Biochemical Pharmacology, Vol. 27, pp. 679–684. Pergamon Press, 1978. Printed in Great Britain.

# PROTEIN CARBOXYMETHYLASE AND METHYL-ACCEPTOR PROTEINS IN HUMAN PLATELETS AND ERYTHROCYTES

ROBERT F. O'DEA*†, O. HUMBERTO VIVEROS‡, ANN ACHESON§, CAROL GORMAN‖ and JULIUS AXELROD

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD 20014, U.S.A.

(*Received* 30 *April* 1977; *accepted* 5 *July* 1977)

**Abstract**—The subcellular distribution and characteristics of protein carboxymethylase (protein methylase II) and its methyl-acceptor proteins have been studied in human platelets and erythrocytes. Human platelets constitute a rich source of protein carboxymethylase with the majority of the active enzyme localized to the cytosol. In contrast, the highest specific activity of protein substrate(s) for the enzyme, i.e. methyl-acceptor proteins, appears to be associated with the particulate fraction containing indoleamine storage vesicles. Thrombin promotes a 60 per cent increase in the carboxymethylation of platelet protein after pulse labeling of the cells with [$^3$H]methionine. Human erythrocytes also contain protein carboxymethylase which is localized almost entirely to the cytosol. Unlike platelet cytosol, erythrocyte cytosol appears to be devoid of methyl-acceptor proteins. Erythrocyte membrane ghosts, however, can be readily methylated and probably constitute the sole source of methyl-acceptor proteins in these cells.

Initially described as an enzyme in pituitary extracts which formed methanol from *S*-adenosyl-L-methionine [1], protein carboxymethylase (*S*-adenosylmethionine: protein-carboxyl methyltransferase, EC 2.1.1.24; protein methylase, II) methylates free carboxyl groups of protein molecules [2–4]. Widely distributed in animal tissues [5, 6], this enzyme catalyzes a reaction resulting in neutralization of negative charges by the formation of protein-methyl esters which undergo rapid spontaneous hydrolysis at physiological pH to liberate methanol [5].

Considerable protein carboxymethylase (PCM) activity was observed in human and rat erythrocytes [7]. It was noted that negligible quantities of enzyme existed in either plasma or the leukocyte-containing fraction. Kim [8] described the kinetic and molecular properties of human and rat erythrocyte protein carboxymethylase and reported that the enzyme in both species had a molecular weight of 25,000 daltons. In further studies on endogenous substrates for the enzyme in rat erythrocytes, Kim *et al.* [6] observed that red blood cells were deficient in substrate protein whereas plasma proteins were good substrates. They suggested that circulating polypeptide hormones, good substrates for a pituitary carboxymethylase [9], could possibly function as natural substrates for the enzyme in red blood cells.

One cellular process where neutralization of negative charges in membrane proteins would be necessary is exocytotic secretion. Recently, Diliberto *et al.* [10] have reported that catecholamine-containing chromaffin vesicles of the adrenal medulla are a rich source of endogenous substrates for the enzyme.

Circulating blood platelets play an essential role in hemostasis [11]. Platelets, which are normally nonadhesive cells, can rapidly undergo profound morphologic and biochemical changes leading to their aggregation and adherence to vascular structures. An initial event in this chain reaction involves the release of stored vesicular contents into plasma in response to various stimuli, such as thrombin [12]. In an effort to further investigate the physiological significance of protein carboxymethylase in cellular elements where release phenomena are critical in health and disease, we examined the subcellular distribution of protein carboxymethylase and its endogenous substrates in human platelets. This report provides evidence for the presence of this enzyme in platelets. Protein carboxymethylase is predominantly localized to the platelet cytosol whereas the highest specific activity of its methyl-acceptor proteins (MAP) reside within membranes localized to the vesicle-containing fraction. In addition, erythrocyte plasma membranes, i.e. ghosts, appear to be the sole source of methyl-acceptor protein in red blood cells.

