Studies on the Collagen Glucosyltransferase Activity Present in Platelets and Plasma

By SUZANNE MENASHI* and MICHAEL E. GRANT†

Department of Medical Biochemistry, University of Manchester Medical School,
Oxford Road, Manchester M13 9PT, U.K.

(Received 20 October 1978)

1. Collagen glucosyltransferase was demonstrated to be associated with pig platelets by using a specific assay for the synthesis of [^{14}C]glucosylgalactosylhydroxylysine. 2. This enzyme from pig platelets required denatured collagen as substrate and the reaction was not inhibited by the presence of triple-helical collagen. These observations indicate that the platelet enzyme cannot form either an enzyme–substrate complex or an enzyme–inhibitor complex with triple-helical collagen. 3. Platelets were fractionated by sucrose-density-gradient centrifugation after either lysis by a glycerol-loading technique or homogenization. Assays of subcellular fractions for collagen glucosyltransferase activity indicated that the enzyme was localized predominantly in the cytosolic fraction and less than 5% of the activity was associated with the membrane fractions. 4. Enzyme assays were carried out on platelet-rich plasma and platelet-poor plasma prepared from pig and human blood. These analyses indicated that most of the collagen glucosyltransferase activity of platelet-rich plasma was in a soluble form and only about 10% was associated with platelets. 5. Comparative studies on the enzyme activity in plasma and platelets of various animal species revealed marked variation, with the guinea pig exhibiting the highest activity. In most cases there was a correlation between the activity found in platelets and plasma, but little species variation was noted in enzyme amounts detected in bone-marrow preparations. 6. The results described here are discussed in the context of the proposal that collagen glucosyltransferase might play a role in mediating collagen–platelet adhesion.

The primary step in haemostasis after vascular injury is generally considered to involve the adhesion of circulating platelets to collagen in the exposed sub-endothelial connective tissue. In an effort to elucidate the nature of this collagen–platelet interaction several workers have investigated various features of the collagen structure that may be responsible for this important interaction (for review see Beachey *et al.*, 1978). However, as has been pointed out by Jamieson *et al.* (1975), experiments in which collagen fibrils, modified collagen preparations and collagenous peptides have been used to promote platelet aggregation do not provide a direct experimental approach to the mechanism of collagen–platelet adhesion.

In an attempt to distinguish membrane-associated events of collagen adhesion from intracellular events associated with platelet aggregation, Jamieson and co-workers investigated platelet plasma-membrane fractions and reported the presence of collagen

glucosyltransferase (Barber & Jamieson, 1971*a*) and collagen galactosyltransferase (Barber & Jamieson, 1971*b*) on the outer surface of the plasma membrane of human platelets. On the basis of these observations and other experiments in which glucosyltransferase activity was inhibited by a range of reagents known to inhibit collagen–platelet adhesion, Jamieson *et al.* (1971) proposed that adhesion may be mediated by the formation of an enzyme–acceptor complex between collagen glucosyltransferase on the platelet surface and galactosylhydroxylysine residues present in collagen or basement membranes. This theory can be considered to be an extension of the mechanisms of intercellular adhesion proposed by Roseman (1970), involving the interaction of glucosyltransferases and incomplete glycoproteins at cell surfaces.

The enzyme assays used to study collagen glucosyltransferase activities associated with human platelets (Barber & Jamieson, 1971*a,b*; Bosmann, 1971) have been criticized for their lack of specificity (Spiro & Spiro, 1971; Myllylä *et al.*, 1975*a*), and methods designed to measure specifically the synthesis of hydroxylysine [^{14}C]glycosides have subsequently

* Present address: Department of Biochemistry, Royal College of Surgeons, Lincoln's Inn Fields, London WC2A 3PP, U.K.

