Rat Liver D-β-Hydroxybutyrate Dehydrogenase. III. Inhibition by Topical Anesthetics*

Gerald S. Gotterer†

ABSTRACT: A series of topical anesthetic agents of quite diverse structure was found to inhibit a solubilized and partially purified preparation of the phosphatidylcholine-requiring enzyme, D-β-hydroxybutyrate dehydrogenase. Except for the tertiary amines, dibucaine, tetracaine, and quinine, the enzyme inhibitory potency of the various agents correlated well with their anesthetic potency.

The same results were obtained whether the enzym was activated with purified phosphatidylcholine or a mixture of mitochondrial lipids. The inhibition was competitive with respect to β-hydroxybutyrate or acetoacetate, but not with respect to nicotinamide–adenine dinucleotide. The inhibition by tetracaine was completely reversible; the inhibition by propanol was only partially reversible.

The mitochondrial enzyme, D-β-hydroxybutyrate dehydrogenase, is a member of a unique class of membrane-bound enzymes which show an absolute requirement for lipid. In previous studies it was shown that a solubilized, partially purified preparation of D-β-hydroxybutyrate dehydrogenase must be preincubated in the presence of a thiol, its coenzyme (NAD), and lipid before maximum reactivation of the enzyme occurs (Sekuzu *et al.*, 1963; Jurtshuk *et al.*, 1963; Gotterer, 1967a, b). The requirement for thiol and lipid is absolute and the lipid requirement can be supplied specifically by phosphatidylcholine or lipid preparations containing phosphatidylcholine. In the course of these studies it was found that alcohols inhibit the activity of the solubilized, activated enzyme and that the inhibitory potency of the alcohol increases with increasing chain length. Since this characteristic is also representative of the anesthetic activity of alcohols, a study was undertaken to determine whether the correlation between enzyme–inhibitory potency extended to other classes of topical anesthetics. This paper reports the results of these studies and kinetic studies examining the effects of the anesthetic agents on the enzymatic activity of D-β-hydroxybutyrate dehydrogenase.

Materials and Methods

Enzyme Preparation. D-β-hydroxybutyrate dehydrogenase was solubilized and partially purified as described previously (Gotterer, 1967a). The following additional step was added which consistently removed inactive protein as determined by enzyme assay and disc electrophoresis, but led to an inconsistent increase in the specific activity of the final enzyme preparation. The solubilized enzyme precipitating between 20 and 60% saturation with ammonium sulfate was suspended in the minimum volume of 0.01 M potassium phosphate buffer (pH 7.4) sufficient to result in a slightly opalescent solution. This solution of enzyme was desalted by passage over a Sephadex 25 column equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). After passage through the column the fractions with protein were pooled and made 0.1 M in thioglycerol and 2 mM in NAD by the addition of appropriate volumes of concentrated solutions of these reagents. The protein was then absorbed onto an hydroxylapatite gel. Bio-Rad Bio-Gel HT (0.28 dry g/ml of settled bed), 0.2 ml/mg of protein, was added to the solution. The suspension was then centrifuged after incubation for 15 min at ice-bath temperatures. The supernatant solution was then assayed for enzymatic activity and an additional 0.1 ml/mg of original protein was added if enzymatic activity remained. The hydroxylapatite was then washed repeatedly with 0.05 M phosphate (K^+)–0.002 M NAD–0.1 M thioglycerol (pH 8.0) until no further protein was eluted from the gel. The washing process was monitored by measuring the absorbance of the wash solutions at 280 mμ. The gel was then washed with 0.05 M phosphate (K^+)–0.002 M NAD–0.1 M thioglycerol (pH 10.5). The enzymatic activity was eluted from the gel by successive washes at this pH. The eluted enzyme was then concentrated by precipitation from a solution 20 and then 60% saturated with ammonium sulfate. The precipitated enzyme preparation was stored at −20° and in this form was stable for several weeks. Prior to use the enzyme was suspended in appropriate buffer and centrifuged for 10 min at 24,000*g* to remove insoluble, enzymatically inactive material.

