

Some Common Characteristics of the Acetylcholinesterase of Human, Bovine and Porcine Erythrocytes¹⁾

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More than 80% of the acetylcholinesterase present in porcine erythrocyte membrane could be released from it by repeated washing with hypotonic buffer, as was true in the case of bovine erythrocyte. Density-gradient centrifugation revealed that the released enzyme particles of either species of mammals have a similar density of about 1.07 which is significantly lower than that of the original stroma. It has phospholipid and cholesterol contents higher than those of the stroma.

Introduction of dinitrophenyl residue to the outer surface of the erythrocyte membrane caused about 60% inhibition of the acetylcholinesterase activity in all of human, bovine and porcine erythrocytes. Penicillin bound to the erythrocyte membrane exerted almost complete inhibition of the enzyme activity in all cases.

These results suggest that acetylcholinesterase of similar nature is present in a form of lipoprotein on the outer surface of the erythrocyte membrane, with a density lower than that of the main part of the membrane.

It was reported previously³⁾ that acetylcholinesterase (EC 3.1.1.7 acetylcholine acetylhydrolase) of bovine erythrocyte is easily released from the erythrocyte membrane by repeated washing with hypotonic buffer and is recovered in a form of lipoprotein particles, and also that divalent cations such as Ca^{2+} and Mg^{2+} in very low concentration is effective in preventing the release. No such a release occurs in the case of human erythrocytes.³⁾ It was recently discovered by the present authors that porcine erythrocyte membrane also releases this kind of enzyme by the same hypotonic treatment. It would therefore be interesting to know whether or not there is any fundamental difference between the characteristics of the acetylcholinesterase proteins of these two groups of mammals, human on one hand and pig and cow on the other hand.

In this paper, the acetylcholinesterase from human, bovine and porcine erythrocytes was investigated and the characteristics were compared each other.

Experimental

Preparation of Washed Erythrocyte Suspension—Freshly-Drawn blood, with trisodium citrate added as anticoagulant, was centrifuged at $900 \times g$ for 15 minutes. After removing the plasma and buffy coat, the precipitated red blood cells were washed three times with physiological saline (0.85% NaCl), and re-suspended in the saline to give desired hematocrit value (usually 5%).

Preparation of Erythrocyte Stroma and Release of Acetylcholinesterase from the Stroma—Washed erythrocytes were hemolyzed in about 20 volumes of hypotonic veronal buffer (30 imOsM, pH 7.4) to obtain hemoglobin-free stroma, according to the method of Dodge, Mitchell and Hanahan.⁴⁾ The stroma were then subjected to three successive treatments with the same hypotonic buffer for total 42 hours at 4° and centrifuged each time at $15600 \times g$ for 30 minutes, according to the method of Burger, Fujii and Hanahan.³⁾

Density-Gradient Centrifugation of Stroma and Acetylcholinesterase-Containing Particles—On the top of sucrose solution with step-wise density-gradient in a centrifuge tube (Fig. 1), stroma suspension or suspen-

1) Part of the present report was presented at the 43 rd Annual Meeting of the Japanese Biochemical Society, Tokyo, October 1970.

2) Location: *Yagoto-Urayama 15, Tenpaku-cho, Showa-ku, Nagoya.*

3) S.P. Burger, T. Fujii, and D.J. Hanahan, *Biochemistry*, **7**, 3682 (1968).

4) J.T. Dodge, C. Mitchell, and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).

sion of the acetylcholinesterase particles detached from the stroma was layered. The tube was put in a horizontal-type rotor of a preparative ultracentrifuge and spun down at $98600 \times g$ for 90 minutes. A pin-hole was opened in the center of the bottom of the tube, and the content was allowed to drop down. The aliquots were collected by means of a fraction collector and were then subjected to the enzyme assay.

