *N*-methylethanolamine-supplemented phospholipids showed a 3-fold higher K_m for NADH than with choline-supplemented phospholipids (Table VI, experiment 2). There was no difference between the two for the K_m for acetoacetate as found in the original membranes (data not shown).

In another experiment, BDH was reconstituted into vesicles containing only phosphatidylmethylethanolamine. This phospholipid did not reactivate the enzyme. This result is consistent with the observation of Isaacson et al. (1979), where only choline analogues with a quaternary nitrogen in the polar head group were found to optimally activate BDH.

Discussion

Before the effect of the zwitterionic polar head group composition on the enzyme kinetics of BDH in a biological membrane could be investigated, supplementation conditions had to be defined for altering the phospholipid composition of primary rat hepatocytes. In the standard Dulbecco's modified Eagle's medium, which contained serine and methionine, the unnatural analogue *l*-2-amino-1-butanol was incorporated into phospholipids to the greatest extent. Efforts to supplement the natural analogues ethanolamine, *N*-methylethanolamine, or *N*,*N*-dimethylethanolamine were unsuccessful since these analogues were methylated to give phosphatidylcholine with only small changes in the phospholipid composition. Many other cells, such as LM cells, have very low levels of the enzymes in the methylation pathway so that all of these analogues are incorporated unchanged into phospholipids (Glaser et al., 1974).

Bremer & Greenberg (1959, 1960) proposed that phosphatidylcholine was synthesized by successive methylation of phosphatidylethanolamine. Methionine was putatively identified as a methyl-group donor (Bremer & Greenberg, 1961; Sundler & Akesson, 1975). Another contributing substrate was phosphatidylserine, which is decarboxylated to form phosphatidylethanolamine (Wilson et al., 1960; Rehbinder & Greenberg, 1965). Phosphatidylethanolamine is the first committed phospholipid substrate in the methylation pathway. Phosphatidylmethylethanolamine and phosphatidyldimethylethanolamine do not exist in appreciable quantities in normal membranes (Katyal & Lombardi, 1976). Therefore, the first step in the methylation pathway is believed to be rate limiting (Schneider & Vance, 1979; Maeda et al., 1980).

In this study, the levels of serine, methionine, and ethanolamine had a large effect on phospholipid metabolism in primary rat hepatocytes. When all three were added to the medium with a radiolabel in ethanolamine, greater than 25% of the phosphatidylcholine was radiolabeled after 2 days of supplementation. When cells were placed in medium lacking serine and methionine, the natural analogues *N*-methyl-

ethanolamine and *N*,*N*-dimethylethanolamine were incorporated into membrane phospholipids without being methylated. Ethanolamine did not supplement well under these conditions for reasons that are not clear.

The enzymes of the methylation pathway were still present in membranes from cells whose phospholipid composition was altered by supplementation. Similar rates of incorporation of [^{3}H]methionine were observed in cells whose membrane composition was altered by choline, ethanolamine, *N*-methylethanolamine, or *N*,*N*-dimethylethanolamine supplementation (unpublished results). Thus, the absence of choline or the buildup of methylation pathway intermediates did not induce the methylation pathway. A similar conclusion was reached by Schneider & Vance (1978), who monitored the enzymes that synthesize phosphatidylcholine in rats which were fed choline-containing or choline-deficient diets. This is in contrast to the situation in *Saccharomyces cerevisiae*, for example, where the presence of *N*-methylethanolamine, *N*,*N*-dimethylethanolamine, or choline in the medium reduced the levels of the *N*-methyltransferases (Waechter & Lester, 1973).