† To whom reprint requests should be addressed.

been described. In a preliminary study of the collagen glucosyltransferase of pig platelets using the assay procedure described by Harwood *et al.* (1975), we observed that this enzyme had some properties in common with the collagen glucosyltransferase isolated from connective tissues (for review see Kivirikko & Myllylä, 1978); in particular, native triple-helical collagen was not a substrate for the pig platelet enzyme (Menashi *et al.*, 1976). However, transferase activity was not considered to be a prerequisite for collagen-platelet adhesion (Jamieson *et al.*, 1975), and the possibility of the formation of an enzyme-substrate complex could not be ruled out in our earlier studies (Menashi *et al.*, 1976). We now report further on the collagen glucosyltransferase associated with platelets and plasma and provide evidence that this enzyme is unlikely to mediate collagen-platelet adhesion.

Experimental

Preparation of pig platelet lysate

Blood was collected in a plastic container by using EDTA (final concn. 5.4 mM) as anticoagulant. Platelet-rich plasma was prepared by centrifugation of whole blood at 370g for 40 min at 12°C and the platelets were then sedimented at 1000g for 50 min at 12°C and resuspended in 0.02 M-Tris/HCl buffer (pH 7.4 at 4°C), containing 0.15 M-NaCl, 5 mM-glucose and 6 mM-EDTA. The platelet preparation was freed of residual erythrocytes by repeated centrifugation at 1000g for 1 min at 4°C. The platelets were collected by centrifugation at 1800g for 15 min at 4°C and washed twice in the above buffer. The pellet obtained in the final wash was resuspended in 4 vol. of 0.02 M-Tris/HCl buffer (pH 7.4 at 4°C) containing 0.15 M-NaCl and 10 μ M-dithiothreitol and the suspension sonicated (3 $\times$ 5 s) at 4°C by using an MSE sonicator at auto-setting 6. The sample was then dialysed overnight at 4°C against 200 vol. of 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 10 μ M-dithiothreitol.

Subcellular fractionation of platelets

Collagen glucosyltransferase activity was assayed on subcellular fractions obtained by two different methods.

Method 1. Platelets were isolated from pig blood by the differential-centrifugation technique described above, and the fractionation procedure was that described by Barber & Jamieson (1970) for human platelets involving glycerol lysis and subsequent sucrose-density-gradient centrifugation. The glycerol lysis yielded a platelet lysate in 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose, and this suspension was layered on top of a solution of 27% (w/v) sucrose in 0.01 M-Tris/HCl buffer and centrifuged at 63 500g for 4 h at 4°C in the 3 $\times$ 25 ml titanium swing-out rotor of an MSE Superspeed

65 ultracentrifuge. Unbroken platelets, granules and other debris sedimented to the bottom of the tubes, whereas the membrane fraction remained as a narrow band at the interface between the cytosol and concentrated sucrose solution. The cytosol fraction was removed with a Pasteur pipette and diluted 1:10 with 0.01 M-Tris/HCl buffer, pH 7.4. Cytosolic proteins were precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to 65% saturation, collected by centrifugation at 15 000g for 1 h, resuspended in 0.02 M-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 10 μ M-dithiothreitol, and dialysed overnight at 4°C against three changes of this buffer. The membrane fraction was isolated, diluted 1:10 with 0.01 M-Tris/HCl buffer, pH 7.4, containing 0.25 M-sucrose and centrifuged at 100 000g for 1 h at 4°C. This membrane pellet and the granule-containing pellet were resuspended in the Tris/NaCl/dithiothreitol buffer.

Method 2. The isolation, homogenization and fractionation of pig platelets were carried out as described by Harris & Crawford (1973). These procedures were kindly carried out by Dr. D. G. Taylor in the University of Birmingham and yielded membrane, granule and cytosol fractions, which were processed as described in Method 1.

