Rat Liver D-β-Hydroxybutyrate Dehydrogenase. I. Partial Purification and General Properties*

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ABSTRACT: D-β-Hydroxybutyrate dehydrogenase has been partially purified from rat liver mitochondrial membrane fragments by solubilization at elevated pH, followed by ammonium sulfate fractionation. The enzyme preparation is inactive as isolated and must be activated by preincubation in the presence of thiol, pyridine nucleotide, and lipid in order to recover enzymatic activity. The requirement for thiol is absolute over a wide pH range; the requirement for added lipid and pyridine nucleotide is less marked at low pH values. The optimum pH for both the activation process and the catalytic activity of the enzyme is about pH 8. The thiol requirement is satisfied by very high concentrations (0.1 M) of either thioglycerol, mercaptoethanol, glutathione, or cysteine. The activation process is optimal at 29°; at higher temperatures the final level of activity obtained is less, although the initial rate of activation is greater. Pyridine nucleotide analogs (inactive as electron acceptors) activate the enzyme when present during the preincubation.

The nicotinamide–adenine dinucleotide linked D-β-hydroxybutyrate dehydrogenase (βOHDH)[1] (EC 1.1.1.30, D-3-hydroxybutyrate:NAD oxidoreductase) of mammalian tissues is tightly bound to mitochondrial membranes and has been found to be present in almost all tissues examined (Lehninger *et al.*, 1960). Utilizing a cholate-solubilized preparation of this enzyme from beef heart, Sekuzu *et al.* (1961, 1963; Jurtshuk *et al.*, 1963) demonstrated that after separation from the membrane the enzyme developed a lipid requirement for enzymatic activity. The lipid requirement was specifically fulfilled by phosphatidylcholine and full activation required the additional presence of pyridine nucleotide and thiol. Most of these findings were confirmed in studies of a preparation from rat liver mitochondria, in which the nonionic detergent Lubrol was used for solubilization (Gotterer, 1964). Because the use of detergents complicates evaluation of the mechanism of activation of this enzyme, a method for solubilization was sought which did not require the use of detergents.[2] A method has been worked out for the extraction and partial purification of the enzyme from rat liver mitochondria in which treatment at elevated pH is utilized to solubilize the enzyme. This paper describes this new method and also the general properties of the partially purified enzyme preparation. The following paper (Gotterer, 1967) describes the studies concerned with the mechanisms by which lipids influence the state of activity of the isolated enzyme. A future paper will describe detailed studies dealing with the effect of pyridine nucleotides.

Experimental Procedures

Materials. Pyridine nucleotide analogs were purchased from P-L Laboratories. NAD was obtained from Sigma Chemical Co.; the sodium salt of DL-β-hydroxybutyrate from Mann Research Laboratories; thioglycerol from Aldrich Chemical Co.; and β-mercaptoethanol, cysteine, GSH, and GSSG from Calbiochem.

Lipids were extracted and washed by the methods of Folch *et al.* (1957) and Ways and Hanahan (1964). Lipid classes were separated by column chromatography on silicic acid. The purity of all lipids was verified by thin layer chromatography on silicic acid, using chloroform–methanol–water (95:35:4, v/v) and/or chloroform–methanol–acetic acid–water (25:15:4:2, v/v) as solvent systems.

Total phosphorus was determined by the method of Gomori (1942) after prior digestion with H_2SO_4 and H_2O_2. Protein was determined by the method of Lowry *et al.* (1951). Concentrations of sulfhydryl reagents were estimated by the nitroprusside method, according to Stadtman (1957).

Lipids were added to the enzyme system in the form of sonic dispersions prepared by removing the organic solvent under a stream of nitrogen, suspending the lipids in the appropriate buffer at a concentration

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[1] Abbreviations used: βOHDH, D-β-hydroxybutyrate dehydrogenase; BOH, β-hydroxybutyrate; thioAD, thionicotinamide–adenine dinucleotide; 3-AcPAD, 3-acetylpyridine–adenine dinucleotide; NAD, nicotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide; GSH, glutathione; GSSG, oxidized glutathione; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates.

[2] During the course of these studies Fleischer *et al.* (1966) have succeeded in solubilizing the enzyme from beef heart mitochondria by the action of phospholipase A.

of 5–10 mg dry weight of lipid/ml of buffer, sonicating in an MSE apparatus at 0–4° for 10 min, and finally clarifying the solution by centrifugation at 105,000*g* for 30 min.

NADH dehydrogenase activity was measured by the method of Minakami *et al.* (1962); succinic dehydrogenase by a slight modification of the method of Arrigoni and Singer (1962) after 10-min activation at 37°. Transhydrogenase activity was measured in a medium containing Tris-Cl buffer (pH 8.1), 100 mM; NADH, 0.5 mM; and analog acceptor, 0.1–2.5 mM, in a total volume of 1.0 ml. The reaction was followed at 395 mμ for thioAD and 375 mμ for 3-AcPAD, with ϵ_{mM} 11.3 and 5.85, respectively.

