# Some Factors Influencing the Hydrolysis of Benzoyl-l-Arginine Ethyl Ester by Human Plasma. (27522)

THOMAS P. FORDE, RALPH A. O'CONNELL, JAMES W. RYAN AND JOSEPH REILLY
*Department of Psychiatry, Cornell University Medical College, and the Payne Whitney Clinic, New York Hospital*

Human serum and plasma contain enzymes capable of hydrolyzing the synthetic arginine esters, Benzoyl-l-arginine amide (BAA) and Benzoyl-l-arginine ethyl ester (BAEE). The use of these substrates was proposed by Nardi (1,2,3), and Brown(4) as a test to measure increased proteolytic activity of serum in pancreatic disease. Other investigators have shown that trypsin(5), plasmin(6), thrombin (7), and plasma kinin releasers(8) also catalyze the hydrolysis of these synthetic arginine esters.

We have observed that hydrolysis of these esters can be increased by several exogenous factors. It is the purpose of this report to show the influence of heparin, Mepesulfate and glass on hydrolysis of BAEE.

*Method.* Blood was drawn in siliconized syringes from the antecubital veins of healthy medical and nursing students and hospital personnel. The blood was placed in polyethylene tubes containing potassium oxalate (2 mg/ml whole blood). The procedure using BAEE (Mann Laboratories) as a substrate for the enzymatic activity was carried out immediately according to the method of Brown (9). Polyethylene tubes were used throughout the incubation, with the exception of experiments demonstrating the effect of glass contact on enzymatic activity. Heparin and Mepesulfate were added to the reaction mixture in various concentrations with constant total volume maintained. Spectrophotometric readings were done at 540 mμ. Enzyme activity is reported in units of BAEE hydrolyzed, a unit being defined as 1 micromole of BAEE hydrolyzed/hour/ml of plasma(9).

*Results.* Initial observations on the enzymatic activity of plasma on synthetic arginine esters revealed a marked discrepancy between the values obtained using heparin and using potassium oxalate as anticoagulants. Both potassium oxalate and heparin were then observed as reactants in the test system in varying concentrations. It was found that potassium oxalate from 0.5-4.0 mg/ml whole blood did not influence the enzymatic activity of plasma on BAEE. Heparin, however, was active in increasing the hydrolysis of BAEE by plasma. The concentration-response relationship of the heparin activation is shown in Fig. 1. Heparin alone had no effect on BAEE hydrolysis.

Fig. 2 shows that addition of heparin to oxalated plasma markedly augments the enzymatic activity of plasma and that this catalytic effect acts gradually during the incubation period.

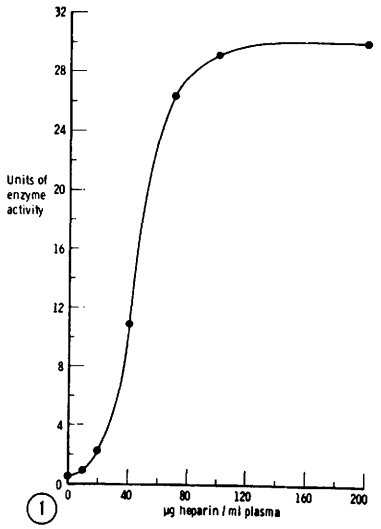
Mepesulfate (Hoffmann-La Roche), a sulfated polygalacturonic acid methyl ester methyl glycoside, is a synthetic anticoagulant similar to heparin in structure and in antithrombin activity(10). It can be seen in Fig. 3 that the activity of Mepesulfate in enhancing plasma hydrolysis of BAEE is similar to that of heparin but that it produces its effect in lower concentrations.