Preparation of Chemically-Modified (Dinitrophenylated) Erythrocytes—The outer surface of erythrocyte membrane was chemically modified by the introduction of dinitrophenyl residues as described by Berg, Diamond and Marfey.⁵⁾ Washed erythrocytes were resuspended in 67 mM phosphate buffer at pH 8.0 to make a 8% suspension, which was then incubated with 20 mM 1-fluoro-2,4-dinitrobenzene (FDNB) solution in methanol at 25° for 30 minutes. In the control experiment, methanol was added in place of the reagent solution. After the incubation, the erythrocytes were centrifuged and washed three times with the phosphate buffer.

Preparation of Penicillin-Binding Erythrocytes—Washed erythrocyte suspension was incubated at 37° for 5 minutes in isotonic phosphate buffer-saline solution (pH 7.4) containing penicillin in a final concentration of 300 mM. The erythrocytes were then washed with the buffered saline.

Assay of Acetylcholinesterase Activity—Acetylcholinesterase activity was measured by the pH drop method of Michel.⁶⁾ The sample (erythrocyte or stroma suspension, etc.) was incubated at 25° in a barbital-KCl buffer solution containing 0.022 mmole of acetylcholine in a total volume of 2.2 ml. The pH was measured to the nearest 0.005 unit before the addition of the substrate and again at the end of the incubation (usually 30 minutes). The pH drop due to acetic acid released from the substrate is proportional to the enzyme concentration in a range of about 0.1—0.6 pH unit, which is brought, for example, by an addition of 0.1—0.5 ml of 5% human erythrocyte suspension to the reaction mixture.

Determinations of Protein and Lipids—Protein was determined by the method of Lowry.⁷⁾ Lipids were extracted from erythrocyte stroma, or acetylcholinesterase-containing particles as described by Ways and Hanahan.⁸⁾ Phospholipid-phosphorus and total cholesterol in the lipid extract were determined by the method of Bartlett⁹⁾ and by the method of Courchaine,¹⁰⁾ respectively.

Result

Acetylcholinesterase Activity in Human, Bovine and Porcine Erythrocytes

Table I indicates the relative activity of erythrocyte acetylcholinesterase from three different mammalian species, expressed by the amount of acetic acid released from the substrate acetylcholine under the defined experimental condition per fixed volume of erythrocytes. There are marked differences in the activity among these species, with human erythrocytes having the activity three to five times higher than that in bovine or porcine cells.

TABLE I. Acetylcholinesterase Activity in Mammalian Erythrocytes

Mammalian species	Activity ^{a)}	
	Mean value ^{b)}	Range ^{b)}
Human	10.5	(8.8—10.7)
Bovine	3.3	(2.9— 3.7)
Porcine	2.0	(1.9— 2.0)

a) activity expressed as μ mole acetic acid released under the condition described in the text

b) data obtained on erythrocyte specimen from 4 individual animals

Acetylcholinesterase Release from Human, Bovine and Porcine Erythrocyte Membrane by Hypotonic Treatment

Erythrocytes were subjected to hypotonic hemolysis and then to repeated washing, as described in the experimental part. The combined supernatant which contains acetyl-

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TABLE II. Species Difference in the Acetylcholinesterase Release from Erythrocyte Membrane

Mammalian species	Activity released ^{a)}	
	by repeated washing with hypotonic buffer (total 32 hr)	by sodium dodecyl sulfate treatment (final conc. 0.01%, for 30 min)
Human	0.5 %	6.5 %
Bovine	76.8	12.6
Porcine	87.0	18.5

a) percentage release of total stromal enzyme activity

cholinesterase released from the membrane and the whole cell hemolysate of the corresponding volume of the erythrocytes were subjected to the enzyme assay. Percentage of the enzyme release was thus calculated as indicated in Table II.

