ACYLATION REACTIONS IN THE PRESENCE OF ACETYLCHOLINESTERASE OF HUMAN ERYTHROCYTES*

by

H. BRUCE COLLIER AND PETER F. SOLVONUK

Department of Biochemistry, University of Alberta, Edmonton (Canada)

HESTRIN[1,2] has shown that purified acetylcholinesterase (AChE) from the electric eel, *Electrophorus electricus*, can mediate the reversible hydrolysis of acetylcholine (ACh) and of propionyl choline. Catalysis by this enzyme of acethydroxamic acid (AHA) formation in the presence of hydroxylamine was also demonstrated, by HESTRIN's photometric method[3]. WILSON, BERGMANN, AND NACHMANSOHN[4] also obtained AHA formation by AChE from *Gymnotorpedo occidentalis*. Thus it is evident that the AChE of nerve tissue can catalyze acylation of O (as in ACh synthesis) and of N (as in AHA formation).

HESTRIN[2] found that in the pH range 5.1–7.0 a lower pH favored ACh formation and a higher pH promoted its hydrolysis. This might be expected from the nature of the reversible reaction:

$$(CH_3)_3N^+CH_2CH_2OAc + HOH \rightleftharpoons (CH_3)_3N^+CH_2CH_2OH + Ac^- + H^+$$

HESTRIN suggested that synthesis of ACh by this mechanism might take place at the neuronal membrane. BERGMANN AND SHIMONI[5] proposed that the AChE system in nerve might act as a buffer: the reverse reaction could neutralize the H^+ that is produced during the conductive process in the nerve membrane.

Mammalian erythrocytes contain an active AChE that is associated with the cell stroma. We have found that this enzyme can catalyze the formation of ACh and also the acetylation of hydroxylamine.

METHODS

Erythrocytes from blood supplied by the Red Cross Blood Transfusion Service were used as the source of AChE. The cells were washed 3 times in the centrifuge with 1% NaCl and were hemolyzed by the addition of 10 times their volume of water. After 12 hours at 8° C the "ghosts" or stromata settled out readily without acidification. The supernatant was removed by decantation and the stromata were washed a total of 4–5 times by settling and decantation. Finally the suspension was centrifuged and the packed stromata were resuspended in 5 times their volume of water. This suspension was kept frozen as "stock enzyme" and thawed just before use. Various preparations were used during the course of the work, so that enzyme activities are not always comparable.

The appropriate substrate was prepared fresh each time, and enzyme was added in the form of the suspension of stromata, diluted as required. When buffering was necessary, tris(hydroxymethyl)aminomethane (TRIS) + HCl was used. All incubations were carried out at 25° C. Simultaneous controls contained enzyme that had been inactivated by boiling about 2 min. During incubation the reaction mixtures containing the suspensions of stromata were agitated continuously in small flasks attached to a Warburg shaker.

* Supported in part by Grant no. 352 from the Defence Research Board of Canada.

References p. 588.

Acethydroxamic acid (AHA) in the reaction mixtures was estimated by the photometric method of HESTRIN[3]. To 1.0 ml of an aliquot was added 2.0 ml of a solution of HCl containing 7.5% trichloroacetic acid as protein precipitant. The strength of the HCl was such as to result in a final pH of 1.0–1.4 in each case. Then 2.0 ml of 5% $FeCl_3$ in 0.1 *N* HCl was added and after filtration the absorbancy of the purple-brown ferric-AHA complex was measured in the Beckman DU spectrophotometer at 540 mμ.

Acetylcholine treated with excess NH_2OH was used to obtain the standard curve, as it is converted quantitatively into AHA under these conditions. Absorbancy varied directly with AHA concentration within the range of 0–5 μmoles/ml of sample.

RESULTS

Effect of pH on synthesis and hydrolysis of ACh

The procedure of HESTRIN[2] was followed in examining the effect of pH upon synthesis and hydrolysis of ACh in the presence of erythrocyte AChE, within the range of pH 5.1–7.0. For the study of ACh synthesis the reaction mixture contained 0.7 *M* Na acetate and 0.7 *M* choline chloride (replaced by 0.7 *M* NaCl in the controls). To 6.0 ml of this solution was added 0.5 ml of the stock enzyme suspension. Aliquots were removed at various time intervals and the ACh concentration was determined by the acethydroxamic acid method[3].