## METHODS

*Materials.* *S*-adenosyl-L-[methyl-$^3$H]methionine, 12.3 Ci/m-mole, and 1-[methyl-$^3$H]methionine, 14.6 Ci/m-mole, were purchased from New England Nuclear Corp., Boston, MA. Gelatin (swine skin, type 1) was obtained from Sigma Chemical Co., St. Louis, MO. Human thrombin (Lot H-1) was a kind gift from Dr. David Aronson of the Bureau of Biologics, National Institutes of Health. QAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, NJ. All other chemicals were obtained from commercial sources.

*Enzymic assay.* Protein carboxymethylase activity was assayed by a modification of a method previously

* Postdoctoral fellow in the Pharmacology Research Associate Training Program.

† Current address: Division of Clinical Pharmacology, University of Minnesota Medical School, 105 Millard Hall, Minneapolis, MN 55455.

‡ Visiting scientist on leave from Catholic University, Santiago, Chile. Current address: Wellcome Research Labs., Department of Medicinal Biochemistry, Research Triangle Park, NC.

§ Undergraduate summer student in LCS, currently at Oberlin College, Oberlin, OH.

‖ Predoctoral student, Department of Physiology, George Washington University, Washington, DC.

described [5, 9, 10]. In this assay, protein methyl esters, formed enzymically through the transfer of the methyl group from *S*-adenosyl-L-[methyl-$^3$H]methionine to carboxyl groups of protein, are precipitated with trichloroacetic acid. After hydrolysis of the precipitated protein-methyl ester in alkaline buffer, the released [$^3$H]methanol is extracted into an organic solvent. The isotonic incubation mixture contained 7.5 μmoles of sodium acetate buffer, pH 6.5, 20 μl gelatin at a saturating concentration (50 mg/ml) as exogenous substrate; 0.22 nmole *S*-adenosyl-L-[methyl-$^3$H]methionine, 12.3 Ci/m-mole, and NaOH to neutralize the $H_2SO_4$ in the *S*-adenosyl-L-[methyl-$^3$H]methionine solution; and variable amounts of subcellular fraction in a final incubation volume of 100 μl. In experiments designed to measure methyl-acceptor proteins (e.g. Fig. 3), 20 μl (5.6 μg protein) of a partially purified adrenal medullary protein carboxymethylase was substituted for the gelatin (see purification details below). Unless stated otherwise, the mixtures were incubated at 37° for 10 min and the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. After centrifugation for 10 min at 20,000 *g*, the precipitated protein-methyl-esters were hydrolyzed with 200 μl of 1.0 M borate buffer at pH 11.0, containing methanol (2.6% v/v) as carrier. The [$^3$H]methanol was extracted with 3 ml of a 3:2 mixture (v/v) of toluene and isoamyl alcohol. After centrifugation for 10 min at 9500 *g*, two 1-ml aliquots of the organic phase were transferred to vials. The radioactivity in one was determined directly after the addition of 10 ml Aquasol (New England Nuclear Corp.). The [$^3$H]methanol in the second vial was evaporated for 1 hr at 80° in a chromatography oven and the radioactivity measured. The difference in radioactivity for samples before and after evaporation was taken as a measure of carboxymethylation. Control assays consisted of incubations in the absence of either protein substrate (endogenous or exogenous) or enzyme.

*Enzyme purification.* Protein carboxymethylase was partially purified from bovine adrenal medulla by a procedure similar to one previously described [9] for bovine pituitary carboxymethylase. Fresh bovine adrenal glands were dissected free of cortex, and the medullary tissue was homogenized in 0.3 M sucrose at pH 7.4 as a 20% (w/v) homogenate. After centrifugation at 26,000 *g* for 30 min, the supernatant was re-centrifuged at 150,000 *g* for 60 min and the pellet discarded. Ammonium sulfate (Schwarz-Mann, Orangeburg, NY. special enzyme grade) was added to 50% saturation and the precipitate recovered by centrifugation at 100,000 *g* for 60 min. After dialysis overnight against 100 vols. buffer containing 5 mM EDTA, 5 mM ethanolamine, and 2.4 mM β-mercaptoethanol, pH 9.8, the dialyzed protein was applied to a 40 × 2.6 cm column of QAE Sephadex A-50 previously equilibrated with the above dialysis buffer and eluted with a linear gradient of EDTA (5–40 mM). The peak fractions were pooled, aliquoted and frozen at −20° for future use. The final partially purified preparation had a specific activity of 2200 pmoles of methyl groups transferred/mg of protein/10 min in the presence of the exogenous substrate gelatin. In the absence of added substrate, the activity was 14.6 pmoles/mg of protein/10 min, a 100-fold purification of exogenous activity relative to endogenous. Over a period of 7 months, no appreciable loss of activity was noted upon storage.