Assay of Enzymatic Activity. Enzymatic activity of βOHDH was measured spectrophotometrically by following the change in absorbancy of NAD at 340 mμ with a Zeiss spectrophotometer coupled to a Sargent log recorder. Most studies were performed in the direction of NAD reduction with β-hydroxybutyrate as substrate. The few studies performed in the reverse direction agreed with the results obtained with β-hydroxybutyrate as substrate.

As will be detailed below, maximum activity of the solubilized enzyme was obtained only after preincubation of the enzyme under special conditions. In general, the activation step was carried out in a concentrated medium and the enzyme was then diluted, usually 20-fold, for assay of enzymatic activity. The preincubation and assay were usually performed in the same cuvet. In those experiments where the conditions of assay were being investigated, the preincubation was carried out in a test tube; the tube was then placed in an ice bath and aliquots were then added to the assay medium, the volume of which was at least 20 times greater than that of the aliquot. Under these conditions, the activity of the activated enzyme remained constant for at least 30 min.

Amytal was added to the assay medium in those studies utilizing unfractionated mitochondria as the enzyme source, in order to prevent the reoxidation of NADH. Sonicated membrane fragments, as obtained by the procedure outlined, contained no NADH oxidase activity and, therefore, no respiratory inhibitor was added during the assay of enzyme preparations at later stages in the isolation procedure.

Unless stated otherwise, the following standard conditions were utilized for assay of enzymatic activity: 75 mM Tris-Cl, pH 8.1; 1 mM NAD, and 11 mM BOH; temperature, 22°.

Results

Preparation of Enzyme. Mitochondria were isolated from 0.25 M sucrose homogenates of livers from Wistar albino rats by the method of Hogeboom *et al.* (1948), washed three times with 0.25 M sucrose, and then stored at −20° until further use. The frozen mitochondria were suspended to a concentration of 50 mg of protein/ml with 0.01 M potassium phosphate buffer (pH 8.2) containing 0.001 M EDTA. The mitochondrial suspension was sonicated in an MSE sonicator at maximum intensity for 10 min in approximately 25-ml batches. Temperature was maintained at 0–4° during sonication by the use of an alcohol–Dry Ice bath. KCl (20 ml of 2.5 M) was added for each 100 ml of sonicated suspension and the suspension was then centrifuged, 10 ml/tube at 105,000*g* for 60 min. The recovered pellets were either used directly or stored at −20°. The optimum pH for extracting the enzyme from the membrane fragments was found to vary between pH 9.5 and 10.5. Below the optimum pH enzymatic activity remained in the membrane fraction; above the optimum pH the enzymatic activity was lost. The presence of NAD in the extraction medium resulted in considerable, though not complete, protection against inactivation at elevated pH and permitted extraction at higher pH values with consequently greater efficiency.

Because of the variability a small trial extraction was carried out with each preparation of sonic membrane fragments to determine the optimum pH. One pellet was first muddled in 0.2 ml of 0.02 M NAD. The volume was then brought to 4.0 ml with 0.1 M potassium phosphate–0.1 M sodium carbonate–0.01 M thioglycerol (pH 9.1). A small (0.1 ml) aliquot was removed for assays and 1.0 ml was removed for centrifugation. The pH of the suspension was raised by the addition of KOH, successively to 9.5, 10.0, 10.5, and 11.0. At each pH an aliquot was removed for assays and centrifugation. When the titration was completed the aliquots were centrifuged at 105,000*g* for 60 min and the supernatants were assayed for enzymatic activity and protein content. For most preparations the optimum was at pH 10.3. Having determined the optimum pH, a large-batch extraction of the enzyme was carried out. The membrane fragments were initially muddled with 0.02 M NAD, 0.2 ml/pellet, and then diluted with 0.1 M potassium phosphate–0.1 M sodium carbonate–0.01 M thioglycerol (pH 9.1) to a volume of 4.0 ml/pellet. The suspension was then centrifuged at 105,000*g* for 60 min and the supernatant was discarded. This preliminary extraction removed little if any of the βOHDH activity, but did remove some protein and thereby resulted in a slight increase in the specific activity of the membrane fraction. The prewashed membrane fragments were then resuspended in NAD and buffer, as above, with the pH of the buffer at the optimum established by the trial run. Slight adjustment to the optimum pH was at times necessary after the suspension of the fragments and was carried out by the addition of 0.1 M KOH. After 10 min the suspension was centrifuged at 105,000*g* for 60 min. The pellets were resuspended in one-half volumes, relative to the initial volumes of NAD and buffer at the optimum pH, and the extraction was repeated until no further activity was extracted. Those extracts with the most activity and the highest specific activities were pooled and then fractionated with ammonium sulfate. The fraction precipitating between 20 and 50% saturation was used as the source of enzyme for the studies reported in this paper. No

TABLE I: Partial Purification of D-β-Hydroxybutyrate Dehydrogenase.