To follow the hydrolysis of ACh, the substrate was added (initial concentration = 30 μmoles/ml) to solutions containing 0.7 *M* NaCl in 0.25 *M* TRIS buffer at pH 5.1, 5.9, and 7.0. The same amount of enzyme suspension was added as in the experiment on synthesis, and ACh in the mixture was determined at the same time intervals.

The results of these experiments are recorded in Table I where it is seen that erythrocyte AChE can catalyze the formation of ACh from acetate and choline, and that the rate of formation is increased as the pH is lowered. The rate of hydrolysis, on the other hand, increases as the pH is raised. These results are similar to those obtained by HESTRIN[2] with the *Electrophorus* enzyme.

TABLE I

THE EFFECT OF pH ON SYNTHESIS AND HYDROLYSIS OF ACETYLCHOLINE

For synthesis: 0.7 *M* Na acetate and 0.7 *M* choline chloride (replaced by 0.7 *M* NaCl in the blanks).
For hydrolysis: 0.7 *M* NaCl, 0.03 *M* ACh, 0.25 *M* TRIS buffer.
0.5 ml of stock enzyme + 6.0 ml of reaction mixture.

Time (hours)	*Acetylcholine (μmoles/ml)*					
	Synthesis			*Hydrolysis*		
	pH = 5.1	*5.9*	*7.0*	*5.1*	*5.9*	*7.0*
0	0	0	0	30.0	30.0	30.0
0.5	0.08	0.02	0.01	22.0	12.0	6.65
1.0	0.09	0.06	0.02	14.3	1.63	0.33
2.0	0.22	0.13	0.06	4.70	0.26	0.22
3.0	0.25	0.15	0.07	1.13	0.22	0.22
4.0	0.35	0.17	0.15	0.40	0.17	0.17

Acethydroxamic acid formation

The acylation of NH_2OH to form AHA in the presence of the red-cell enzyme was followed photometrically. The mixtures were incubated at pH 6.3 and 25° C. Experiments with stromata inactivated by boiling indicated that in no case was there non-

References p. 588.

enzymic formation of AHA. Table II illustrates the relationship between concentration of the enzyme and AHA synthesis from Na acetate and NH_2OH. The stock enzyme in this case was diluted 1:4 and varying amounts of this suspension were diluted to 1.0 ml with water before addition to the 2.0 ml of substrate solution. The degree of AHA formation in 2 hours varied directly with the enzyme concentration.

Ethyl acetate could also be used as a substrate for AHA formation, as shown in Table III. One ml of stock enzyme suspension was added to 5 ml of the substrate solution, and again the rate of AHA formation varied directly with enzyme concentration. The velocity of acylation is appreciably greater with ethyl acetate than with Na acetate, as WILSON *et al.*[4] had also found.

HESTRIN[2] observed that choline inhibited AHA formation from Na acetate, and our results in Table IV show that 0.5 *M* choline decreased the rate of AHA formation by about one-half in the presence of the red-cell AChE.

TABLE II

ENZYME CONCENTRATION AND AHA SYNTHESIS FROM SODIUM ACETATE

The reaction mixture contained 0.7 *M* Na acetate and 0.7 *M* NH_2OH. Stock enzyme was diluted 1:4. Two hours incubation at pH 6.3 and 25°.

Enzyme (ml)	*AHA (μmoles/ml)*
0.25	0.13
0.50	0.25
0.75	0.37
1.00	0.50

TABLE III

ENZYME CONCENTRATION AND AHA SYNTHESIS FROM ETHYL ACETATE

The reaction mixture contained 0.14 *M* ethyl acetate and 0.7 *M* NH_2OH. The pH was adjusted to 6.3 with 0.25 *M* TRIS. 1.0 ml enzyme in 6.0 ml total volume.