As is clear from the Table II, porcine erythrocytes as well as bovine erythrocytes allowed the release of as much as about 80% of the acetylcholinesterase activity originally present in the intact membrane. In contrast, almost no release was observed from human erythrocytes, as demonstrated previously.³⁾

Attempts to detach the acetylcholinesterase from erythrocyte membrane by a treatment with a detergent, sodium dodecyl sulfate, did not bring any success. Only a slight portion of this enzyme could be released, as shown in the Table II. In this case, too, human erythrocytes allowed the release to only a very slight extent (6.5% of the total stromal activity) in comparison with the bovine and porcine red blood cells (12.6 and 18.5%, respectively). It had been confirmed already that this enzyme is quite stable in a 0.01% of the detergent solution employed in the present experiment.

Density-Gradient Centrifugation of Erythrocyte Stroma and Acetylcholinesterase Released from the Erythrocyte Membrane

The above-mentioned supernatant containing released acetylcholinesterase was spun down by an ultracentrifuge at $118000 \times g$ for 60 minutes and the sedimented acetylcholinesterase-containing particles were resuspended in a small amount of water. This suspension as well as the freshly-prepared stroma suspension were subjected to step-wise density gradient centrifugation separately. Fig. 1 shows the bands appeared after the centrifugation at $98600 \times g$ for 90 minutes.

In the case of acetylcholinesterase from bovine cells, a main band appeared in the region of the sucrose solution with the density of 1.07, which proved to have high specific activity of the enzyme as the result of the enzyme assay on the fractions collected from the tube. Similarly, the enzyme from porcine erythrocyte was detected in the corresponding region, though with slightly higher density. In either case, another faint band appeared under the main band, but it had no enzyme activity.

In contrast, both bovine and porcine erythrocyte stroma similarly gave each one main band between the density 1.15 and 1.22. The other faint band or bands observed in the upper region may probably correspond to the stroma debris.

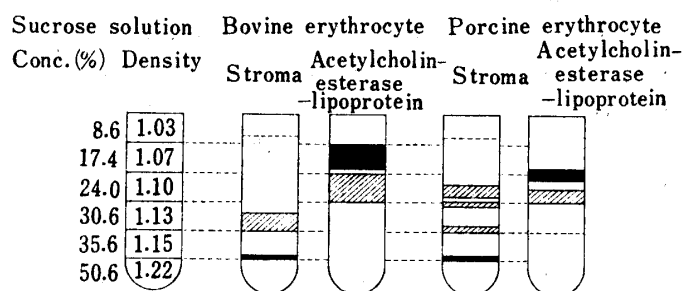


Fig. 1. Density Gradient Centrifugation of Erythrocyte Stroma and Acetylcholinesterase-Lipoprotein (Centrifuged $98600 \times g$, for 90 min)

The above results clearly indicate that the acetylcholinesterase-containing particles released from bovine and porcine erythrocyte membrane have a similar density which is significantly lower than those of the original stroma.

Lipids and Protein Contents of the Bovine Erythrocyte Stroma and the Acetylcholinesterase-Lipoprotein Release from the Stroma

Protein, phospholipid and cholesterol contents were determined on the bovine stroma and on the acetylcholinesterase-containing particles released from the stroma by hypotonic treatments. The amounts were expressed as percentage of the dry weight.

Table III shows that the acetylcholinesterase preparation contains only 30% of protein, while the original stroma contain as much as 57% of protein. The former is rich in phospholipid and cholesterol which occupies 42 and 27% of the dry weight, respectively. Thus, it was confirmed that the enzyme under examination is released from erythrocyte membrane in a form of relatively lipid-rich lipoprotein particles.

TABLE III. Lipid and Protein Contents of Bovine Erythrocyte Stroma and of Acetylcholinesterase-Lipoprotein Released from the Stroma

Contents (% of dry weight)	Stroma (%)	Acetylcholinesterase- lipoprotein (%)
Protein	57	30
Phospholipid	32	42
Cholesterol	10	27

This result coincides with the finding obtained above that the acetylcholinesterase-containing particles have appreciably lower density than the original stroma.