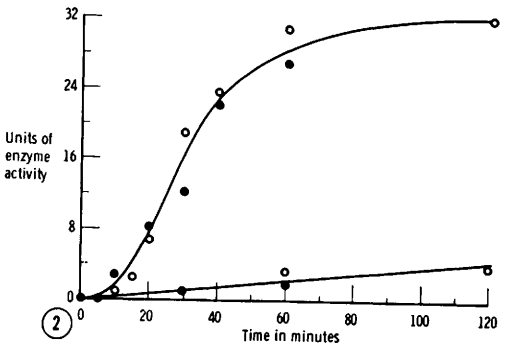
The glass activation of BAEE hydrolysis by plasma is illustrated in Table I. In these experiments the plasma samples were divided between polyethylene and glass tubes for the incubation period. When the glass tubes were

TABLE I. Comparison of Enzymatic Activity of the Same Plasma Sample Incubated in Glass and Polyethylene Tubes.

| | Units BAEE hydrolyzed | |
|---|---|---|
| Subject | Polyethylene | Glass |
| 1 | 1.2 | 6.4 |
| 2 | 1.2 | 26.8 |
| 3 | 1.6 | 34.8 |
| 4 | 4.4 | 3.6 |
| 5 | 2.0 | 11.6 |
| 6 | 3.2 | 37.2 |
| 7 | 4.8 | 30.0 |
| 8 | 2.4 | 21.6 |
| 9 | 2.8 | 24.0 |
| 10 | 7.2 | 26.8 |
| 11 | 1.6 | 5.6 |
| Avg ± S.E. | 2.9 ± .57 | 23.5 ± 3.72 |

(p value of difference $<.001$)



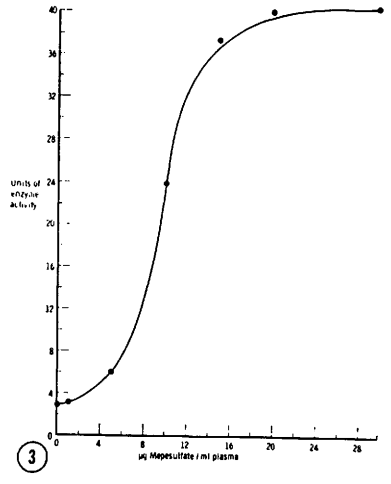




FIG. 1. Relation of varying concentrations of heparin to micromoles of BAEE hydrolyzed. Each point represents the average of 5 determinations. Micrograms of heparin per ml of plasma are plotted against micromoles of BAEE hydrolyzed.

FIG. 2. Relation of time of incubation to micromoles of BAEE hydrolyzed. Upper curve represents oxalated plasma to which 100 micrograms heparin/ml plasma has been added. Lower curve represents oxalated plasma without heparin. Open and solid circles refer to different plasma samples.

FIG. 3. Relation of varying concentrations of Mepesulfate to micromoles of BAEE hydrolyzed. Micrograms of Mepesulfate per ml of plasma are plotted against micromoles of BAEE hydrolyzed.

siliconized there was no increase in enzymatic activity and the values obtained were the same as those of the plastic tubes. Also, when plasma was omitted from the reaction mixture, no spontaneous hydrolysis of the substrate by the glass surface was observed.

*Discussion.* The results outlined above suggest that plasma hydrolysis of BAEE is a complex enzymatic mechanism sensitive to a number of factors which are capable of altering the results obtained. It is likely that factors other than glass and heparin and its analogues are also capable of influencing the reaction. Investigations involving the use of this method must be carefully standardized, and efforts made to rule out activating and inhibiting factors.

The mode of action of heparin and its analogues is incompletely understood. Heparin is a polymer of high molecular weight composed of disaccharide units made up of acetylated glucosamine and glucuronic acid combined with sulfuric acid. The latter groups account for the strong electronegative character of heparin which enables it to combine with many proteins, including thrombin, prothrombin, fibrinogen and albumin(11). Buluk has postulated that heparin inhibits antifibrinolysin and thereby enhances fibrinolysis (12). It may be that the role of heparin in augmenting the hydrolysis of BAEE is similar, *i.e.*, the complexing of an enzymatic inhibitor present in plasma, with resultant increase of enzymatic activity.

Margolis(13) has shown the existence in plasma of a component which reacts with glass to form "contact factor". This factor takes part in the initiation of clotting through its interaction with P.T.A. (plasma thromboplastin antecedent) and also reacts with another plasma component (component B) to release plasma kinins. Of interest are the ob-

servations that fibrinolysin(6) and plasma kinin releasers(8) are both present in plasma and capable of catalyzing the hydrolysis of BAEE.