Treatment	Total Protein (mg)	Total Act. (μmoles/min)	Sp Act. (μmoles/min mg)	Yield (%)
Sonicated mitochondria	2100	1200	0.6	100
Sonic fragments	840	960	1.1	80
pH 9.1 supernatant	175	23	0.1	—
pH 9.1 extracted fragments	670	900	1.3	75
pH 10.5 extract I	137	350	2.6	29
Extracted residue	450	320	0.7	27
pH 10.5 extract II	33	110	3.3	9
Extracted residue	370	130	0.4	11
pH 10.5 extract III	11	21	1.9	2
$(NH_4)_2SO_4$ fractions, extracts I–III				
0–20%	72	100	1.4	8
20–50%	63	230	3.7	19

TABLE II: Requirements for Activation as a Function of pH of Preincubation Medium.[a]

	Activity (A_{340}/min × 10^3)		
	pH 6.25	pH 8.0	pH 9.5
Complete system	130	440	205
Component omitted			
Thioglycerol	0	0	0
NAD	80	170	17
Lipid	100	60	12

[a] Preincubation was for 30 min at 29°. The complete preincubation medium contained: 76 μg of protein; 20 mM Tris–sodium phosphate–sodium carbonate buffer, adjusted to appropriate pH with minimum amounts of NaOH or HCl; 4 mM NAD; 200 mM thioglycerol, adjusted to appropriate pH with NaOH; and 44 mμmoles of mitochondrial phosphatidylcholine in total volume of 0.1 ml. After preincubation the enzymatic activity was assayed under standard conditions in a total volume of 2.0 ml.

significant activity was ever recovered at higher levels of ammonium sulfate saturation.

A typical purification is presented in Table I. It is seen that approximately one-third of the total enzymatic activity was lost with each extraction at pH 10.5. The solubilized activity was stable at pH 10.5 over the entire period required to complete the extraction. This is in contrast to the loss of activity which occurred when the membrane fragments were exposed to the elevated pH. Such results could be due either to a greater susceptibility of the membrane-bound enzyme, to inactivation at the elevated pH, or to an immediate reduction in the activity of the enzyme on release from the membrane. The latter interpretation in turn could be due to irreversible inactivation of the solubilized enzyme or to the absence of optimum conditions for complete reactivation of the enzyme during preincubation. Experimental evaluation of these alternatives remains to be carried out.

It is to be pointed out that the isolation of the enzyme was subject to some variation from one batch to the next. The variability in optimum pH for extraction has been discussed. The specific activity of the enzyme in the various extracts from a single membrane preparation was also found to vary; in most instances the specific activity of the second and third extracts was higher than that of the first extract.

In some preparations ammonium sulfate fractionation resulted in a lowering of the specific activity. The reason for this occasional reduction of activity is as yet not understood. Despite this occasional loss of activity the ammonium sulfate precipitation step was retained because it consistently removed a significant amount of protein with low specific activity. More importantly, ammonium sulfate precipitation permitted more prolonged storage of the extracted enzyme. The enzyme could be stored at −20° in the form of an ammonium sulfate precipitate for several months without significant loss of enzymatic activity. When stored in solution, at pH 10.5, 8.1, or 7.4, from temperatures of −20 to 25°, the enzyme rapidly lost activity over the course of a few days.

Enzyme preparations isolated in this manner contained no flavin or cytochrome detectable by spectroscopy. The following enzymatic activities were either absent or present at levels less than 1% of that of βOHDH: succinic dehydrogenase, NADH dehydrogenase, NADH oxidase, and transhydrogenase (NADH, donor; 3-acetylpyridine–adenine dinucleotide or thionicotinamide–adenine dinucleotide, acceptor).

Attempts at further purification by salt and solvent extraction and chromatography have as yet been unsuccessful. The enzyme passed through a CM-cellulose column and was irreversibly bound to a DEAE column and, therefore, is presumably negatively charged in the pH range 9–10.5.