*Platelet preparation and fractionation.* Thirty ml of whole human blood, obtained from healthy male and female donors, was collected by venipuncture and immediately mixed with 4.8 ml of a solution containing 75 mM sodium citrate, 37.5 mM citric acid and 6.25 mM EDTA. Platelet-rich plasma was prepared by a modification of the method described by Murphy *et al.* [13]. After centrifugation of the anticoagulated whole blood at 175 *g* for 10 min, the platelet-rich supernatant was collected and the tube containing packed red blood cells was re-centrifuged at 300 *g* for 10 min. The two platelet-rich supernatants were pooled and the platelets pelleted by centrifugation at 860 *g* for 10 min. The pellet was gently resuspended to the original plasma volume ($2 \times 10^8$ to $5 \times 10^8$ cells/ml) in an isotonic buffer containing 116 mM NaCl, 4.2 mM KCl, 1.8 mM $KH_2PO_4$, 25 mM Tris–HCl, 10.9 mM sodium citrate, 1.2 mM $MgSO_4$ and 6 mM dextrose at a final pH of 7.40. These and all subsequent centrifugations were performed at 4°.

Platelet subfractions were prepared by differential centrifugation according to the method of Robblee *et al.* [12] after limited sonication (60 per cent intensity for 30 sec in a Branson sonicator) of 5-ml aliquots of the resuspended platelets. Fractions sedimenting at 14,000 *g* ($P_1$) followed by 42,000 *g* ($P_2$) and their corresponding supernatants ($S_1$ and $S_2$) were obtained. Particulate preparations were gently resuspended in the above isotonic buffer to achieve a protein concentration of approximately 1 mg/ml.

*Preparation of erythrocyte ghosts.* After removal of platelet-rich plasma by the method outlined above, and of the buffy coat, human erythrocyte ghosts were prepared by the method of Dodge *et al.* [14]. After washing and resuspension of the intact cells three times in 5 vols. of isotonic buffer containing 50 mM sodium phosphate and 90 mM NaCl, pH 7.4, the cells were lyzed by the addition of 5 mM sodium phosphate buffer, pH 7.4, and the ghosts obtained as a pellet by centrifugation at 30,000 *g* for 15 min. After four additional washings in hypotonic buffer to remove hemoglobin, the membranes were resuspended in the above isotonic phosphosaline buffer to achieve a protein concentration of approximately 1 mg/ml and used immediately. The first lyzate (cytosol) was saved and employed in the experiments described below. Proteins were measured by the method of Lowry *et al.* [15].

## RESULTS

Figure 1 presents the time course of human platelet protein carboxymethylase in the 14,000 *g* supernatant of sonicated platelets. The reaction appears linear both in the presence and absence of added exogenous substrate. The methylation of particulate platelet proteins in the presence of added adrenal medullary protein carboxymethylase is also linear (not shown). The linearity of the data suggests that both the contribution of inhibitors or activators in this relatively crude preparation and the spontaneous hydrolysis of protein-methyl esters to liberate methanol are minimal.