Preparation of plasma, platelets and bone marrow from various species

Blood was anticoagulated with EDTA (final concentration 5.4 mM) and platelet-rich plasma was prepared by centrifugation of the blood (2–10 ml) at 370g for 20 min at 12°C. Platelets were sedimented by centrifugation of platelet-rich plasma for 50 min at 2500g and 12°C to yield a supernatant of platelet-poor plasma. The platelet pellet was resuspended in 0.02 M-Tris/HCl buffer (pH 7.4, at 4°C) containing 0.15 M-NaCl and 10 μ M-dithiothreitol. Platelet-rich plasma, platelet-poor plasma and the platelet suspension were assayed directly for collagen glucosyltransferase activity.

Fresh bone-marrow tissue obtained from bovine sternum and the femurs of a rat, rabbit and guinea pig was suspended in Ca^{2+} -free Hanks balanced salt solution (Gibco-Biocult Ltd., Paisley, Scotland, U.K.) containing 10 μ g of deoxyribonuclease (BDH Chemicals, Poole, Dorset, U.K.)/ml and heparin (4 units/ml). The samples were dispersed as described by Levine & Fedorko (1976) and the marrow cells were poured through a stainless-steel sieve (100 mesh, 150 μ m diameter). The cells were washed three times with the above solution and collected by centrifugation at 600g for 5 min. The cells were resuspended in 0.02 M-Tris/HCl buffer (pH 7.4 at 4°C) containing 0.15 M-NaCl and 10 μ M-dithiothreitol, sonicated as above and assayed for collagen glucosyltransferase.

Determination of collagen glucosyltransferase

Preparation of substrates. Substrates for the assay

of collagen glucosyltransferase were derived from corneas dissected from bovine eyes obtained from a local abattoir within 1–2 h of slaughter. Corneal collagen was isolated by pepsin-digestion procedures described previously for the solubilization of anterior-lens-capsule collagen (Kefalides & Denduchis, 1969). Amino acid analyses were almost identical with those described for highly purified bovine corneal collagen (Freeman *et al.*, 1968) and indicated the presence of approx. 11 residues of hydroxylysine/1000 amino acid residues, of which approx. 65% were glycosylated. Polyacrylamide-gel-electrophoretic analyses, by the method of Sykes & Bailey (1971), demonstrated that the corneal collagen had a chain composition consistent with it being a type-I collagen.

In experiments to investigate the effects of substrate conformation on transferase activity, the corneal collagen was used in the native triple-helical form and also after denaturation at 60°C for 10 min. Under the isolation conditions described above the isolated collagen retained its triple-helical conformation and had an optical rotation, $[\alpha]_D^{20}$, of -430° , whereas after denaturation the optical rotation was -135° . In all other experiments the substrate used in the collagen glucosyltransferase assay was prepared by the selective removal of glucose from the corneal collagen by hydrolysis in 0.025 M-H₂SO₄ for 20 h at 100°C. After hydrolysis the sample was neutralized with 2 M-NaOH and the gelatinized substrate used directly in the assay.

Standard enzyme assay and characterization of products. The reaction was carried out in polypropylene tubes (5 ml) for 90 min at 37°C in a total volume of 200 μ l, which contained 15 μ mol of Tris/HCl buffer, pH 7.4, 2 μ mol of MnCl₂, 0.4 μ mol of 2-mercaptoethanol, 10 μ l of 0.1% (v/v) Triton X-100, 0.67 μ Ci of UDP-[¹⁴C]glucose (260 mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) and 2.5 mg of glucose-free corneal gelatin. The reaction was stopped by rapidly cooling to 4°C followed by the addition of 15 vol. of acetone. The protein precipitate was collected by centrifugation and the pellet washed a further three times. The isolation and partial purification of the [¹⁴C]glucosylgalactosylhydroxylysine was carried out by alkaline hydrolysis and ion-exchange chromatography and final purification achieved by paper electrophoresis (Harwood *et al.*, 1975). Up to 90% of the radioactivity in the alkaline hydrolysate was eluted with water from the ion-exchange columns, but all the labelled glycoside was recovered when the column was eluted with 1.5 M-NH₃. When the platelet enzyme was assayed, electrophoresis of this glycoside-containing fraction revealed the presence of two minor contaminants (peaks A and B) as well as the major peak C of radioactivity corresponding to [¹⁴C]glucosylgalactosylhydroxylysine (Fig. 1a). In all