Assay of Enzymatic Activity. Appropriate aliquots of enzyme and lipid were preincubated at 28° for 45 min in the presence of 60 mM phosphate (K^+)–200 mM thioglycerol–4 mM NAD (pH 8.1), unless indicated otherwise. The enzyme so activated was either assayed immediately or kept at ice-bath temperatures until assayed, usually within 20 min. A 0.1-ml aliquot of the activated

* From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland. *Received October 7, 1968.* This work was supported by grants from the U. S. Public Health Service (HE 10638 and GM 05919).

† Recipient of the Elliott P. Joslin Research and Development Award, 1968, American Diabetes Association.

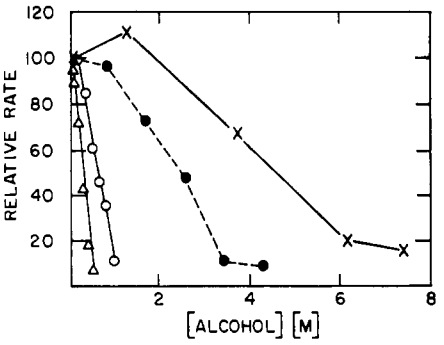

FIGURE 1: Inhibition of D-β-hydroxybutyrate dehydrogenase by normal alcohols. Methanol (×), ethanol (●), propanol (○), butanol (△).

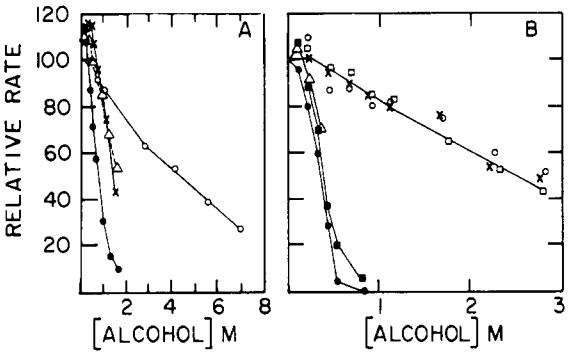

FIGURE 2: Inhibition of D-β-hydroxybutyrate dehydrogenase by alcohols. (A) 1-Propanol (●), 1-propen-3-ol (×), isopropyl alcohol (△), 1,3-propanediol (○); (B) 1-butanol (●), isobutyl alcohol (■), 2-buten-1-ol (△), 1,3-butanediol (×), 1,4-butanediol (○), 2,3-butanediol (□).

enzyme was dilute 20-fold and assayed in the presence of 75 mM potassium phosphate buffer (pH 8.1), 15 mM β-hydroxybutyrate, and 1.2 mM NAD, except in those experiments in which the substrate concentrations were systematically varied. The reaction was followed spectrophotometrically by monitoring the 340-mμ absorption of reduced NAD.

Additions to the assay medium of compounds with such low water solubility that concentrated stock solutions could not be made were made from concentrated ethanolic solutions. The ethanol concentration of the assay medium after such additions was well below the minimum concentration necessary to cause any effect on enzymatic activity.

Materials. Egg lecithin was purchased from Sylvana Chemical Co., Orange, N. J. Mitochondrial lipids were extracted and washed by the method of Folch *et al.* (1957), using 0.05 M KCl as the washing solution. Aqueous suspensions of the lipids were prepared by removing the volatile organic solvent under a stream of nitrogen, suspending the lipids in water at a concentration of about 8 μmoles of lipid-phosphorus/ml, and sonicating at 0° for 10 min. In some cases the sonicated suspension was clarified by centrifugation at 105,000*g* for 30 min.