The altered zwitterionic phospholipid composition of the hepatocytes had a large impact on the enzymatic activity of BDH. There was a 3-fold increase in the K_m for NADH of BDH in membranes from *N*-methylethanolamine-supplemented cells relative to BDH in membranes from choline-supplemented cells. When BDH was assayed in membranes from *l*-2-amino-1-butanol-supplemented cells, a 2-fold increase in the K_m for NADH was observed. There was a correlation between the extent of incorportion of the choline analogue into the phospholipid, or the decrease in phosphatidylcholine content, and the effect on the kinetic parameters of BDH. The K_m for acetoacetate was unaffected by the altered phospholipid compositions. When BDH was solubilized from phosphatidylmethylethanolamine membranes, the reconstituted enzyme activity was identical with that of control membranes. This shows that the effect on the activity of BDH was mediated by the lipid environment with no alteration in the enzyme itself. Several membrane lipid properties could have been responsible for the altered BDH kinetics.

BDH which catalyzes a reaction involving negatively charged substrates has been shown to be sensitive to membrane surface charge (Clancy et al., 1981). In that study, phospholipase D was utilized to manipulate the surface charge in submitochondrial membranes. The kinetic parameters for the negatively charged substrates of BDH were modulated by the electrostatic potential. Although BDH in rat liver submitochondrial membranes is influenced by an electrostatic potential at low ionic strength, the ionic strength in the assay mixture used in this study was adjusted to neutralize the electrostatic effects. For example, in the absence of NaCl in the enzyme assay mixture, the apparent K_m for NADH of BDH in submitochondrial membranes isolated from choline-supplemented cells was 88 mM. The addition of 25 mM NaCl to the assay mixture reduced the K_m to 30 mM. This ionic strength eliminated the electrostatic potential because further increases in the salt concentration had no further effect on the K_m. In the present study, 25 mM NaCl was routinely added to the assay mixture so that the electrostatic potential would not contribute to the results.

The K_m of BDH was also shown to be sensitive to the zwitterionic composition of the phospholipids in a reconstituted system (Clancy et al., 1981). For purified BDH, the K_m for NADH increased as the phosphatidylcholine content decreased in unilamellar vesicles comprised of beef heart phosphatidylcholine and *Escherichia coli* phosphatidylethanolamine. The K_m for acetoacetate was unaffected by the changes in the zwitterionic phospholipid composition. There are a number of problems in interpreting the results of reconstitution studies such as properly evaluating the efficiency of the reconstitution and the integrity of the lipid vesicles. The results in the reconstituted system were basically similar to those obtained in this study, however, where a decrease in phosphatidylcholine concentration due to the incorporation of an analogue resulted in an increase in the K_m for NADH. Consequently, the K_m appears to depend on the concentration of phosphatidylcholine per se rather than a change in another specific phospholipid species.

Fleischer and co-workers have shown in a reconstituted system that BDH will not bind NADH (and presumably NAD^+) unless phosphatidylcholine is present in the membrane (Fleischer et al., 1974b; Gazzotti et al., 1974). Since the reaction mechanism is ordered sequential, NADH or NAD^+ must bind to the enzyme before the second substrate (D-β-hydroxybutyrate or acetoacetate) can be bound (Nielson et al., 1973). This explains why no change in the K_m for acetoacetate was observed when the phosphatidylcholine content decreased. It has also been assumed that only a few phosphatidylcholine molecules are necessary to activate the enzyme (Fleischer et al., 1974b).

One interpretation of the results from this study is that when the content of phosphatidylcholine in the membranes of hepatocytes is reduced by polar head group supplementation there is not enough phosphatidylcholine left to activate the enzyme. If it is assumed that BDH comprises 0.1% of the total membrane protein in mitochondria, there are 32 pmol of BDH/mg of protein. Mitochondria contain approximately 0.7 μmol of phospholipid/mg of protein, and phosphatidylcholine comprises 50% of the phospholipids under normal conditions. Hence, the molar ratio of phosphatidylcholine to BDH in mitochondria is approximately 11 000 to 1. When cells were supplemented with *N*-methylethanolamine, the phosphatidylcholine content dropped approximately in half so that the molar ratio of phosphatidylcholine to BDH was now 5500 to 1. Consequently, the lipid modifications obtained in this study altered the enzyme kinetics despite the fact that large quantities of phosphatidylcholine were still present. It is unlikely that other proteins in the mitochondrial membrane would have a higher affinity for phosphatidylcholine than BDH and sequester all the phosphatidylcholine. In the reconstituted system where no other proteins are present, the K_m of BDH clearly reflects the overall composition of the phospholipids [see Table VI and Clancy et al. (1981)].