Enzyme (ml)	*AHA (μmoles/ml)*		
	15'	*30'*	*60'*
0.25	0.11	0.23	0.44
0.50	0.23	0.47	0.94
1.00	0.46	0.90	1.81

TABLE IV

THE EFFECT OF CHOLINE ON AHA FORMATION FROM SODIUM ACETATE

The reaction mixture contained: 0.5 *M* Na acetate, 0.5 *M* NH_2OH, 0.5 *M* choline chloride (replaced by 0.5 *M* NaCl in the controls). pH 6.3.
1.0 ml stock enzyme in 2.0 ml total volume.

Time (hours)	*AHA (μmoles/ml)*	
	— choline	*+ choline*
0.5	0.18	0.06
1.0	0.36	0.13
3.0	1.06	0.42
5.0	1.26	0.63

References p. 588.

To test the effect of various enzyme inhibitors upon AHA formation, ethyl acetate was used as the substrate. The results of this experiment, as summarized in Table V, indicate that the activity was inhibited almost completely by eserine and not at all by *p*-chloromercuribenzoate or by $CuSO_4$. MOUNTER AND WHITTAKER[6] and MARKWARDT[7] have concluded that AChE is probably not a thiol enzyme; this would explain the lack of effect of the thiol inhibitors.

Ethyl acetate was also used in testing the effect of various cations on AHA formation. Na^+, K^+, NH_4^+, Mg^{++}, and Ca^{++} were added, individually, at 0.01 *M* concentration, but none showed any effect on the rate of AHA formation.

TABLE V

EFFECT OF ENZYME INHIBITORS ON AHA FORMATION FROM ETHYL ACETATE

The reaction mixture contained: 0.2 *M* ethyl acetate, 0.7 *M* NH_2OH, 0.25 *M* TRIS buffer, pH 6.3. 60′ incubation.
1.0 ml stock enzyme in 10 ml total volume.

Inhibitor	*Conc. (M)*	*AHA (μmoles/ml)*
None	—	0.36
Eserine	0.00015	0.01
p-chloromercuribenzoate	0.0001	0.18
$CuSO_4$	0.001	0.36

Estimation of choline acetylase activity

HOLLAND AND GREIG[8] reported that they had demonstrated choline acetylase activity of human erythrocytes, but MATHIAS AND SHEPPARD[9] failed to observe any measurable activity in their preparations. We have been unable to detect choline acetylase in acetone powder of human erythrocytes, by a method that does show activity in rat-brain acetone powder.

Acetone powders of human erythrocytes from fresh blood were prepared and extracted by the method of HOLLAND AND GREIG. These extracts were added to a reaction mixture containing the following (final concentration as given): KCl (0.08 *M*), K acetate (0.05 *M*), adenosine triphosphate (0.005 *M*), cysteine (0.02 *M*), coenzyme A concentrate (Krishell Laboratories, 5% CoA, 2 mg/ml), NH_2OH (0.05 *M*), and TRIS buffer, pH 7.2 (0.02 *M*). Incubation for 4 hours at 37° resulted in no detectable AHA formation, whereas an extract of rat-brain acetone powder produced 0.84 μmoles of AHA/ml reaction mixture.

It was found that addition of eserine, as employed by HOLLAND AND GREIG to inhibit AChE, permitted formation of rubroeserine and that this gave rise to high blanks in the determination of AHA.

DISCUSSION

Our experiments indicate that the AChE of the human erythrocyte is able to catalyze certain acylation reactions: acylation of the O atom as in ACh formation, and of the N atom as in hydroxamic acid formation. The results were essentially similar to those obtained by HESTRIN[1,2] with AChE of nerve tissue. No evidence of a separate choline acetylase in erythrocytes was obtained.

References p. 588.

It is not clear whether the reversal of the usual hydrolytic role of AChE in the erythrocyte is of any physiological significance. Since there is no definite evidence that the erythrocytes can synthesize ACh *in vivo*, the function of the esterase remains obscure. However, BERGMANN AND SHIMONI[5] have suggested that the AChE system acts as a buffer in the nerve membrane, catalyzing the formation of ACh as H-ions accumulate during the conductive process. It is possible that AChE plays a similar role in the erythrocyte: it may thus prevent a local accumulation of H-ions (such as would result from glycolysis), which in turn would alter the permeability of the membrane.