Fig. 1. Paper electrophoresis of the products of the enzymic reaction

After alkaline hydrolysis, neutralization and desalting of the products of the enzyme reaction, a portion was applied to Whatman no. 1 chromatography paper (30 cm $\times$ 25 cm) and electrophoresis was carried out in 0.1 M-pyridine/acetate buffer, pH 5.0, at 250 V for 4.5 h. After drying at 100°C the strips were cut into 1 cm sections and the radioactivity was determined (Harwood *et al.*, 1975). (a) Activity in platelet lysate (2.9 mg of protein from approx. 4 ml of pig platelet-rich plasma). The shaded histogram shows the control experiment, where no enzyme was added to the incubation mixture. (b) Activity in platelet-poor plasma (2.5 mg of protein, corresponding to 40 μ l of pig plasma). Peaks A and B are unknown contaminants and peak C is [¹⁴C]glucosylgalactosylhydroxylysine.

experiments on platelet fractions the radioactivity in peaks A and B remained fairly constant and showed no correlation with that in the [¹⁴C]glucosylgalactosylhydroxylysine peak. The identification and purity of the labelled disaccharide unit was confirmed by elution of peak C from the electrophoresis strip,

followed by amino acid analysis (Askenasi & Kefalides, 1972). All the ^{14}C was found to co-chromatograph with an authentic standard of glucosylgalactosylhydroxylysine. It is also noteworthy that after hydrolysis of peak C in 2M-HCl for 2 h at 100°C, all the radioactivity was recovered in [^{14}C]glucose.

When the plasma enzyme was assayed by these procedures the synthesis of hydroxylysine glycoside was linear with enzyme amounts over the range 10–60 μl of plasma enzyme. A distinct peak of [^{14}C]glucosylgalactosylhydroxylysine was always obtained on the electrophoretogram, but peaks A and B were found to represent a relatively large proportion of the radioactivity in the sample (Fig. 1b). This observation emphasizes the need to include the final electrophoresis step in the enzyme assay and is contrary to the findings of Anttinen (1977), who reported that, when human serum and plasma were assayed for collagen glucosyltransferase, about 80% of the radioactivity found in the glucosylation reaction was present as the specific product [^{14}C]glucosylgalactosylhydroxylysine.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Substrate conformation and enzyme activity

The effect of substrate conformation on the glucosylation reaction was studied by using preparations of pig platelets lysed and sonicated as described above. In previous studies comparing the efficiency of native triple-helical corneal collagen and heat-denatured corneal collagen as substrates for the pig platelet enzyme (Menashi *et al.*, 1976), a marked difference was observed in that when the reaction was

studied as a function of time and enzyme concentration no synthesis of glucosylgalactosylhydroxylysine was observed with the native collagen. However, the possibility of a non-productive interaction of the enzyme with the triple-helical collagen could not be excluded in our previous experiments. Accordingly, further studies have been conducted in which the platelet enzyme preparation was incubated with the denatured substrate in the absence and presence of various amounts of native triple-helical collagen (Table 1). Because the collagen triple helix is readily denatured above 38°C, these experiments were conducted at 30°C, to ensure that the native collagen

Table 1. *Effect of native triple-helical collagen on collagen glucosyltransferase activity of platelets*

Samples of platelet lysate were assayed for collagen glucosyltransferase activity by using the standard assay procedures, except that before the addition of the denatured collagen substrate the samples were preincubated for 10 min at 22°C with various concentrations of native collagen. The incubation was carried out for 90 min at 30°C instead of 37°C to ensure that the native collagen retained its triple-helical conformation. The synthesis of [^{14}C]glucosylgalactosylhydroxylysine ([^{14}C]Glc-Gal-Hyl) was determined as described in the text.