The conclusion that the K_m of BDH reflects the overall phospholipid composition says that there is not a limited number of phosphatidylcholine molecules that stay tightly bound to the enzyme and activate it. The phosphatidylcholine molecules necessary to activate the enzyme must exchange with the bulk lipid environment, and this exchange must be faster than the turnover time of the enzyme (milliseconds) in order to alter the K_m. This conclusion, which is based on the activity of the enzyme, concerns the presumably few high-affinity sites that activate the enzyme and expands conclusions based on physical studies that suggest the large number of boundary lipids that surround an integral membrane protein undergoes exchange (Oldfield et al., 1978; Seelig & Seelig, 1978; Jost & Griffith, 1980).

When hepatocytes were supplemented with *N*-methylethanolamine, they incorporated the analogue and reduced the amount of phosphatidylcholine in the membranes fairly rapidly. There was, however, a lag before this change was re-

flected in the K_m of BDH. This type of lag has also been observed in the activity of adenylate cyclase in LM cells (Engelhard et al., 1978) and some transformation-associated changes in Rous sarcoma virus transformed chicken embryo fibroblasts (Hale et al., 1977). One explanation for these observations is that the components exist in localized lipid regions that do not change their composition as rapidly as the general membrane. It has been shown by using methods to manipulate the phospholipid composition similar to those used in this study that enveloped viruses bud from localized lipid regions that do not reflect the average properties of the plasma membrane (Pessin & Glaser, 1980).

In conclusion, the activity of BDH can be significantly influenced by the content of phosphatidylcholine in the membranes as well as by an electrostatic potential. A change in any number of membrane parameters, such as membrane fluidity, for example, could alter the exchange of phosphatidylcholine between the enzyme and the bulk lipid and alter the enzymatic activity. This emphasizes the complexity in determing how specific phospholipids activate membranous enzymes.

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Registry No. NADH, 58-68-4; D-β-hydroxybutyrate dehydrogenase, 9028-38-0; choline, 62-49-7; ethanolamine, 141-43-5; *N*-methylethanolamine, 109-83-1; *N,N*-dimethylethanolamine, 108-01-0; *l*-2-aminobutanol, 5856-63-3; *N,N*-diethylethanolamine, 100-37-8; L-serine, 56-45-1; L-methionine, 63-68-3.

References

Akesson, B. (1977a) *Biochem. Biophys. Res. Commun. 76*, 93.

Akesson, B. (1977b) *Biochem. J. 168*, 401.

Akesson, B. (1978) *FEBS Lett. 62*, 177.

Ames, G. F. (1968) *J. Bacteriol. 95*, 833.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.

Boch, H. G., & Fleischer, S. (1974) *Methods Enzymol. 31*, 374.

Bremer, J. & Greenberg, D. M. (1959) *Biochim. Biophys. Acta 35*, 287.

Bremer, J., & Greenberg, D. M. (1960) *Biochim. Biophys. Acta 37*, 173.

Bremer, J., & Greenberg, D. M. (1961) *Biochim. Biophys. Acta 46*, 205.

Clancy, R. M., Wissenberg, A. R., & Glaser, M. (1981) *Biochemistry 21*, 6060.

Davis, R. A., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B., & Steinberg, D. (1979) *J. Biol. Chem. 254*, 2010.

Engelhard, V. H., Glaser, M., & Storm, D. R. (1978) *Biochemistry 17*, 3191.

Ferguson, K. A., Glaser, M., Bayer, W. H., & Vagelos, P. R. (1975) *Biochemistry 14*, 146.