Comparison between the specific activities of protein carboxymethylase in platelet cytosol ($S_1$) or par-

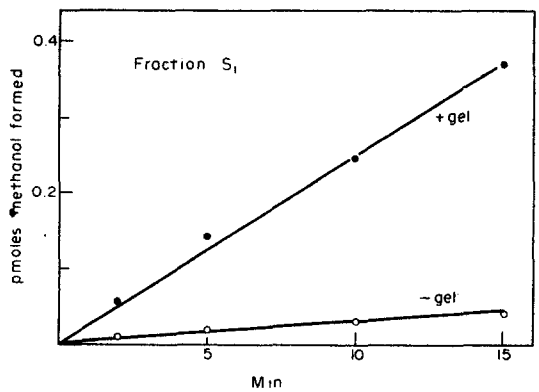


Fig. 1. Time course of human platelet protein carboxymethylase activity. A 14,000 *g* supernatant ($S_1$) of a sonicated platelet preparation was prepared as described in Methods. Approximately 10 μg of supernatant protein was incubated at the times indicated in the presence (●——●) and absence (○——○) of 1 mg gelatin. Protein carboxymethylase was assayed as described in Methods. The values shown are representative of four separate experiments.

ticulate proteins ($P_1$) (Fig. 2) revealed a striking difference between the two fractions. Employing a saturating concentration of gelatin, there is an 8-fold greater activity of protein carboxymethylase in the soluble fraction. In contrast, proteins present in platelet fraction $P_1$ were better methyl-acceptor proteins of methyl groups transferred than the proteins in the soluble fraction (Fig. 3). These data are summarized in Table 1, where both PCM and MAP data in both large vesicle ($P_1$) and small vesicle ($P_2$) fractions are compared. The specific activity of PCM is much higher in both ($S_1$ and $S_2$) soluble fractions than in the particulate proteins. However, the endogenous enzyme activity (assayed in the absence of gelatin), which is a function of the enzyme and substrate concentrations present in the tissue, was quite similar for all fractions. The ratio of endogenous to exogenous enzyme activity was much larger in the particulate fractions. This increase reflects the greater contribution of endogenous MAP in these fractions relative to the cytosol. Approximately 95 per cent of the total platelet PCM activity is present in the soluble fraction (data not shown). Thus this enzyme appears to be localized primarily in the cytosol of human platelets, a pattern of distribution qualitatively similar to that reported in rat pituitaries [5], bovine adrenal medulla [10], rat erythrocytes [6] and human erythrocytes (see below). In contrast, the specific activity of MAP is 65 per cent greater in the $P_1$ than in the soluble $S_1$ fraction. In spite of the greater substrate specific activity of the particulate proteins, 82 per cent of the total MAP activity remains in the soluble fraction (88 per cent of the total proteins in the platelet sonicate are soluble). The specific activity of the acceptor proteins in the supernatant greatly increases after removing the $P_2$ fraction (Table 1). It is possible that the lower specific activity of $S_1$ relative to $P_1$ is due to the presence of an endogenous inhibitor which is removed by high-speed centrifugation.

Recently, Diliberto *et al.* [10] have reported that lysis of pituitary synaptosomes and adrenal medulla chromaffin vesicles releases soluble PCM and MAP respectively. After subjecting human-platelet 14,000 *g* particles to freeze–thaw lysis, PCM activity increased more than 2-fold and a very high specific activity for MAP was noted in the lyzate (Table 2). This large increase in MAP activity in the lysed $P_1$ fraction presumably reflects the release of soluble protein which under normal conditions may be exposed to only a small fraction of endogenous protein carboxymethylase.

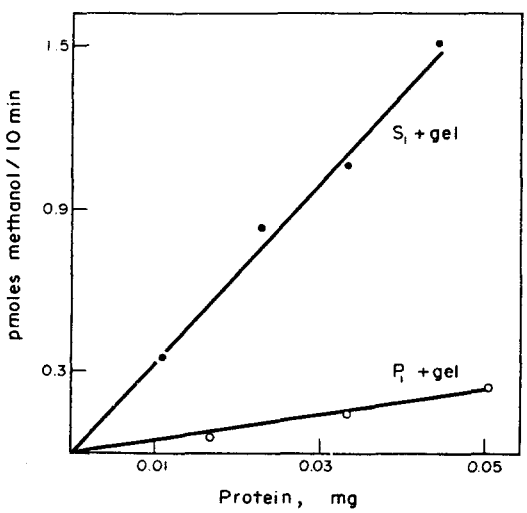


Fig. 2. Protein carboxymethylase activity in soluble and particulate fractions of human platelets. Fractions of sonicated human platelets [14,000 *g* soluble ($S_1$) and particulate ($P_1$)] were prepared as described in Methods. Incubations were performed for 10 min at 37° in the presence of 1 mg gelatin. Values shown are representative of four separate experiments. Values on the abscissa refer to the quantities of cell fraction protein utilized.