Inhibition of Acetylcholinesterase Activity of Human, Bovine and Porcine Erythrocytes by Introduction of Dinitrophenyl Residues to the Membrane Outer Surface

For the purpose of introducing dinitrophenyl residues to the outer surface of erythrocyte membrane, erythrocytes were treated with monofluorodinitrobenzene solution as described in the experimental part. The acetylcholinesterase activity of the red blood cells thus treated was determined and compared with the activity in untreated cells. The percentage inhibition obtained on the erythrocytes of three different mammalian species is cited in Fig. 2.

In agreement with the results obtained by Herz, Kaplan and Gleiman¹¹⁾ on human erythrocyte acetylcholinesterase, introduction of dinitrophenyl radicals into membrane components brought about remarkable inhibition on the enzyme activity. The inhibition percentage is all about 60% on espective species examined.

Inhibition of Acetylcholinesterase Activity of Human, Bovine and Porcine Erythrocyte by Penicillin Bound to the Membrane

It was reported by Herz¹²⁾ that acetylcholinesterase activity of human erythrocyte is greatly reduced in the presence of penicillin. It was also demonstrated by the present authors¹³⁾ that erythrocyte which had been kept in contact with penicillin solution and then was washed, showed increased resistance against hypotonic hemolysis. This fact suggests that penicillin bound to the erythrocyte membrane can actually exert a stabilizing effect on the membrane. Therefore, effect of membrane-bound penicillin on the acetylcholinesterase activity of human, bovine and porcine erythrocytes was investigated.

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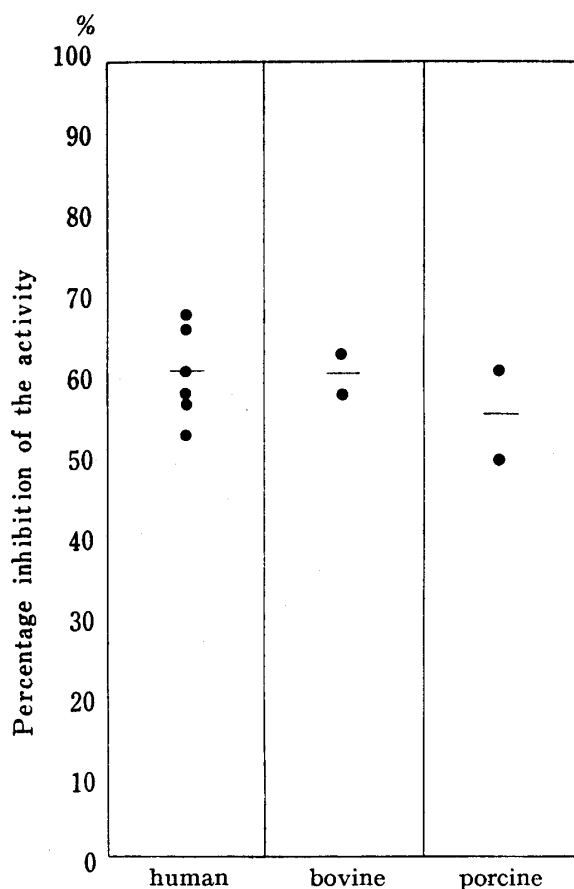


Fig. 2. Inhibitory Effect of Monofluorodinitrobenzene on Erythrocyte Acetylcholinesterase