Fleischer, S., Meissner, G., Smigel, M., & Wood, R. (1974a) *Methods Enzymol. 31*, 292.

Fleischer, S., Boch, H. G., & Gazzotti, P. (1974b) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M. E., Quagliariello, E., & Siliprandi, N., Eds.) pp 125–136, North-Holland Publishing Co., Amsterdam, The Netherlands.

Gazzotti, P., Boch, H. G., & Fleischer, S. (1974) *Biochem. Biophys. Res. Commun. 58*, 309.

Gennis, R. B., & Jonas, A. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 195.

Gilmore, R., Cohn, N., & Glaser, M. (1979) *Biochemistry 18*, 1042.

Glaser, M., Ferguson, K. A., & Vagelos, P. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4072.

Goetterer, G. S. (1967) *Biochemistry 6*, 2147.

Grover, A. K., Slotboom, A. J., DeHaas, G. H., & Hammes, G. G. (1975) *J. Biol. Chem. 250*, 31.

Hale, A. H., Pessin, J. E., Palmer, F., Weber, M. J., & Glaser, M. (1977) *J. Biol. Chem. 252*, 6190.

Isaacson, Y. A., Deroo, P. W., Rosenthal, A. F., Bittman, R., McIntyre, J. O., Boch, H. G., Gazzotti, P., & Fleischer, S. (1979) *J. Biol. Chem. 254*, 117.

Jost, P. C., & Griffith, O. H. (1980) *Ann. N.Y. Acad. Sci. 348*, 391.

Kates, M. (1972) in *Techniques in Lipidology*, p 354, Elsevier/North-Holland, New York.

Katyal, S. L., & Lombardi, B. (1976) *Lipids 11*, 513.

Lee, T. C., Blank, M. L., Piantadosi, C., Ishag, K. S., & Snyder, F. (1975) *Biochim. Biophys. Acta 409*, 218.

Leffert, H. L., Koch, K. S., Moran, T., & Williams, M. (1979) *Methods Enzymol. 58*, 536.

Lehninger, A. L., Sudduth, H. C., & Wise, J. B. (1960) *J. Biol. Chem. 235*, 2450.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maeda, M., Tanak, Y., & Akamatsu, Y. (1980) *Biochem. Biophys. Res. Commun. 96*, 876.

McIntyre, O., Bock, H. G., & Fleischer, S. (1978) *Biochim. Biophys. Acta 513*, 255.

Moore, C., Blank, M. L., Lee, T.-C., Benjamin, B., Piantadosi, C., & Snyder, F. (1978) *Chem. Phys. Lipids 21*, 175.

Nielson, N. C., Zahler, W. L., & Fleischer, S. (1973) *J. Biol. Chem. 248*, 2556.

Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H. S., Hshung, J. C., Kang, S. Y., King, T. E., Meadows, M., & Rice, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657.

Patterson, M. K., Jr. (1979) *Methods Enzymol. 58*, 141.

Pessin, J. E., & Glaser, M. (1980) *J. Biol. Chem. 255*, 9044.

Rehbinder, D., & Greenberg, D. M. (1965) *Arch. Biochem. Biophys. 108*, 110.

Schneider, W. J., & Vance, D. E. (1978) *Eur. J. Biochem. 85*, 181.

Schneider, W. J., & Vance, D. E. (1979) *J. Biol. Chem. 254*, 3886.

Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747.

Sekuzu, I., Jurtshuk, P., & Green, D. E. (1961) *Biochem. Biophys. Res. Commun. 6*, 71.

Sundler, R., & Akesson, B. (1975) *J. Biol. Chem. 250*, 3359.

Waechter, C. J., & Lester, R. L. (1973) *Arch. Biochem. Biophys. 158*, 401.

Wilson, J. D., Gibson, K. D., & Udenfriend, S. (1960) *J. Biol. Chem. 235*, 3539.