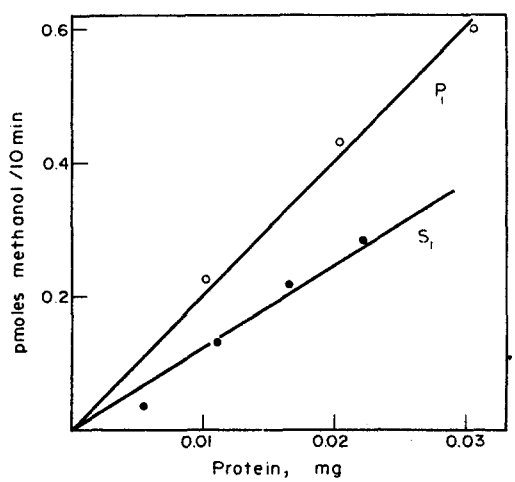


Fig. 3. Methyl-acceptor protein activity in soluble and particulate fraction of human platelets. Fractions of sonicated platelets [14,000 *g* soluble ($S_1$) and particulate ($P_1$)] were incubated as described in Methods in the presence of 5.6 μg of partially purified bovine adrenal medullary protein carboxymethylase. Values shown are representative of four separate experiments.

Table 1. Protein carboxymethylase (PCM) activity and methyl-acceptor protein (MAP) in human platelet subcellular fractions*

| Fraction | PCM | | Ratio§ | MAP‖ |
|---|---|---|---|---|
| | Exogenous activity† | Endogenous activity‡ | | |
| $S_1$ (14,000 *g* super.) | 26 ± 2.7¶ | 0.6 ± 0.16 | 0.02 | 8.7 ± 0.7 |
| $P_1$ (14,000 *g* pellet) | 3.3 ± 0.8 | 0.9 ± 0.16 | 0.27 | 14.5 ± 1.4** |
| $S_2$ (40,000 *g* super.) | 29.3 ± 2.9¶ | 0.6 ± 0.12 | 0.02 | 15.1 ± 1.6†† |
| $P_2$ (40,000 *g* pellet) | 1.9 ± 0.5 | 1.0 ± 0.13 | 0.53 | 7.3 ± 1.6 |

* These values represent the means ± S. E. M. for duplicate determination in four separate experiments. All assays were done at four different concentrations of each cell fraction.

† Exogenous PCM activity (pmoles/10 min/mg of protein) was measured in the saturating portion of the platelet fraction concentration curve, employing gelatin (10 mg/ml) as the exogenous protein substrate.

‡ Endogenous PCM activity (pmoles/10 min/mg of protein) was measured in the absence of added gelatin or purified PCM.

§ Ratio of endogenous activity to activity in presence of exogenous substrate.

‖ MAP activity (pmoles/10 min/mg of protein) was measured by adding partially purified PCM (56 μg/ml of incubation); MAP activity equals the total substrate activity minus the endogenous activity.

¶ Supernatant PCM activity was significantly greater than pellet activity with a $P < 0.001$, by Student's *t*-test.

** MAP activity in $P_1$ was significantly greater than $S_1$ with a $P < 0.01$ and greater than $P_2$ with a $P < 0.05$.

†† MAP activity in $S_2$ was significantly greater than $P_2$ with a $P < 0.05$.

The ability of platelet particulate protein to function as a substrate for protein carboxymethylase was subjected to kinetic analysis (data not shown). The apparent $K_m$ of $P_1$ as substrate was 0.4 mg/ml, which compares quite favorably to the value reported by Diliberto *et al.* [10] for the adrenal chromaffin vesicle membrane.

