heart (H. Ladinsky, personal communication), did not alter the absence of observable calcium uptake by the microsomal fraction from the medulla.

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Department of Pharmacology,
State University of New York,
Downstate Medical Center,
Brooklyn, N.Y., U.S.A.

BARRY GOZ*

* Trainee under U.S. Public Health Service Pharmacology Training Grant 5 TI GM 163. The research reported here represents part of the work carried out by the author in fulfillment of the requirements for the Ph.D. degree at the State University of New York Downstate Medical Center. Present address: Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.

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Dependence of rat serum lactonase upon calcium

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RECENTLY we reported that rat plasma and liver contain an enzyme that very rapidly catalyzes the hydrolysis of γ-butyrolactone (GBL) to γ-hydroxybutyric acid (GHB).[1] The fact the diisopropylfluorophosphate, prostigmine, and physostigmine did not markedly inhibit this hydrolysis suggested that the enzyme might be more specifically a lactonase than an esterase. This was further borne out by the ability of rat plasma and serum to hydrolyze the homologue, γ-valerolactone, but at a slower rate than GBL. This paper presents further evidence that supports this contention.

When simple attempts were made to purify the lactonase in rat serum, it was observed that dialysis and Sephadex treatment completely destroyed all the enzymatic activity. These observations together with our finding that EDTA completely blocks GBL hydrolysis by rat plasma suggested that one or more reversibly bound metal ions might be involved in the function of this enzyme. Therefore, the dependence of the lactonase on various cations was investigated.

Since we had established that plasma could hydrolyze GBL to GHB and that further metabolism by this tissue was negligible, a simpler method than measuring the disappearance of GBL and appearance of GHB by gas chromatography was sought to follow the rate of hydrolysis. It is known that GBL yields the corresponding hydroxy acid on hydrolysis;[2] therefore, it was considered feasible to utilize a titrimetric assay, based on the amount of acid formed during hydrolysis, to follow the lactonase activity. Similar types of titrimetric assays have been employed to measure activity of esterases.[3] Titration of the GHB formed in the reaction under consideration was found to be a very simple and reliable method for our purposes. The details of the method as well as some preliminary findings are presented below.

All incubations in these studies were carried out at 37° by means of a constant-temperature bath coupled to a jacketed incubation vessel. The internal temperature of the reaction mixture was monitored by means of a YSI model 4ZSC telethermometer and did not deviate from 37° by more than 0·2° All reactions were followed with a Radiometer Titrigraph type SBR23SBUI. Sodium hydroxide (*ca.* 0·3 M) was prepared from carbon dioxide-free distilled water and standardized with potassium acid phthalate. The concentration of GBL routinely used in this study was $1{\cdot}3 \times 10^{-2}$ M, which

approximates the level found in the blood of rats 1 min after the intravenous administration of an anesthetic dose of GBL (500 mg/kg).[4] The incubation volume was 10 ml and contained 0·5 ml of rat serum. In all experiments the reaction was followed for at least 15 min, and in most experiments, to completion. When EDTA was added to the reaction mixture it was preincubated with the enzyme for 5 min prior to addition of the substrate. In addition, when cation-activation was studied, the various cations were incubated with the EDTA–enzyme mixture for another 5 min before addition of the substrate. All cations employed were used as chloride salts. Since the rate of hydrolysis by serum lactonase under the above conditions was found to be linear for at least the first 2 min, the rates of hydrolysis have been calculated during this initial 2-min period.

With this method it was found that GBL was stable in isotonic saline for periods exceeding 1 hr (i.e. no acid was formed from GBL during this period). Furthermore, in the absence of GBL as a substrate, no detectable acid formation was observed in the presence of rat, human, rabbit or guinea pig serum over a 30-min period (cf. control experiment, Fig. 1). With this technique we also confirmed