Denatured collagen substrate (mg)	Native collagen (mg)	Collagen glucosyltransferase activity	
		$10^{-4} \times$ Synthesis of [^{14}C]Glc-Gal-Hyl (d.p.m.)	Relative activity (%)
0.3	—	6.46	100
0.3	0.1	6.61	102
0.3	0.2	5.94	92
0.3	0.3	6.50	101
—	0.3	0.03	2

Table 2. *Distribution of collagen glucosyltransferase in fractions obtained from pig platelets by glycerol lysis*

Platelets were obtained from approx. 3 litres of blood. Subcellular fractions were prepared as described in the text and were assayed for protein content and collagen glucosyltransferase activity. One unit of enzyme activity is defined as the amount of enzyme required to synthesize 6.79×10^5 d.p.m. of [^{14}C]glucosylgalactosylhydroxylysine, corresponding to 1 nmol in 1 h at 37°C by using 2.5 mg of gelatin substrate. Proportions of protein and enzyme activity in subcellular fractions are reported as a percentage of the respective values in the lysate. Specific activity is defined as units of activity per mg of protein and the relative specific activity is reported as the ratio of the specific activity in the subcellular fraction to that in the lysate.

Fraction	Total protein		Total activity		Collagen glucosyltransferase	
	(mg)	(%)	(units)	(%)	$10^2 \times$ Specific activity	Relative specific activity
Lysate	765	100	30.17	100	3.94	1.0
Cytosol	146	19	15.75	52	10.79	2.74
Membrane	22	2.9	1.02	3	4.64	1.18
Debris	540	71	10.0	33	1.85	0.47

Table 3. *Distribution of collagen glucosyltransferase in fractions obtained from pig platelets by homogenization*

Platelets were obtained from approx. 3 litres of blood. Subcellular fractions were prepared as described in the text and were assayed for protein content and collagen glucosyltransferase activity. One unit of enzyme activity is defined as the amount of enzyme required to synthesize 6.79×10^5 d.p.m. of [^{14}C]glucosylgalactosylhydroxylysine, corresponding to 1 nmol in 1 h at 37°C by using 2.5 mg of gelatin substrate. Proportions of protein and enzyme activity in subcellular fractions are reported as a percentage of the respective values in the homogenate. Specific activity is defined as units of activity per mg of protein and the relative specific activity is reported as the ratio of the specific activity in the subcellular fraction to that in the homogenate.

Fraction	Collagen glucosyltransferase					
	Total protein		Total activity		$10^2 \times$ Specific activity	Relative specific activity
	(mg)	(%)	(units)	(%)		
Homogenate	732	100	24.90	100	3.40	1.0
Cytosol	455	62	19.47	78	4.28	1.26
Membrane	39.5	5.4	1.05	4	2.66	0.78
Granules	79.2	10.8	1.88	8	2.36	0.69

Table 4. *Comparison of collagen glucosyltransferase activity in platelet-rich plasma and platelet-poor plasma prepared from pig and human blood*

Samples (40 μl) of platelet-rich plasma and platelet-poor plasma, prepared from pig and human blood, were assayed for collagen glucosyltransferase activity under standard conditions. The results reported are expressed in d.p.m. of [^{14}C]glucosylgalactosylhydroxylysine synthesized, and each value represents a mean of duplicate determinations on six animals of the same species ± 1 s.d.

Species	Collagen glucosyltransferase activity		
	$10^{-3} \times$ Activity in platelet-rich plasma (d.p.m.)	$10^{-3} \times$ Activity in platelet-poor plasma (d.p.m.)	Proportion of total activity in platelets (%)
Pig	42.5 ± 4.7	39.2 ± 4.7	8 ± 5
Human	9.5 ± 1.0	8.1 ± 0.9	15 ± 3

retained its triple-helical conformation. No inhibition of collagen glucosyltransferase activity was observed to occur even when equal amounts of native and denatured collagen were present, thus demonstrating that the triple-helical substrate does not inhibit this reaction.