The enzyme preparation still contained about 50 mμmoles of lipid phosphorus/mg of protein. This amount of lipid represents about one-sixth that present in intact mitochondria. Thin layer chromatography of lipids extracted from the isolated enzyme demon-

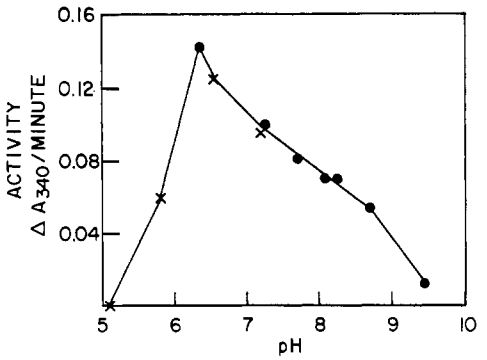

FIGURE 1: Activation in the absence of added lipid as a function of pH. Preincubation medium: Buffer: Tris–phosphate–carbonate (20 mM) adjusted to appropriate pH by addition of minimum amounts of NaOH or HCl, NAD (4 mM), thioglycerol (200 mM) adjusted to appropriate pH with NaOH, and enzyme (55 μg), in a total volume of 0.11 ml. Preincubated at 29° for 30 min. Assayed in total volume of 2.0 ml under standard conditions. The figure is a composite of two experiments.

strated the presence of the same classes of lipids as are present in isolated mitochondrial membranes.

Preincubation: Its Need and General Characteristics. After pH solubilization no enzymatic activity could be demonstrated when the enzyme was assayed directly in the standard medium, *i.e.*, in the presence of NAD and β-hydroxybutyrate. On the other hand, considerable activity was found after the enzyme had been preincubated under certain specified conditions. Pyridine nucleotide, lipid, and thiol must all be present in order to achieve maximum activation of the enzyme. As seen in Table II, the relative requirement for these components varied with the pH of the preincubation medium. Thiol was required at all pH values. On the other hand, the requirement for lipid and pyridine nucleotide was significantly less at lower pH values. The activity of the enzyme preparation, preincubated in the absence of added lipid, as a function of the pH of the preincubation medium is illustrated in more detail in Figure 1. This experiment was performed with an enzyme preparation which could be stimulated as much as 17-fold by preincubation at pH 8.1 in the presence of saturating amounts of added lipid. The figure demonstrates that the activity in the absence of added lipid reached a peak at about pH 6.3 and abruptly fell to zero by pH 5.1. This activity in the absence of added lipid is thought to be due to the residual lipid present in the enzyme preparation. Since the apparent loss of activity could be reversed when the pH was returned to higher values, it would appear that between pH 5.1 and 6.3 a functional group was titrated which was critical for activation of the enzyme.

The temperature at which the preincubation was carried out influenced the rate and extent of activation of enzymatic activity. As seen in Figure 2A the initial rate of activation of enzymatic activity increased with

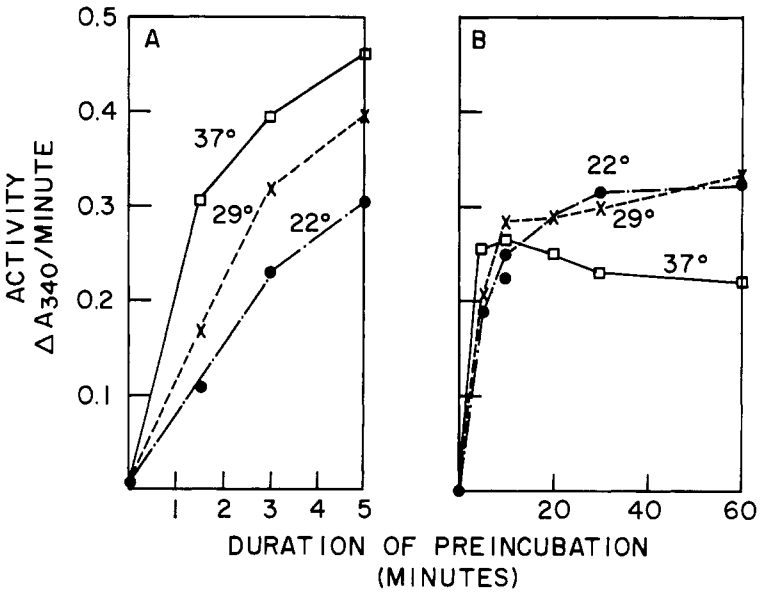

FIGURE 2: Effect of time and temperature on the activation process. (A) Medium for preincubation: Tris-Cl (pH 8.1), 60 mM; thioglycerol (pH 8.1), 200 mM; NAD, 8.0 mM; mixed mitochondrial lipids, 41.5 mμmoles of lipid phosphorus; and enzyme, 175 μg, in total volume of 0.5 ml. At appropriate times 0.1-ml aliquots were removed and assayed at 22° in volume of 2.0 ml under standard conditions. (B) Medium for preincubation: Tris-Cl, 75 mM; thioglycerol (pH 8.1), 200 mM; NAD, 1.0 mM; mixed mitochondrial lipids, 730 mμmoles of lipid phosphorus; and enzyme, 525 μg, in total volume of 0.7 ml. At appropriate times 0.1-ml aliquots were removed and assayed in total volume of 2.0 ml under standard conditions.