NAD was purchased from Sigma Chemical Co., NADH from Calbiochem, DL-β-hydroxybutyrate (sodium) from Mann Research Laboratories. Lithium acetoacetate was prepared by the method of Hall (1962) using ethyl acetoacetate obtained from Eastman Organic Chemicals as starting material. Allyl alcohol, 1,3-butanediol, 1,4-butanediol, and 1,3-propanediol were obtained from Matheson Coleman and Bell; 2-buten-1-ol and 2,3-butanediol from K & K Laboratories. Tetracaine-HCl (Pontocaine-HCl) was obtained as a 1% aqueous solution from Winthrop Laboratories and dibucaine-HCl (Nupercaine-HCl) as a 0.25% solution in 5% dextrose in water from Ciba Pharmaceutical Co. The dextrose content of the latter reagent was found to have no effect on the reactions studied. All other reagents were purchased from standard sources, were of the highest purity available, and were used without further purification.

Results

Effect of Simple Alcohols. Inhibition of D-β-hydroxybutyrate dehydrogenase activity by a series of normal alcohols is illustrated in Figure 1. It is seen that alcohols with longer chain length inhibit enzymatic activity at lower alcohol concentration. The stimulation seen at low concentrations of methanol was found with several other alcohols in numerous other experiments. This stimulation at low alcohol concentration is a variable phenomenon which seems to depend on the lot and age of both the lipid preparation and the enzyme preparation. The stimulation appears to be most reproducible when both the enzyme and the lipid preparations are used immediately after preparation,

The significance of the hydrophobic character of the carbon chain in determining enzyme inhibitory potency is shown in experiments using substituted alcohols. With a series of three-carbon alcohols the normal chain alcohol is the most inhibitory (Figure 2A). Change from the normal to the isopropyl isomer or the addition of a double bond into the chain results in a halving of the inhibitory potency, as determined by the concentrations required to cause 50% inhibition. Addition of an hydroxyl group, as in 1,3-propanediol, results in even a more marked reduction in potency. In other experiments it was found that addition of a third hydroxyl group (glycerol) caused no significantly greater effect than the addition of the second hydroxyl group.

With the series of four-carbon alcohols, the change from the normal to the isobutyl isomer or the insertion of a double bond in the 2–3 position has little effect on the inhibitory potency (Figure 2B). On the other hand the addition of a second hydroxyl group profoundly reduces the effectiveness of the alcohol.

Effect of Anesthetic Agents. Compounds of quite varied chemical structure are able to block nerve conduction (Agin *et al.*, 1965). The ability of such compounds to inhibit the enzymatic activity of D-β-hydroxybutyrate dehydrogenase is illustrated in Table I. With the exception of tetracaine, dibucaine, and quinine, a correlation is found between the concentration required

TABLE I: Inhibition by Anesthetics.

Compound	Concn (mM) Causing 50% Inhibn of Enzymatic Act. A	Min Concn (mM) Required to Block Nerve Conduction[a] B	A/B
Methanol	4600	1230	3.7
Ethanol	2500	562	4.4
Isopropyl alcohol	1300	355	3.7
Acetone	1000	398	2.5
Propanol	630	251	2.5
Urethan	400	100	4.0
Butanol	230	60	3.8
Benzyl alcohol	65	20	3.2
Acetanilide	35	14.8	2.4
Benzimidazole	25	6.5	3.9
Phenol	12.8	10	1.3
Hydroquinone	8.4	25.1	0.3
o-Phenanthroline	1.6	0.16	10
8-Hydroxyquinoline	1.4	2.0	0.7
β-Naphthol	1.1	1.0	1.1
Tetracaine	0.60	0.00126	475
Dibucaine	0.58	0.000063	9200
Quinine	0.11	0.00025	440

[a] Data from Agin *et al.* (1965).

to cause 50% inhibition of enzymatic activity and the concentration required to block nerve conduction. Despite a range of absolute concentration spanning three orders of magnitude, the ratio of enzyme inhibitory potency to anesthetic potency of these compounds averages 3.2, with a standard deviation of 2.3. Tetracaine, dibucaine, and quinine, all tertiary amines, on the other hand, require from 440 to 9200 times higher concentrations to block enzymatic activity than those concentrations required to block nerve conduction.