*Effect of thrombin on human platelet protein carboxymethylation.* Thrombin, a proteolytic enzyme, plays a pivotal role in the formation of fibrin in the blood coagulation reaction and also is a potent inducer of the platelet release reaction and aggregation [11]. Recently, it has been shown that thrombin interacts with the platelet plasma membrane and liberates a small glycoprotein [16]. In view of the recent proposal that protein carboxymethylase could be involved in the process of exocytotic release [10], we examined the extent of protein carboxymethylation in platelets after exposure to thrombin. As shown in Table 3, the addition of 2 units/ml of human thrombin to platelets preincubated in the presence of 3 μM [$^3$H]L-methionine, the amino acid precursor of *S*-adenosyl-L-methionine, resulted in a 50–60 per cent increase in protein-methyl ester formation at 5 and 10 min. At times less than 5 min, increases were also noted, although they were not statistically significant (data not shown). Considerable variations in basal carboxymethylation activity were observed from one experiment to another. Physiological differences between human platelet donors might partially explain this phenomenon. Differences in total acid-precipitable or acid-soluble tritium were not observed between thrombin-treated and control cells. It appears, therefore, that increased carboxymethylation of platelet protein(s) is associated with the stimulation of platelets by thrombin. The precise nature of this association has not been defined yet.

*Human erythrocyte protein carboxymethylase.* Erythrocytes constitute the major source of protein carboxymethylase in the blood [6, 7]. Furthermore, Kim *et al.* [6] observed that hemoglobin was a poor substrate for this enzyme. We, therefore, examined the distribution of PCM and MAP activity in human erythrocytes after fractionation of the cells into cytosol and membrane "ghost" fractions. Human erythrocyte cytosol is essentially free of endogenous sub-

Table 2. Protein carboxymethylase (PCM) and methyl-acceptor protein (MAP) in lysed particulate fraction*

| Fraction | PCM | | MAP† |
|---|---|---|---|
| | Exogenous activity† | Endogenous activity† | |
| $S_1$ | 19.7 | 3.2 | 6.2 |
| $P_1$ | 6.9 | 1.3 | 10.7 |
| $P_1$ super. after lysis | 16.0 | 1.5 | 40.5 |
| $P_1$ pellet after lysis | 2.5 | 1.6 | 6.5 |

* Values shown are averages for triplicate determinations in two separate experiments. Lysis was accomplished by means of rapid freezing and thawing three times.

† Specific activity is expressed as pmoles/10 min/mg of protein.

Table 3. Effect of thrombin on human platelet protein carboxymethylation*

| Group | Per cent of control |
|---|---|
| Control (boiled thrombin) | 100 (6) |
| Five min after thrombin | 167 ± 26.6†(6) |
| Ten min after thrombin | 146 ± 8.3‡(5) |

* Values shown are the mean ± S. E. M. of the per cent change in carboxymethylation of platelet protein relative to a boiled-thrombin control which was assayed at the same time point. Control activity has been arbitrarily expressed as 100 per cent due to variations in the response of platelet preparations from different donors. Washed human platelets (*ca.* $3 \times 10^8$ cells/ml) were resuspended in buffer (see Methods) and preincubated for 5 min at 37° in the presence of $3 \times 10^{-6}$ M [$^3$H]-L-methionine (5 $\mu$Ci) in a final volume of 100 $\mu$l. At zero time, reactions were started by the addition of 10 $\mu$l of a 2-unit/ml H-1 human thrombin solution and terminated at the appropriate time by the addition of 100 $\mu$l of ice-cold buffer. The tubes were immediately centrifuged at 850 *g* for 10 min at 4° and the medium was removed. One ml of 10% trichloroacetic acid was added to the platelet pellet and vigorously mixed. The precipitated protein was then assayed for methylester formation as described in Methods. The numbers in parentheses refer to the number of separate experiments.

† Significantly greater than control with a $P < 0.05$, by paired *t*-test.