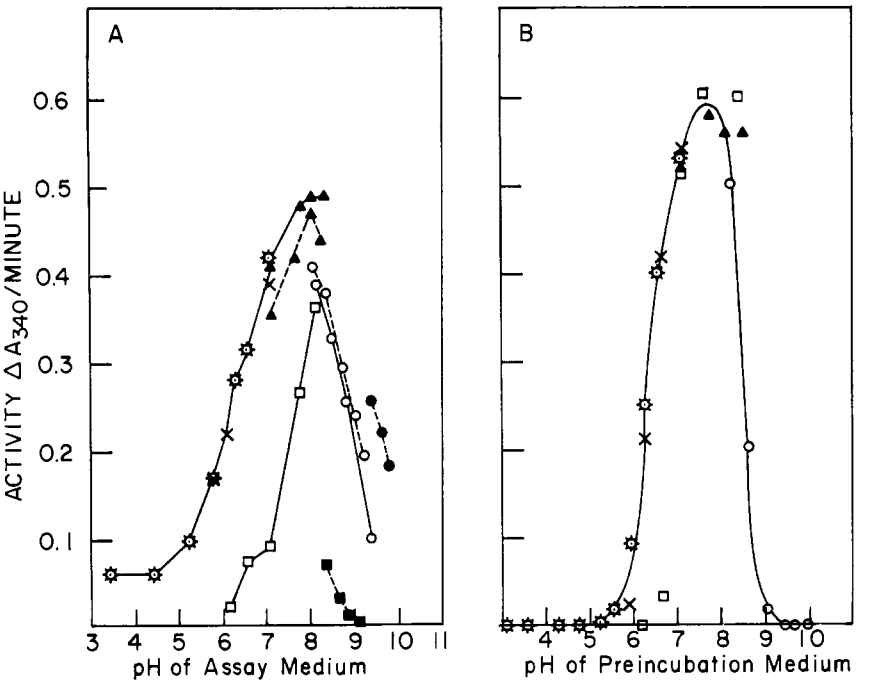

FIGURE 3: Effect of pH on enzymatic activity and activation. (A) Preincubation medium contained Tris-Cl (pH 8.1), 75 mM; NAD, 10 mM; thioglycerol (pH 8.1), 200 mM; mixed mitochondrial lipids, 2.5 μmoles of lipid phosphorus; and enzyme, 2.16 mg, in total volume of 2.4 ml. After preincubation for 30 min at 29°, the mixture was iced and 100-μl aliquots were assayed in medium containing 42.5 mM buffer, 1 mM NAD, and 10 mM BOH in total volume of 2.0 ml. The final pH of the assay medium was verified after each assay. (B) Preincubation medium: buffer (25 mM), NAD (1 mM), thioglycerol (200 mM), mixed mitochondrial lipids, 104 mμmoles of lipid phosphorus, and enzyme (90 μg) in total volume of 0.1 ml. Preincubation was for 30 min at 29°. Assays were performed under standard conditions at pH 8.1 in total volume of 2.0 ml. The final pH of the assay medium was verified after each assay. (☼) Citrate–phosphate, (×) phosphate, (□) Tris-maleate, (△) Tris-Cl, (○) glycine, (●) sodium carbonate, and (■) borate.

increasing temperature. Figure 2B shows that the total activation achieved was less when the temperature was raised to 37°. It will be shown in a subsequent paper (G. S. Gotterer, in preparation) that during preincubation at 37° a concomitant inactivation of the enzyme occurred and that the presence of NAD at least partially prevented this inactivation process.

The effect of pH on both the activation and the enzymatic processes was examined. As seen in Figure 3, the optimum pH for both processes was found to be in the range from 8.0 to 8.5.

Thiol Requirement. Activation of the isolated enzyme showed an absolute requirement for thiol, as seen in Table II. Figure 4 demonstrates that as much as 0.1 M thiol was required for maximum activation and that several thiols were equally effective in their ability to support the activation. The presence of 0.4 M thiol in the *assay* medium, on the other hand, resulted in a 20% inhibition of enzymatic activity.

The need for such high concentrations of thiol raised the question of whether the active component was a trace contaminant, such as the oxidized form of the thiol. That the oxidized form of the thiols is not the active component is suggested by the results in Table III, in which it is seen that enrichment of the preincubation medium with oxidized glutathione resulted in the diminution rather than the enhancement of activation.

