Biocytinase, an Enzyme Concerned with Hydrolytic Cleavage of Biocytin. (21090)

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Biocytin refers to a conjugate of biotin isolated from yeast extract and identified as ε-N-biotinyl-L-lysine(1). Biocytin is available as a source of biotin for most biotin-requiring microorganisms, for example *Lactobacillus casei*. Biocytin is unavailable as a source of biotin for a few biotin-requiring microorganisms, for example, *Lactobacillus arabinosus*. Preliminary experiments have indicated that the ability of a microorganism to utilize biocytin as a source of biotin is correlated with the occurrence of an enzyme that cleaves biocytin yielding biotin as one moiety (2). The amount of enzyme present in such bacterial extracts as judged by the amount of biocytin hydrolyzed, was very small and rendered experimentation difficult. With the availability of synthetic biocytin in quantity (3), and with the indication that it is safe for administration in man(4), it has been administered to patients in an effort to discover some therapeutic use for this agent. In connection with these studies it has been found quite unexpectedly that blood and urine samples from such individuals appeared to contain biotin rather than biocytin as determined by differential microbiological assay. Subsequent study has shown that there exists in blood, and presumably other tissues as well, an enzyme in considerable quantity that is active in hydrolyzing biocytin to yield biotin.

Since the linkage involved in the conjugation of biotin with lysine (ε-acylation) appears to be unique as far as compounds derivable from natural material are concerned, it is suggested that a previously unrecognized enzyme may be involved in this hydrolysis. Biocytinase is suggested as a tentative name for this enzyme.

Methods. Enzymatic hydrolyses were carried out in open glass tubes without shaking at 37° for 1 hour in a constant temperature water bath. The basal reaction mixture usually consisted of 1 ml of 0.1 M phosphate buffer of pH 6.6 containing 0.01 γ of added biocytin as biotin per ml and 0.2 ml of a 1:10 dilution of blood, plasma, or serum in distilled water. Where variations in pH were studied this was accomplished by varying the proportions of M NaH_2PO_4 and M Na_3PO_4 used to prepare the buffer. In some preliminary experiments, especially where comparatively large amounts of blood were used, no buffer was employed beyond that of the blood itself. Where addenda were studied they were dissolved in the buffer-biocytin mixture if neutral or were added to the buffer-biocytin mixture in the form of neutralized solutions if acid or alkaline. Usually the blood samples studied were collected with sufficient heparin to prevent clotting. Citrated blood was also satisfactory. The enzymatic reactions were terminated by autoclaving the tubes for 5 minutes or by immersing in a boiling water bath for 10 minutes. The reaction mixtures were then diluted to 20 ml and aliquots assayed microbiologically for biotin with *Lactobacillus arabinosus*(5). Suitable controls established that the biocytin, phosphate buffer, blood, plasma, serum, and addenda studied were essentially free of interfering biotin. It was also demonstrated that the various addenda studied, in the concentrations employed, did

TABLE I. Data Demonstrating Quantitative Hydrolysis of Biocytin by Biocytinase of Plasma.

Enzymatic reaction time, min.	Hydrolysis of biocytin, %
0	4
2	46
5	84
10	98
15	100
30	107
45	101
60	99

Reaction mixtures: 1 ml biocytin sol. in water (0.01 γ biocytin as biotin/ml). 1 ml of normal human plasma. Enzymatic activity was stopped at indicated times by immersion of appropriate tube in boiling water bath for 10 min.

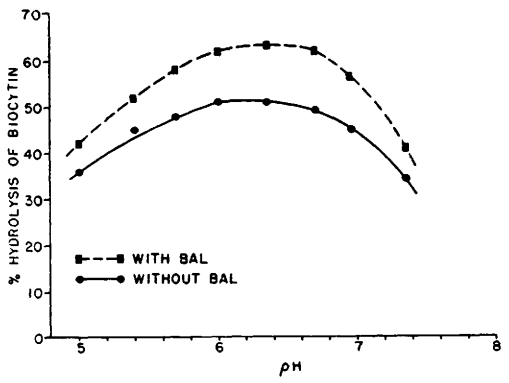

FIG. 1. pH optimum of biocytinase. Reaction mixture: 1 ml biocytin solution (0.01 γ biocytin as biotin/ml) in 0.1 M phosphate buffer of the indicated pH, 0.2 ml of a 1:10 dilution of whole blood in distilled water. BAL where used was present in the biocytin-buffer mixture to saturation. Reaction stopped by autoclaving tubes at 120°C for 5 min.

not influence the response of *Lactobacillus arabinosus* to biotin. Data are presented in terms of the per cent of the 0.01 γ of biocytin hydrolyzed under the conditions of a particular experiment.

Results. The experiment summarized in Table I demonstrates that biocytin is quantitatively hydrolyzed under appropriate conditions of time and enzyme concentration. For more critical studies of the reaction the enzyme concentration was lowered and the reaction time arbitrarily set at 60 min. Data summarized in Fig. 1 demonstrated that the pH optimum is in the range of 6.0-6.7. Additional experiments in which "tris" (tris-(hydroxymethyl) aminomethane) buffer was employed showed that no other maximum exists up to pH 9. Stability studies of biocytinase in which the enzyme content of blood samples stored at 5°C was determined showed that the enzyme appeared to vary in an unpredictable manner such as to suggest a "cyclic" type of activity. In an effort to "stabilize" the reaction, additions of the following compounds were tested and found inactive: niacinamide, manganese sulfate, yeast extract, glycine, magnesium sulfate, glucose, adenosine triphosphate, catechol disulfonate, 'Sequestrene', oxygen, nitrogen, carbon dioxide, ascorbic acid, cystine, potassium ferricyanide, potassium ferrocyanide, and sodium chlorate.