The nature of the lipid used to activate the enzyme does not seem to affect the relative potency of the anesthetic agent. Figure 3 illustrates an experiment in which the potency of propanol and tetracaine was examined with an enzyme preparation activated with either purified egg lecithin or a crude mixture of lipids isolated from rat liver mitochondria. It is seen that the change in composition of the lipid activator causes no significant differences in the inhibitory potency of these agents.

Reversibility of Inhibition. One characteristic of topical anesthetics is the reversible nature of their anesthetic action. The reversible nature of the inhibition of the activity of D-β-hydroxybutyrate dehydrogenase by the anesthetic agents was examined by preincubating for 30 sec the activated enzyme with a concentration of anesthetic which caused about 50% inhibition. The short preincubation time was selected in order to make the experiment most similar to the other experiments reported in this paper in which the enzyme was

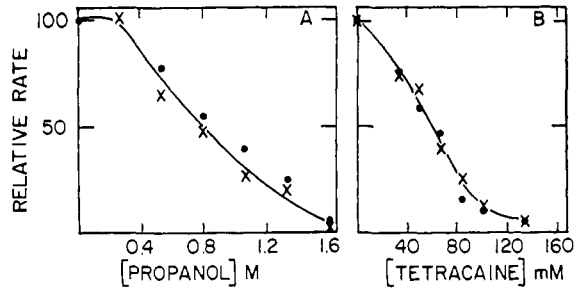

FIGURE 3: Effect of the nature of the lipid activator on inhibition of D-β-hydroxybutyrate dehydrogenase by anesthetics. Amount of lipid used to activate the enzyme was that which resulted in about 90% of maximum activation. (●) Lecithin, 900 mμmoles of lipid phosphorus/mg of protein; (×) mitochondrial lipids, 290 mμmoles of lipid phosphorus/mg of protein.

assayed immediately on exposure to the anesthetic agents. The anesthetic was then diluted to a concentration which caused negligible inhibition and the activity of the enzyme then measured immediately. Table II shows that

TABLE II: Reversibility of Inhibition.

Expt	Compound	Concn during Preincubation (mM)	Concn during Assay (mM)	Rel Act.
1	Tetracaine	0	0	100
		0.65	0.032	100
		0.65	0.65	52
2	Propanol	0	0	100
		670	33	47
		670	670	32
		0	33	112

inhibition by tetracaine is immediately and completely reversible by dilution. Inhibition by propanol under the same conditions, however, was only partially reversible by dilution. The additional control run in experiment 2 shows that the concentration of alcohol remaining after dilution is not sufficient to account for the residual inhibition resulting from the prior exposure of the activated enzyme to the higher inhibitor concentration. The time dependence of the reversal of the inhibition caused by propanol was not further examined.

Kinetics of Inhibition. Inhibition by propanol, benzimidazole, and tetracaine was examined as a function of substrate concentration. Results with β-hydroxybutyrate, plotted as double-reciprocal plots, are presented in Figure 4. It is seen that the anesthetic agents all inhibit competitively with respect to β-hydroxybutyrate. When the reaction is studied in the reverse direction,

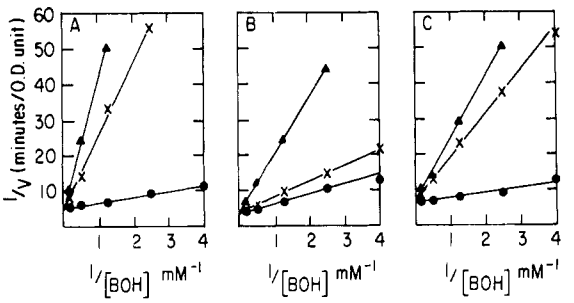

FIGURE 4: Kinetics of inhibition by anesthetics. (●) No additions; (A) propanol, 500 mM (×), 667 mM (▲); (B) benzimidazole, 15 mM (×), 30 mM (▲), in 0.17 M ethanol; (C) tetracaine, 0.415 mM (×), 0.665 mM (▲).

with acetoacetate and reduced NAD as substrates, the inhibition by the agents is also strictly competitive with respect to acetoacetate. On the other hand, inhibition with respect to NAD was found to be of the mixed type.