‡ Significantly greater than control with a $P < 0.01$.

strates, whether assayed by addition of adrenal medulla protein carboxymethylase or by measuring PCM activity in the absence of gelatin (Fig. 4). In the presence of exogenous substrates, however, the cytosol affords a rich source of protein carboxymethylase. In contrast, the erythrocyte ghost itself can function as a protein substrate of high specific activity. The carboxymethylase activity, associated with the membrane, however, is lower. These data are summarized in Table 4. These results suggest that the protein carboxymethylase in human erythrocytes could be involved in the methylation and, therefore, charge neutralization of the erythrocyte membrane itself.

## DISCUSSION

The platelet plasma membrane is the primary site of action of many agents that affect the function of this dynamic cell. It is well known that the plasma membrane and glycocalyx of the platelet contain a number of specific protein recognition sites, which, upon interaction with other molecules in the environment, contribute to the activation of this cell [11, 17]. Once activated, the platelet may contract and secrete stored intracellular substances followed by dramatic changes in its adhesive and adherent properties. Although not completely understood, it is believed that the release of stored materials from the platelets proceeds via either one of two routes, or possibly both. The first route, described by White [18], consists of a surface-connected canalicular system which has been implicated as the channel through which released substances leave the platelet. The second system, proposed by Warren and Vales [19], is that vesicles arising from the tips of platelet pseudopods sep-

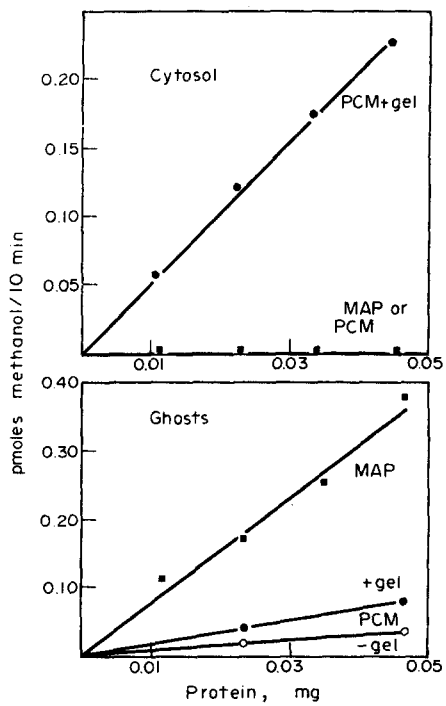


Fig. 4. Protein carboxymethylase activity and methyl-acceptor protein in human erythrocytes. Erythrocyte cytosol and membrane ghosts were prepared as described in Methods. Protein carboxymethylase (PCM) activity was assayed in the presence and absence of 1 mg gelatin. Methyl-acceptor protein (MAP) was measured in the presence of 5.6 $\mu$g of partially purified bovine adrenal protein carboxymethylase.

Table 4. Protein carboxymethylase (PCM) activity and methyl-acceptor protein (MAP) in human erythrocytes (RBC)*

| Fraction | PCM | | Ratio‡ | MAP† |
|---|---|---|---|---|
| | Exogenous activity† | Endogenous activity† | | |
| RBC 30,000 *g* super. | 5.2 | ND§ | ND | ND |
| RBC "ghosts" | 1.7 | 0.74 | 0.45 | 7.4 |

* Values shown are the averages of triplicate determinations in two separate experiments.

† Specific activity is expressed as pmoles/10 min/mg of protein.

‡ Ratio of endogenous activity to exogenous PCM activity (+gelatin).

§ Not detectable.

arate from the cell upon contact with subendothelial vascular structures. These processes, whether they involve changes in membrane adhesiveness (stickiness), cytoskeletal architecture or vesicle membrane–plasma membrane interaction, could conceivably be dependent on alterations in protein charge or structure catalyzed by protein carboxymethylase.