TABLE III: Effect of Oxidized Glutathione on Activation.[a]

Concn of GSSG (mM)	Act. (A_{340}/min × 10^3)
0	345
5	325
10	315
40	320
80	220

[a] Preincubation: 70 μg of enzyme, 220 mμmoles of mixed mitochondrial lipids, 1 mM NAD, 15 mM Tris-Cl (pH 8.1), 600 mM thioglycerol, oxidized glutathione as indicated in total volume of 0.1 ml, preincubated at 25° for 45 min. Assayed in total volume of 2.0 ml in the presence of 1.0 mM NAD, 75 mM Tris-Cl (pH 8.1), and 10 mM BOH.

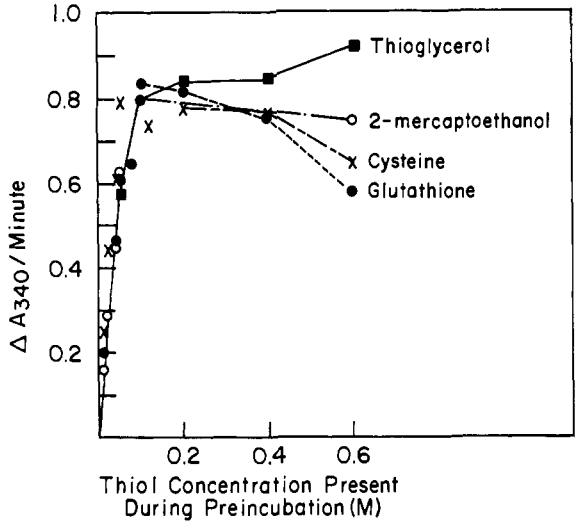

FIGURE 4: Thiol requirement for activation. Preincubation: 1.0 mM NAD, 15 mM Tris-Cl (pH 8.1), 220 mμmoles of mixed mitochondrial lipids, 140 μg of enzyme, and thiol, as indicated in a total volume of 0.1 ml, preincubated at 25° for 45 min. Assayed in 2.0 ml in the presence of 1.0 mM NAD, 75 mM Tris-Cl (pH 8.1), and 10 mM BOH.

Pyridine Nucleotide Requirement. The relative effectiveness of various pyridine nucleotide analogs and simple adenine compounds to serve as activators or as electron acceptors is compared in Table IV. It is seen that only NAD and to a lesser extent acetylpyridine–adenine dinucleotide served as electron acceptors. The use of higher concentrations of analogs did not change the results.

On the other hand, several of the analogs and even ADP and AMP caused significant activation. The 3-pyridine aldehyde and the thionicotinamide derivatives, which were totally inactive as electron acceptors, in particular produced very significant activation of the enzyme. It should be pointed out that these results reflect minimum effectiveness in activation, since no attempt was made to correct for the inhibition of the catalytic activity caused by the residual presence of the analogs in the assay medium.

Table IV suggests that NADH was less effective as an activator than oxidized NAD. The lesser effectiveness of NADH was in part due to product inhibition (compare lines 1 and 3, Table V). It would appear, however, that NADH is in fact less effective than NAD since the enzyme preincubated with NADH was less active than that preincubated with NAD when the preparations were assayed under identical conditions (lines 3 and 4, Table IV). Figure 5 demonstrates the effect of altering the NAD:NADH ratio present during preincubation in an experiment in which all assays were performed in the presence of the same NAD and NADH concentrations. Independent experiments, utilizing purified alcohol dehydrogenase, showed that NADH was stable and catalytically active after incubation under the conditions required for activation.

TABLE IV: Pyridine Nucleotide Requirements.[a]

Addition	Relative Activity: Activating (addn to preincubn)	Relative Activity: Electron Accepting (addition to assay)
None	11	–
NAD	100	100
NADH	49	–
NADP	25	0
NADPH	12	–
3-Acetylpyridine–adenine dinucleotide	56	11
3-Pyridinealdehyde–adenine dinucleotide	54	0
Thionicotinamide–adenine dinucleotide	55	0
Nicotinamide–hypoxanthine dinucleotide	20	(5)
3-Acetylpyridine–hypoxanthine dinucleotide	16	0
3-Pyridinealdehyde–hypoxanthine dinucleotide	20	0
ATP	22	–
ADP	40	–
AMP	41	–
Nicotinamide	9	–