Discussion

These studies have shown that a series of topical anesthetics of quite diverse chemical structure inhibit the activity of a solubilized, partially purified preparation of D-β-hydroxybutyrate dehydrogenase. In the case of each agent examined, the inhibition was found to be competitive with respect to both β-hydroxybutyrate and acetoacetate. There certainly are no consistent structural analogies between these agents and the enzyme's substrates which would readily explain the competitive nature of the inhibition. Lang (1943) has shown that D-β-hydroxybutyrate dehydrogenase, in a particulate form, is equally active against β-hydroxy acids containing from four to nine carbon atoms. It is therefore possible that the binding site for the hydroxy acids might have a hydrophobic surface. The competitive kinetics could then be explained by the binding of these agents at this specific hydrophobic surface, in a fashion analogous to the binding of such agents at an air–water interface.

Alternatively, the competitive kinetics could represent quasicompetitive inhibition as seen with some allosteric enzymes with which binding at allosteric sites results in enzyme inhibition manifesting competitive kinetics (Stadtman, 1966). Recent work, especially that with aspartyl transcarbamylase (Gerhart and Schachman, 1968), has clarified the nature of such allosteric enzymes and has shown that such enzymes contain a catalytic subunit, which contains the active site, and allosteric subunits which contain the effector binding sites. Changes in enzyme activity result from alterations in subunit conformation brought about by altered subunit interactions attendant upon the binding of substrate or effector at their specific binding sites.

On the basis of the limited experimental evidence available it is possible to propose an allosteric model in which the physical state of the lipid could provide a moderating influence on the catalytic activity of an associated enzyme. It is known that lipids in an aqueous environment form an ordered structure (Luzzati and Husson, 1962). This may take the from of a lamella, a spherical micelle, or structures of other shapes. In this model the nature of the interaction of the enzyme with the ordered lipid structure would influence the conformation of the catalytic protein. The lipid–protein interaction could take place directly with the catalytic unit, in which case the lipid structure would serve a role analogous to effector subunit protein, or the interaction could take place indirectly through the intermediary of an effector protein. An important variable in the system would be the physical state of the ordered lipid structure. Especially important would be the tightness of packing of the lipid molecules and more specifically the relative density of the binding sites at the lipid–protein interface. A change in the packing of the binding sites at the lipid–protein interface would cause alterations in the conformation of the protein and thereby alter catalytic activity.

Such a mechanism is especially appealing in the case of D-β-hydroxybutyrate dehydrogenase. Even with only the partially purified preparations studied to date, there would appear to exist a stoichiometry between lipid and protein (Gotterer, 1967b). More important, the high degree of specificity shown for phosphatidylcholine would support the concept of firm, yet highly specific binding sites between the protein and the ordered lipid structure.

The effect of topical anesthetic agents on enzymatic activity, as presented in this paper, can be explained on the basis of this model. Studies with ordered lipid in the form of a monolayer at an air–water interface have shown that a direct relationship exists between the ability of various substances to penetrate into the lipid monolayer and the anesthetic potency of these agents (Skou, 1958; Hersh, 1967). The correlation between enzyme inhibitory potency and anesthetic potency demonstrated in this paper can therefore be extended to a correlation with the ability of these agents to penetrate into ordered lipid structures. The presence of these agents in the lipid structures affects the packing of the lipids within the structure. In the specific system studied, the agents would alter the density of the phosphatidylcholine binding sites at the lipid–protein interface. These changes could then cause the conformational changes which result in altered enzymatic activity. Analogous to the reciprocal effects shown by allosteric enzymes by the binding of molecules by either the catalytic or the effector subunits, the binding of substrate at the active site could cause conformational changes which would alter the packing at the lipid–protein interface and make penetration by exogenous molecules (*i.e.*, anesthetics) less likely. Such reciprocal effects would account for the competitive kinetics shown by these agents. It might be added that the stimulation of enzymatic activity frequently seen at low alcohol concentrations might be explained by the same scheme: maximum activity of the complex is attained when the packing of the lipids results in an optimum density of the phosphatidylcholine binding sites.