GREIG AND HOLLAND[10] suggested that the erythrocyte AChE system might be necessary for the integrity of the cell, but their interesting experiments were carried out in the presence of relatively high concentrations of ACh, which were certainly unphysiological. It seems more likely that the red-cell AChE may catalyze synthesis of ACh, rather than its hydrolysis (if it can be assumed that acetate and choline are available).

ACKNOWLEDGEMENT

We wish to thank Dr. D. I. BUCHANAN, Director of the Red Cross Blood Transfusion Service, Edmonton, for arranging to supply blood that was no longer useful for transfusion purposes.

SUMMARY

1. The acetylcholinesterase of human erythrocytes was shown to catalyze certain acylation reactions.

2. In the range of pH 5.1–7.0, a lower pH favored synthesis, while a high pH promoted hydrolysis of acetylcholine.

3. The enzyme catalyzed acethydroxamic acid formation from sodium acetate or from ethyl acetate at pH 6.3.

4. Enzymic formation of acethydroxamic acid was inhibited by eserine and by choline, but not by *p*-chloromercuribenzoate or by cupric ions.

5. Acethydroxamic acid formation was not influenced by the presence of the following ions: Na^+, K^+, NH_4^+, Ca^{++}, or Mg^{++}.

6. Choline acetylase activity could not be demonstrated in human erythrocytes.

RÉSUMÉ

1. L'acétylcholinestérase des érythrocytes humains est capable de catalyser certaines réactions d'acétylation.

2. Entre pH 5.1 et pH 7, l'abaissement du pH favorise la synthèse, tandis que son élévation active l'hydrolyse de l'acétylcholine.

3. L'enzyme catalyse la formation d'acide acétohydroxamique à partir d'acétate de sodium ou d'acétate d'éthyle à pH 6.3.

4. La formation enzymatique d'acide acétohydroxamique est inhibée par l'ésérine et par la choline, mais ne l'est pas par le *p*-chloromercuribenzoate ou les ions cuivriques.

5. La formation d'acide acétohydroxamique n'est pas influencée par la présence des ions suivants: Na^+, K^+, NH_4^+, Ca^{++} ou Mg^{++}.

6. On ne trouve pas d'activité choline acétylasique dans les érythrocytes humains.

ZUSAMMENFASSUNG

1. Acetylcholinesterase aus menschlichen Erythrocyten ist imstande, gewisse Acylationsreaktionen zu beschleunigen.

References p. 588.

2. Im pH-Bereich 5.1–7.0 fördert ein niederer pH-Wert die Synthese, ein höherer pH-Wert die Spaltung von Acetylcholin.

3. Bei einem pH-Wert von 6.3 förderte das Enzym die Bildung von Acethydroxamsäure aus Natrium- oder Ethylacetat.

4. Enzymatische Bildung von Acethydroxamsäure wird durch Eserin und Cholin, nicht aber durch *p*-Chloromercuribenzoat und Cu^{++} Ionen gehemmt.

5. Acethydroxamsäurebildung bleibt durch folgende Ionen unbeeinflusst: Na^{+}, K^{+}, NH_4^{+}, Ca^{++} und Mg^{++}.

6. Cholinacetylaseaktivität konnte in menschlichen Erythrocyten nicht festgestellt werden.

REFERENCES

1 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 879.
2 S. HESTRIN, *Biochim. Biophys. Acta*, 4 (1950) 310.
3 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
4 I. B. WILSON, F. BERGMANN AND D. NACHMANSOHN, *J. Biol. Chem.*, 186 (1950) 781.
5 F. BERGMANN AND A. SHIMONI, *Biochim. Biophys. Acta*, 10 (1953) 49.
6 L. A. MOUNTER AND V. P. WHITTAKER, *Biochem. J.*, 53 (1953) 167.
7 F. MARKWARDT, *Z. physiol. Chem.*, 295 (1953) 264.
8 W. C. HOLLAND AND M. E. GREIG, *Arch. Biochem. Biophys.*, 39 (1952) 77.
9 P. J. MATHIAS AND C. W. SHEPPARD, *Proc. Soc. Exptl. Biol. Med.*, 86 (1954) 69.
10 M. E. GREIG AND W. C. HOLLAND, *Am. J. Physiol.*, 164 (1951) 423.

Received November 16th, 1954