[a] Preincubation medium: Tris-Cl (pH 8.1), 185 mM; thioglycerol (pH 8.1), 154 mM; mixed mitochondrial lipids, 84 mμmoles of phosphate; enzyme, 125 μg; additions, 5 mM as indicated; total volume, 0.13 ml. Preincubated at 29° for 30 min. Assayed after addition of 1.9 ml. Tris-Cl (pH 8.1), 75 mM, containing 1 mM NAD. Reaction started with addition of 0.015 ml; 1.5 M β-hydroxybutyrate. Specific activity, activated in the presence of NAD: 3.2 μmoles/min per mg of protein at 22°. Study of electron-accepting effects. Preincubation medium: Tris-Cl (pH 8.1), 63 mM; thioglycerol (pH 8.1), 210 mM; mixed mitochondrial lipids, 1.4 μmole; enzyme, 2.0 mg; total volume, 0.86 ml. Preincubated at 29° for 40 min. Kept in ice bath while 0.100-ml aliquots were assayed in total volume of 2.0 ml containing Tris-Cl (pH 8.1), 75 mM; β-hydroxybutyrate, 12.5 mM; pyridine nucleotide 1 mM, as indicated. Wavelengths (mμ) with ϵ_{mM} in parentheses: NAD, 338 (6.22); NADP, 339 (6.2); 3-acetylpyridine–adenine nucleotide, 358 (9.3); thionicotinamide–adenine dinucleotide, 395 (11.3); nicotinamide–hypoxanthine dinucleotide, 338 (6.2); 3-acetylpyridine–hypoxanthine dinucleotide, 361 (9.0); and 3-pyridinealdehyde–hypoxanthine dinucleotide, 356 (9.4).

TABLE V: Comparison of NAD and NADH: Activation and Assay.[a]

Expt	Concentration of Pyridine Nucleotide (mM)				Act. (A_{340}/min)
	Preincubation		Assay		
	NAD	NADH	NAD	NADH	
1	2.5	0	1.2	0	0.430
2	0	2.5	1.0	0.2	0.290
3	2.5	0	1.2	0.2	0.370
4	0	2.5	1.2	0.2	0.280

[a] Preincubation medium: Tris, 67 mM; thioglycerol, 200 mM; pyridine nucleotide, as indicated; mixed mitochondrial lipids, 39 mμmoles; and enzyme, 122 μg; total volume, 0.15 ml (pH 8.1). Preincubated at 29° for 40 min. Assayed in a total volume of 2.0 ml containing Tris-Cl (pH 8.1), 75 mM; pyridine nucleotide, as indicated. Reaction started with addition of 0.015 ml of 1.5 M β-hydroxybutyrate.

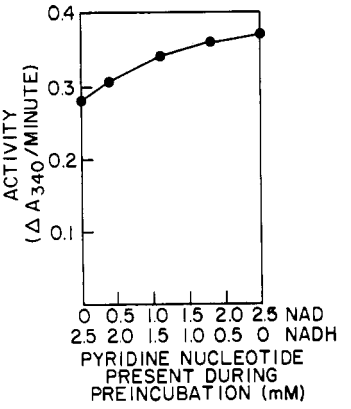

FIGURE 5: Effect of NAD:NADH ratio on activation. Preincubation conditions as in Table V. Assayed in a total volume of 2.0 ml containing Tris-Cl (pH 8.1), 75 mM; NAD, 1.2 mM; NADH, 0.2 mM; and β-hydroxybutyrate, 12.5 mM.

The lower effectiveness of NADH was, therefore, not due to its destruction during the preincubation and presumably reflects a difference in the response of the enzyme to the oxidation state of NAD.

Discussion

Various criteria have been used to evaluate whether an enzyme preparation solubilized from a complex, membranous state has been significantly altered during the process of solubilization (*e.g.*, Watari *et al.*, 1963; King, 1963). D-β-Hydroxybutyrate dehydrogenase, isolated by treatment of membrane fragments at elevated pH, as described in this paper, has the same substrate and electron-acceptor specificity and the same pH–activity profile as that existing when the enzyme was in the membranous state. On the other hand, solubilization has obviously resulted in very significant changes in the properties of the enzyme. After solubilization the enzyme was completely dependent on preincubation with thiol and almost completely dependent on the presence of lipid during the preincubation. Sufficient information is not yet available to evaluate the physiological significance of these studies concerning the properties of the extracted enzyme. The following points are pertinent, however.

Wise and Lehninger (1962) reported the reactivation of oxidatively inactivated, membrane-bound dehydrogenase by incubation in the presence of thiol and NAD. Unlike the reactivation of the membrane-bound enzyme, however, the presence of β-hydroxybutyrate or succinate did not enhance the reactivation of the solubilized enzyme. It remains to be determined whether the mechanism of the reactivation is identical in the two preparations.

The properties of the pH-solubilized preparation from rat liver are very similar to those of the detergent-solubilized preparation from beef heart, as described by Sekuzu *et al.* (1963) and Jurtshuk *et al.* (1963). Assuming a Q_{10} of 2 for the enzymatic activity of the activated complex, the specific activities of the preparations isolated from rat liver by pH treatment were essentially the same as those of the preparations isolated from beef heart by detergent extraction. Both show very similar requirements for activation in the presence of thiol, pyridine nucleotide, and lipid. Attempts to achieve further purification of the rat liver enzyme by ammonium sulfate fractionation of the activated complex, as applied by Jurtshuk *et al.* (1963) to the beef heart enzyme, were unsuccessful. As demonstrated in the following paper, the lipid specificity is the same for both enzyme preparations.