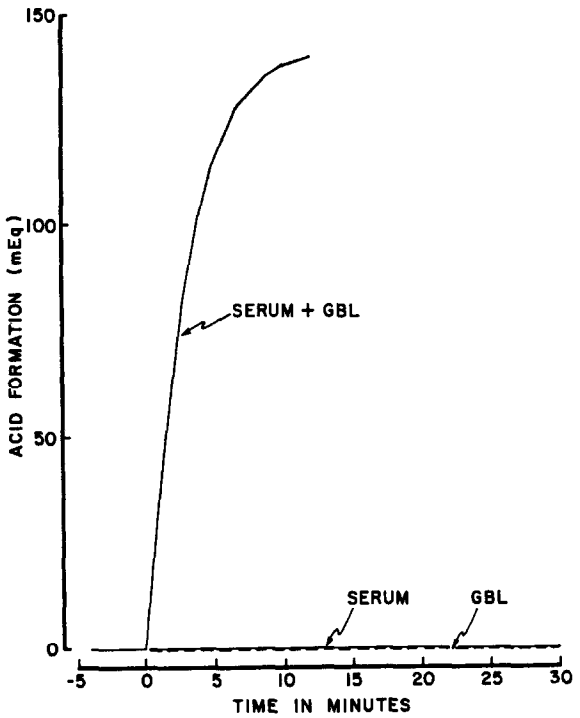

FIG. 1. Hydrolysis of GBL by rat serum (1:10 dilution) at pH 7·4 and 37°.

a number of findings observed[1] when we employed the gas chromatographic technique of measuring the disappearance of GBL to follow lactonase activity, such as the inability of human and cat cerebrospinal fluid (1:10 dilution) and rat brain to catalyze the hydrolysis of GBL to GHB and the ability of EDTA to block the hydrolysis of GBL by rat plasma and serum completely. Table 1 shows the specificity of the EDTA-effect in relation to rat serum. Of the cations investigated, Ca^{++} alone was capable of restoring complete enzymatic activity. This was not an instantaneous effect for, if the substrate was added immediately after the Ca^{++}, only partial activity was restored. However, a preincubation of the EDTA-treated serum with Ca^{++} for 5 min completely restored the enzymatic activity.

The CA^{++}-dependence of the lactonase is yet another difference in properties between serum esterase and serum lactonase. There is no substantive evidence that cholinesterases are metalloproteins, and, as a matter of fact, Svensmark[5] has reported that neither desalting of cholinesterase nor adding chelating agents abolishes esterase activity, indicating that reversibly bound metal ions are not involved in the function of this enzyme.

TABLE 1. CATION DEPENDENCE OF RAT SERUM LACTONASE ACTIVITY

	μmoles of GBL hydrolyzed/min/ml serum ± S.D.	Number of determinations
Blanks		
GBL + isotonic saline	0*	4
Serum + isotonic saline	0*	4
Control		
GBL + serum + isotonic saline	26·2 ± 4·4	16
Additions to controls (Experimental)		
1. EDTA	0*	16
2. EDTA + Ca^{2+}	26·7 ± 3·3	5
3. EDTA + Ba^{2+}	0*	4
4. EDTA + Zn^{2+}	0*	4
5. EDTA + Mg^{2+}	0*	4
6. EDTA + Mn^{2+}	1·7 ± 0·3	4
7. EDTA + Co^{2+}	0*	4

* No detectable acid production observed over at least a 15-min period.

Note: EDTA was used in a concentration of 1×10^{-4}M throughout and was preincubated with serum for 5 min before the addition of the substrate, GBL. All the cations were tested in a concentration of 2×10^{-4}M and were preincubated with serum–EDTA mixture for another 5 min before the addition of GBL.

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Department of Pharmacology,

Yale University School of Medicine,
New Haven, Conn., U.S.A.

R. H. ROTH
R. LEVY
N. J. GIARMAN

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Hydroxylation of cinchophen by microsomal enzymes

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HYDROXYLATION is a common primary metabolic fate of exogenous aromatic substances. The extent of hydroxylation and the orientation of the substituting group have been the subjects of some previous studies with intact animals and with cell-free systems.[1–3] In previous studies on cinchophen detoxication we have found that cinchophen *in vivo* is mainly hydroxylated to 4′- and 8-hydroxycinchophen in dog. This paper describes an enzyme system which hydroxylates cinchophen to 4′-hydroxycinchophen in rabbit and dog.

Male rabbits and dogs were chosen for test animals. In the rabbit the liver and in the dog both the liver and duodenum were used. The tissue samples were homogenized with Ultra-Turrax (TP 18/2) for 5–10 sec on 0·25 M sucrose solution in 0°C (40 g tissue/100 ml 0·25 M sucrose). The homogenate was centrifuged at 9000 *g* for 10 min in 0°C (Servall, SS-1). The supernatant was then centrifuged at 105,000 *g* for 30 min, 0°C (Spinco, L). The precipitate was washed once with cold 0·25 M sucrose