In the human platelet, the vast majority of protein carboxymethylase is localized to the cytosol, a location which is appropriate for its interaction with subcellular organelles, soluble proteins and other membrane components in this cell. Although the major fraction of methyl-acceptor proteins is also soluble, those fractions which are most effective as substrates are localized to the large vesicle particulate fraction, which contains lysosomes and indoleamine storage granules. This fraction, upon lysis, released soluble proteins which were exceptionally good substrates. Although precise characterization of these substrates has not been performed, it appears that the relationship of enzyme to substrate in the platelet is quite similar to that recently described for the adrenal chromaffin tissue [10].

Additional evidence for a possible role for protein carboxymethylase in platelet activation was provided by the data shown in Table 3. Thrombin provoked a 50–60 per cent increase in platelet methyl-ester formation. Under the conditions employed here, significant changes in carboxymethylation were seen at 5 or 10 min after exposure to thrombin. Smaller, but statistically insignificant increases were noted at earlier times. At this point we cannot exclude either the association or participation of protein carboxymethylation in platelet activation phenomena.

Protein carboxymethylase in human erythrocytes is predominantly soluble whereas methyl-acceptor substrates are totally particulate, associated with the plasma membrane. However, the findings reported here suggest that erythrocyte carboxymethylase need not rely upon circulating plasma proteins or polypeptides to function as substrates. It is possible that erythrocyte carboxymethylase could regulate charge distribution or structural changes in the erythrocyte plasma membrane itself.

In summary, the data presented here affirm the presence of protein carboxymethylase in human platelets. The localization of the enzyme and the most effective methyl-acceptor substrates suggests that the enzyme is available for methylation of subcellular membranous structures. Thrombin stimulates a significant increase in carboxymethylase activity, suggesting the role of this enzyme in platelet activation. The ease of its assay plus its relative abundance in this tissue suggests that protein carboxymethylase could provide a useful marker enzyme in those clinical states presenting defects in platelet function. In addition, we have shown that human erythrocyte ghosts constitute an excellent source of methyl-acceptor substrate, a finding of biological significance since the cytosol of this cell possesses abundant enzyme.

## REFERENCES

1. J. Axelrod and J. W. Daly, *Science, Washington* **150,** 892 (1965).
2. S. Kim and W. K. Paik, *Biochemistry* **10,** 3141 (1971).
3. M. Liss, A. M. Maxam and L. J. Cuprak, *J. biol. Chem.* **244,** 1617 (1969).
4. A. M. Morin and M. Liss, *Biochem. biophys. Res. Commun.* **52,** 373 (1973).
5. E. J. Diliberto and J. Axelrod, *J. Neurochem.* **26,** 1159 (1976).
6. S. Kim, L. Wasserman, B. Lew and W. K. Paik, *J. Neurochem.* **24,** 625 (1975).
7. J. Axelrod and C. K. Cohn, *J. Pharmac. exp. Ther.* **176,** 650 (1971).
8. S. Kim, *Archs Biochem. Biophys.* **161,** 652 (1974).
9. E. J. Diliberto and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **71,** 1701 (1974).
10. E. J. Diliberto, O. H. Viveros and J. Axelrod, *Proc. natn. Acad Sci. U.S.A.* **73,** 4050 (1976).
11. H. A. Cooper, R. G. Mason and K. M. Brinkhouse, *A. Rev. Physiol.* **38,** 501 (1976).
12. L. S. Robblee, D. Shepro and F. A. Belamarick, *J. gen. Physiol.* **61,** 462 (1973).
13. S. M. Wolfe and N. R. Shulman, *Biochem. biophys. Res. Commun.* **35,** 265 (1969).
14. J. T. Dodge, C. Mitchell and D. J. Hanahan, *Archs Biochem. Biophys.* **100,** 119 (1963).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
16. D. R. Philips and P. P. Agin, *Biochim. biophys. Acta* **352,** 218 (1974).
17. S. F. Mohammed, C. Whitworth, H. Y. K. Chuang and R. L. Lundblad, *Proc. natn. Acad. Sci. U.S.A.* **73,** 1660 (1976).
18. J. G. White, *Am. J. Path.* **66,** 295 (1972).
19. B. A. Warren and O. Vales, *Br. J. exp. Path.* **53,** 206 (1972).