Distribution of collagen glucosyltransferase activity in subcellular fractions

Two methods were used to disrupt platelets, and the subcellular fractions obtained were assayed for glucosyltransferase activity. In the first series of experiments, the glycerol-lysis technique as previously applied to human platelets by Barber & Jamieson (1970) was used, and the results of a typical experiment are reported in Table 2. It was found that the lysis of pig platelets by this method was not totally

effective, and the debris fraction contained a high proportion of the total protein. The presence in this fraction of up to 40% of the total lactate dehydrogenase activity, a known cytoplasmic enzyme, was consistent with the presence of unlysed cells in the debris pellet. Nevertheless, over half of the total glucosyltransferase activity was found in the cytosol and in three separate experiments only 2–5% of enzyme activity was detected in the membrane fraction.

In the second series of experiments, the procedures described by Harris & Crawford (1973) for the disruption of pig platelets by homogenization were used. This technique has been found to be highly reproducible (Harris & Crawford, 1973) and the distribution of protein in the fractions (Table 3) is similar to that previously described. Assay of lactate dehydrogenase (Wroblewski & La Due, 1955) confirmed that over 70% of the activity of this enzyme was present in the cytosolic fraction. The relatively low recovery of total proteins and lactate dehydrogenase activity can be explained by the fact that, when the membrane fragments were pelleted from the corresponding particulate zone obtained after sucrose-density-gradient centrifugation, the supernatant containing some soluble proteins (Harris & Crawford, 1973) was not assayed. Determination of the collagen glucosyltransferase in the fractions isolated indicated that the enzyme occurred predominantly in the soluble phase, and again, less than 5% of the enzyme activity was found to be present in the membrane fraction.

Determination of collagen glucosyltransferase activity in platelet-rich plasma and platelet-poor plasma

Blood was collected from six pigs and the enzyme activity in platelet-rich plasma was compared with that in platelet-poor plasma (Table 4). The enzyme activity in platelet-rich plasma from all the animals

Table 5. *Comparison of specific activities in plasma, platelets and bone marrow of various species*

Plasma, platelets and bone marrow were prepared as described in the text and assayed for collagen glucosyltransferase activity under standard conditions. Specific activity is defined as units of activity per mg of protein, where 1 unit of enzyme activity corresponds to the amount of enzyme required to synthesize 6.79×10^5 d.p.m. of [^{14}C]glucosylgalactosylhydroxyllysine, corresponding to 1 nmol in 1 h at 37°C by using 2.5 mg of gelatin substrate.

Species	$10^2 \times$ Collagen glucosyltransferase specific activity		
	Plasma	Platelets	Bone marrow
Guinea pig	10.00	27.60	3.01
Cow	2.80	2.73	5.10
Pig	1.74	3.98	—
Rat	1.58	16.58	3.93
Mouse	1.52	2.66	—
Sheep	1.19	2.37	—
Dog	1.16	2.82	—
Human	0.38	3.99	—
Rabbit	0.24	0.46	4.02

was very similar, but when platelets were removed by sedimentation, the platelet-poor plasma was found to contain on average 90% of the activity associated with platelet-rich plasma. A similar study was conducted with blood from six healthy human volunteers, all aged 18 years. The enzyme activity in human platelet-rich plasma varied little from individual to individual, but was markedly lower than that in pig platelet-rich plasma. Assays of human platelet-poor plasma also indicated that only a small proportion of the enzyme activity in platelet-rich plasma was associated with platelets (Table 4).