*Summary.* The hydrolysis of the synthetic substrate BAEE (Benzoyl-l-arginine ethyl ester) by proteolytic enzymes present in plasma is increased by heparin and its synthetic analogue, Mepesulfate. This action of heparin and Mepesulfate is not produced by the calcium binding anticoagulant, potassium oxalate. The hydrolysis of BAEE by plasma is also catalyzed by glass contact resulting in values which are higher and statistically different from those obtained when the reaction is carried out in polyethylene tubes. The implications of these results and possible mechanisms for these findings are considered.

Grateful acknowledgement is made to Dr. Oskar Diethelm for his interest and support of this work. The authors also wish to thank Miss Portia Cutchin for technical assistance.

1. Nardi, G. L., *J. Lab. and Clin. Med.*, 1958, v52, 66.
2. Nardi, G. L., Lees, C. W., *New Eng. J. Med.*, 1958, v258, 797.
3. Nardi, G. L., *Gastroent.*, 1960, v38, 50.
4. Brown, M. E., *New Eng. J. Med.*, 1959, v260, 331.
5. Neurath, H., *Ann. N. Y. Acad. Sci.*, 1957, v68, 11.
6. Troll, W., Sherry, S., Wachman, J., *J. Biol. Chem.*, 1954, v208, 85.
7. Sherry, S., Troll, W., *ibid.*, 1954, v208, 95.
8. Webster, E., Pierce, J. V., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 186.
9. Brown, M. E., *J. Lab. and Clin. Med.*, 1960, v55, 616.
10. Manggieri, C. N., Engelberg, R., Randall, L. O., *J. Pharmacol. and Exp. Therap.*, 1951, v102, 157.
11. Quick, A. J., *Hemorrhagic Disseases,* Lea and Febiger, Phila., 1957, p326.
12. Buluk, K., Januszko, T., *Pathol. Polska.*, 1952, v8, 107.
13. Margolis, J., *J. Physiol.*, 1958, v144, 1.

Received March 28, 1962. P.S.E.B.M., 1962, v110.

## Vasopressin and ACTH Release. (27523)

RICHARD E. GRINDELAND,*† FRANCES E. WHERRY, AND EVELYN ANDERSON†
*Section on Endocrinology, Nat. Inst. Arthritis and Metabolic Diseases, NIH, and Dept. Physiology, Howard University, Bethesda, Md., and Washington, D. C.*

The hypothesis that vasopressin is the neurohumor from the hypothalamus which stimulates the release of ACTH has had strong support, both in anatomical and physiological studies(1,2,3,4). However, a serious criticism of the physiological evidence has been that very large amounts of vasopressin have been necessary to demonstrate ACTH release. McCann(5) has reported 0.6 U of vasopressin as the effective intravenous dose for depletion of adrenal ascorbic acid in rats with lesions in the median eminence. Casentini *et al.*(4) found that 12 mU produced a comparable depletion in adrenal ascorbic acid when given into the carotid artery of rats blocked with 9-alpha fluorohydrocortisone. These amounts of vasopressin are several thousand times the amount which produces antidiuresis in the rat; according to Dicker (6), the rat is sensitive to the antidiuretic effect of 3.5 $\mu$U/100 g body weight. Kwaan and Bartelstone(7) have set up experiments which approach more closely physiological conditions. They found that 2 mU of vasopressin injected into the IIId ventricle of the dog produced an increased secretion of adrenal steroids.

Using a new method for testing ACTH release, we have found that 1.0 $\mu$U of vasopressin injected into the arterial blood supply of an ACTH-secreting pituitary tumor grafted in the hind leg of a hypophysectomized rat causes release of measurable amounts of ACTH.

* Postdoctoral Fellow of Nat. Cancer Inst., U.S.P.H.S., 1961-62.
† Present address: Ames Research Center, N.A.S.A., Moffet Field, Calif.