It has been suggested that topical anesthetics exert their effect either by altering the water structure at the cell membrane surface (Pauling, 1961) or by directly interacting with the lipids of the cell membrane (Skou, 1961). That the inhibition of enzymatic activity shown

by the anesthetic agents is strictly competitive with respect to β-hydroxybutyrate and acetoacetate indicates that these agents produce a very specific effect on this lipoprotein enzyme. Such an effect could be due to the direct interaction of the molecule within a hydrophobic portion of the lipoprotein complex, as discussed above. Alternatively, the results could be explained on the basis of a unique susceptibility of a special portion of the enzyme molecule to nonspecific changes in water structure at the lipoprotein surface brought about by the inhibitory agents.

The results presented in this paper must be considered in light of the fact that nerve membranes have been shown to have Na^+–K^+-stimulated adenosine triphosphatase activity which has been implicated in the active transport of ions in these cells (Skou, 1957) and that the Na^+–K^+-stimulated ATPase has been found to have a lipid requirement similar in many ways to that of the D-β-hydroxybutyrate dehydrogenase (Tanaka and Strickland, 1965). It is therefore important to consider the effect of anesthetic agents on the enzymes contained in the membranes as well as on the permeability properties of the membrane alone. Such considerations have been offered by Mullins (1968) and others.

The failure of the tertiary amine anesthetics (tetracaine, dibucaine, and quinine) to inhibit D-β-hydroxybutyrate dehydrogenase at the low concentrations which would have been expected on the basis of the correlation between enzyme inhibitory potency and anesthetic potency demonstrated for the other agents is not readily explainable. Pertinent to this question may be the fact that the enzyme has an absolute requirement for phosphatidylcholine, which at the pH of the system exists as a zwitterion and has a positively charged quaternary ammonium ion (Bangham and Dawson, 1958). With p*K*'s of 8.9, 8.4, and 8.0, dibucaine, tetracaine, and quinine would all have a significant portion of their molecules as positively charged ions. The charged groups at the site of interaction of the enzyme and the phosphatidylcholine might interfere with the effective interaction of the tertiary amine anesthetics with the lipoprotein complex.

Acknowledgment

The author wishes to acknowledge the excellent technical assistance of Mrs. Irene Wood.

References

Agin, D., Hersh, L., and Holtzman, D. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 952.

Bangham, A., and Dawson, R. M. C. (1958), *Nature 182*, 1292.

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.

Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.

Gotterer, G. S. (1967a), *Biochemistry 6*, 2139.

Gotterer, G. S. (1967b), *Biochemistry 6*, 2147.

Hall, L. M. (1962), *Anal. Biochem. 3*, 75.

Hersh, L. (1967), *Mol. Pharmacol. 3*, 581.

Jurtshuk, Jr., P., Sekuzu, I., and Green, D. E. (1963), *J. Biol. Chem. 238*, 3595.

Lang, K. (1943), *Hoppe-Seyler's Z. Physiol. Chem. 277*, 114.

Luzzati, V., and Husson, F. (1962), *J. Cell Biol. 12*, 207.

Mullins, L. J. (1968), *Fed. Proc. 27*, 898.

Pauling, L. (1961), *Science 134*, 15.

Sekuzu, I., Jurtshuk, Jr., P., and Green, D. E. (1963), *J. Biol. Chem. 238*, 975.

Skou, J. (1957), *Biochim. Biophys. Acta 23*, 394.

Skou, J. (1958), *Biochim. Biophys. Acta 30*, 625.

Skou, J. (1961), *J. Pharm. Pharmacol. 13*, 204.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Tanaka, R., and Strickland, K. P. (1965), *Arch. Biochem. Biophys. 111*, 583.