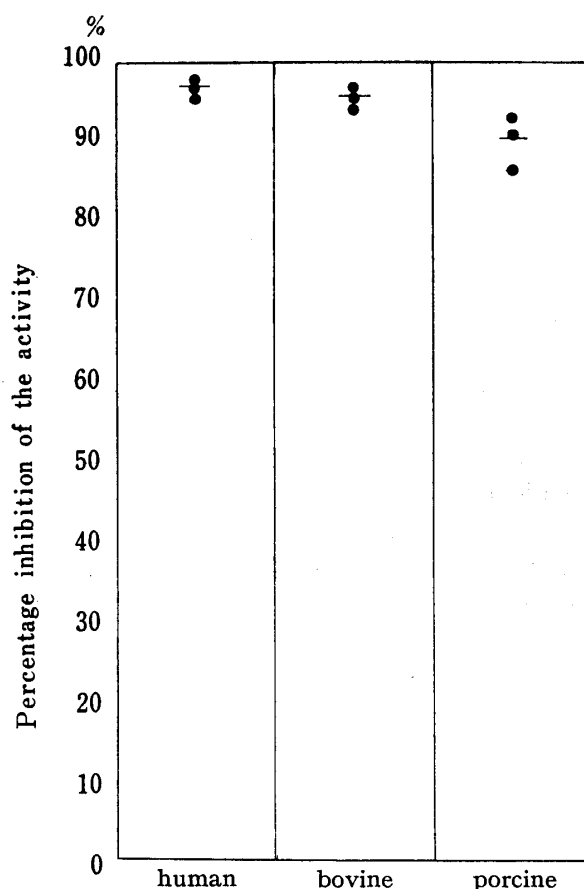


Fig. 3. Inhibitory Effect of Penicillin on Erythrocyte Acetylcholinesterase

As shown in Fig. 3, the erythrocytes from any of the species examined, which had been incubated in 300mM penicillin solution at 37° for 5 minutes and were washed, had only negligible activity of the enzyme remained. The inhibition of the activity by the membrane-bound penicillin is all in an order of 90—97%. Thus, it becomes apparent that inhibition of the activity by penicillin is a common feature of the erythrocyte acetylcholinesterase.

Discussion

It becomes now evident from the foregoing experimental results that in addition to bovine erythrocytes, porcine erythrocytes also easily release their acetylcholinesterase component in a form of lipoprotein from their outer surface of the membrane as the result of repeated washing with hypotonic buffer, whereas human erythrocytes are quite resistant to such a treatment. The detached particles from either bovine or porcine erythrocyte stroma were found to have similar density which is significantly lower than that of the original stroma.

At the same time, some common characteristics of this enzyme in the erythrocytes of these animals were revealed. Namely, the enzyme was all inhibited to a similar extent by introduction of dinitrophenyl groups into the membrane components and also by allowing the absorption of penicillin molecules on the membrane outer surface.

Thus, it is understood that the ease with which erythrocyte membrane of certain species releases its acetylcholinesterase component, does not mean the characteristics of this enzyme in the particular species quite different from those of the other species of the mammals. Most probably, the enzyme under examination is located in a form of lipoprotein on the outer surface of erythrocyte membrane, and the binding of this lipoprotein particles to the main frame-

works of the membrane is sometimes very firm and sometimes rather weak. In the latter case, the acetylcholinesterase-containing particles are easily released from the main part of the membrane by such a mild treatment as washing with hypotonic solution.

A question remains to be solved that what kind of factors are responsible for the firmness of the binding. When the release of this enzyme from bovine erythrocyte membrane was first observed and no release was detected with human red blood cells,³⁾ some species differences in the erythrocyte membrane characteristics were taken into consideration as the possible causes of the difference in the membrane stability, including the difference in membrane phospholipid composition, the sodium-pump activity, divalent cation contents, *etc.* However, as revealed by the present work, the porcine erythrocytes which have the membrane phospholipid distribution¹⁴⁾ and the ATPase activity¹⁵⁾ rather similar to human erythrocytes, still can release the acetylcholinesterase. Furthermore, according to Hanahan,¹⁶⁾ dog erythrocytes which are Na⁺-rich cells with almost no Na⁺-pump activity, as are the case with bovine red blood cells, do not allow such a release of this membrane enzyme. Therefore, neither the membrane phospholipid composition nor Na⁺-pump activity alone is considered to be responsible for the membrane stability in this connection. The most probable cause considered at present will be the Ca or Mg content of the erythrocyte membrane proper (not the whole cells) and atomic absorption studies to determine the contents are now in progress in our laboratory.

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