The enzyme preparation contains sufficient endogenous lipid to account for its activity in the absence of added lipid. In the presence of only the endogenous lipid, however, the pH optimum for activation shifted from 8.1 to 6.4.

An acid extract of the enzyme preparation contains organic phosphorus, has an ultraviolet spectrum compatible with this being pyridine nucleotide, and presumably reflects NAD present in the preparation. The contribution of the NAD added during the solubilization process to this residual NAD remains to be determined. Further studies are, therefore, required to evaluate whether the pyridine nucleotide requirement might be an absolute requirement rather than only a partial requirement, as would appear from the present studies.

Under all circumstances thiol at very high concentrations was required. The equal effectiveness of several lots of thioglycerol as well as of several types of thiol compounds and the inhibition by oxidized thiols make it likely that the reduced thiol is in fact the active agent.

Preliminary experiments utilizing gel filtration indicate that the thiol causes a disaggregation of the enzyme preparation. It is tentatively suggested that a disaggregated form of the enzyme interacts with lipid to form the active enzyme–lipid complex.

The results presented indicate that NAD is a more effective activator than NADH. Because of the known propensity for NADH solutions to form inhibitors of pyridine nucleotide linked enzymes (Dolin, 1962), these results must be interpreted with some caution. Care was taken to minimize the formation of inhibitors. NADH was stored carefully dessicated and solutions were prepared immediately prior to use.

The physiological significance of β-hydroxybutyrate dehydrogenase is as yet undetermined. The acetoacetate formed is not further metabolized in isolated liver mitochondria (Lehninger, 1962). Devlin and Bedell (1960) have suggested that the enzyme may function in a transport system for the passage of reducing equivalents across the mitochondrial membrane. Klingenberg and Häfen (1963) have suggested that the enzyme might function in a mechanism for coordinating the oxidation–reduction levels in the mitochondria from one tissue to another *via* the ratio of β-hydroxybutyrate to acetoacetate in the circulating blood. The finding that the NAD:NADH ratio affects the inherent activity of the enzyme suggests the additional possibility that the enzyme might function as a biological buffer system for NAD and NADH. As more NADH is formed the enzyme becomes less active and thereby slows generation of NADH. The system would work in the reverse sense starting with acetoacetate and NADH; the enzyme would become increasingly more active as the reaction proceeded. Studies are being undertaken to evaluate this possibility.

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References

Arrigoni, O., and Singer, T. P. (1962), *Nature 193*, 1256.
Devlin, T., and Bedell, B. (1960), *J. Biol. Chem. 235*, 2134.
Dolin, M. (1962), *Biochim. Biophys. Acta 63*, 219.
Fleischer, R., Casu, A., and Fleischer, S. (1966), *Biochem. Biophys. Res. Commun. 24*, 189.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Gotterer, G. S. (1964), Ph.D. Dissertation, Johns Hopkins University, Baltimore, Md.
Gotterer, G. S. (1967), *Biochemistry 6*, 2147 (this issue; following paper).
Hogeboom, G. H., Schneider, W. C., and Palade, G. H. (1948), *J. Biol. Chem. 172*, 619.
Jurtshuk, Jr., P., Sekuzu, I., and Green, D. E. (1963), *J. Biol. Chem. 238*, 3595.
King, T. E. (1963), *J. Biol. Chem. 238*, 4037.
Klingenberg, M., and Häfen, H. (1963), *Biochem. Z. 337*, 120.
Lehninger, A. L. (1962), *J. Biol. Chem. 178*, 625.
Lehninger, A. L., Sudduth, H. C., and Wise, J. B. (1960), *J. Biol. Chem. 235*, 2450.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, K. J. (1951), *J. Biol. Chem. 193*, 265.
Minikami, S., Ringler, R., and Singer, T. (1962), *J. Biol. Chem. 237*, 569.
Sekuzu, I., Jurtshuk, Jr., P., and Green, D. E. (1961), *Biochem. Biophys. Res. Comm. 7*, 71, 76.
Sekuzu, I., Jurtshuk, Jr., P., and Green, D. E. (1963), *J. Biol. Chem. 238*, 975.
Stadtman, E. (1957), *Methods Enzymol. 3*, 939.
Watari, H., Kearney, E. B., and Singer, T. P. (1963), *J. Biol. Chem. 238*, 4063.
Ways, P., and Hanahan, D. (1964), *J. Lipid Res. 5*, 318.
Wise, J. B., and Lehninger, A. L. (1962), *J. Biol. Chem. 237*, 1363.