Addition of any one of the following *sulfhydryl compounds* to the reaction resulted in consistent "high" values: thiomalate, thioglycollate, glutathione, and BAL (2,3-dimercapto-1-propanol). Some data demonstrating this effect are summarized in Table II. BAL because of its high activity, low solubility and neutrality was found most convenient to use. Stability studies in which the biocytinase activity of a sample of whole blood stored at 5° was determined at intervals with and without the addition of BAL to the reaction mixture are summarized in Fig. 2. It is apparent that essentially constant and high enzymatic activity of the blood was shown for a period up to 12 days. On the other hand, the activity determined without added BAL varied in an unpredictable manner as previously described.

Although a comprehensive study of the structural specificity of the substrate essential for biocytinase activity was not carried out, some data with biotinamide were obtained. In one experiment involving comparable quantities of the two substrates 47% biotinamide and 32% biocytin were hydrolyzed. In another experiment 56% biotinamide and 45% biocytin were hydrolyzed. If the two compounds are hydrolyzed by the same enzyme then biotinamide is acted upon at a slightly higher rate.

TABLE II. Effect of Certain Sulfhydryl Compounds on the Activity of Biocytinase.

Exp. No.	Addendum	Hydrolysis of biocytin
1	0	27
	Glutathione, .1 mg	32
	1.0	51
	5.0	59
2	0	25
	Thioglycollic acid, .01 mg	26
	.1	40
	.5	56
	BAL (saturated)	77
3	0	24
	Thiomalic acid, .1 mg	27
	1.0	38
	2.0	44
	BAL (saturated)	67

Reaction mixtures: 1 ml pH 6.63 0.1 M phosphate buffer containing 0.01 γ of added biocytin as biotin/ml. Addenda as indicated. 0.2 ml of a 1:10 dilution of whole blood in distilled water. Enzymatic activity stopped after 1 hr by autoclaving tubes at 120°C for 5 min.

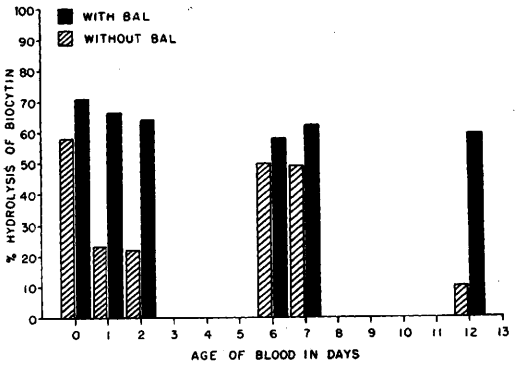

FIG. 2. Stability of biocytinase in stored blood. Reaction mixtures: 1 ml biocytin sol. (0.01 γ biocytin as biotin/ml) in 0.1 M pH 6.6 phosphate buffer, 0.2 ml of a 1:10 dilution of whole blood in distilled water. BAL where used was present in the biocytin-buffer mixture to saturation. Reaction stopped by autoclaving tubes at 120°C for 5 min.

The distribution of biocytinase between plasma and the cellular components of blood has not been extensively studied. In one experiment a sample of whole blood gave 66% hydrolysis of biocytin while the plasma derived from the same sample of blood gave 85% hydrolysis. It would appear that the biocytinase is largely present in the plasma although some activity must be present in the red or white cells to account for the distribution of activity observed.

Discussion. The enzymatic nature of the hydrolysis of biocytin by blood appears to have been established. The essential nature of an appropriate sulfhydryl compound for consistent maximal activity of the enzyme has been pointed out. The optimum pH of biocytinase is within the range of that common to most animal and microbial enzymes. The possible identity of biocytinase with a known amidase, peptidase, or acylase has not been established in this work. It would appear that some concentration and purification of the enzyme is essential before such a point could be established. It has previously been reported that biocytin is unaffected by commercial preparations of pepsin, trypsin, papain, takadiastase, polidase, or mylase(6).

Simple calculations show that there is enough biocytinase in the blood alone of an average adult to hydrolyze approximately 3 mg of biocytin in one hour. The body of an average normal human adult probably does not contain more than 1 mg of biotin in various "combined forms"(7). In addition, if all the biotin contained in a typical diet were in the form of biocytin, only about 0.25 mg calculated as biotin would be consumed per day(8). Thus it is apparent that there may be considerable "physiological pressure" for the conversion of biocytin to biotin. The significance, if any, of this phenomenon remains to be explored.

Summary. The existence in blood of an enzyme in relatively large amount, that accomplishes the hydrolysis of biocytin (ε-N-biotinyl-L-lysine) tentatively termed biocytinase has been pointed out. The enzyme has a pH optimum at 6.0-6.7 and requires for optimum effect the presence of an appropriate sulfhydryl compound.

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