Comparison of enzyme activity in plasma, platelets and bone marrow of various species

The collagen glucosyltransferase activity in platelet-poor plasma and platelets from nine species was determined (Table 5). There were marked differences in the enzyme activity in the various plasma preparations, with that from guinea pig exhibiting the highest activity, which was approx. 40 times that from rabbit, which had the lowest activity among the species investigated. The activity of collagen glucosyltransferase in platelets from these animals showed some correlation with the plasma values, i.e. enzyme activity was highest in guinea-pig platelets and lowest in rabbit platelets. In general the specific activity in platelets was approx. 2–3 times that in plasma, although in human and rat platelets the specific activity was consistently higher by a factor of approx. 10.

Because platelets are derived from megakaryocytes in bone marrow, further studies were carried out on bone-marrow cells from four of the species (Table 5). Comparison of enzyme activities in bone marrow with those in plasma and platelets from the same species showed no apparent correlation. Thus, values for rabbit bone marrow were higher than for guinea-pig bone marrow, which is considered to have a particularly high concentration of megakaryocytes (Levine & Fedorko, 1976).

Discussion

The biochemical basis of the interaction of collagen with platelets is not understood and little is known of the molecular nature of the platelet membrane-associated receptors which mediate this interaction. The suggestion that collagen glucosyltransferase present on the platelet membrane may have a role in these processes (Jamieson *et al.*, 1971) was most attractive and has stimulated a considerable interest in the role of the carbohydrate side chains of collagen in the adhesion and aggregation of platelets (Chesney *et al.*, 1972; Puett *et al.*, 1973; Kang *et al.*, 1974; Brass & Bensusan, 1976; Gordon & Simons, 1977; Santoro & Cunningham, 1977; Chiang *et al.*, 1977). Although it has been possible to confirm that platelets possess collagen glucosyltransferase activity (Menashi *et al.*, 1976; Smith *et al.*, 1977; Anttinen *et al.*, 1977), the role of this enzyme in collagen-platelet adhesion is brought into question by the work presented here and that described earlier (Menashi *et al.*, 1976; Anttinen *et al.*, 1977).

The collagen glucosyltransferase activity from pig platelets has several properties in common with the enzymes isolated from rat kidney cortex (Spiro & Spiro, 1971), embryonic chick tissues (Myllylä *et al.*, 1975a, 1976) and human platelets (Anttinen, 1977; Smith *et al.*, 1977), which have also been investigated by using specific assay systems. These enzymes have very similar pH optima (6.5–7.5), have a preference for Mn^{2+} to fulfil the bivalent-cation requirement, and are inhibited by thiol-blocking agents. The inability of the pig platelet collagen glucosyltransferase to use native triple-helical collagen as substrate provides a further property in common with the connective-tissue enzyme (Myllylä *et al.*, 1975b).

The collagen glucosyltransferase of chick fibroblasts and chondrocytes has been shown to be bound to the membrane of the endoplasmic reticulum (Harwood *et al.*, 1975). The original claim that the platelet enzyme was also membrane-bound and localized predominantly in the outer surface of the human platelet membrane (Barber & Jamieson, 1971a) was one of the major factors on which the original proposal of Jamieson *et al.* (1971) was based. However, more recent studies using a specific assay have

indicated that in human platelets most of the collagen glucosyltransferase activity was present in the cytoplasm and only 5% of the activity was associated with the membrane fraction (Smith *et al.*, 1977). These observations are similar to the results reported here on the distribution of enzyme activity in pig platelets fractionated by two different techniques. In each case it was found that the enzyme was predominantly present in the soluble cytosolic fraction and less than 5% with the membrane fraction.

The observation that the specific activity of the enzyme in the membrane remained at a value similar to that of the homogenate and not much lower than that of the cytosol (Table 3) is a little surprising. This finding might be explained if the membrane contains a different form of the enzyme, which is present in a lower concentration in the intact platelet than the cytosol enzyme. However, the demonstration that only approx. 10% of the collagen glucosyltransferase activity in plasma is associated with platelets (Table 4) suggests that the enzyme associated with the membrane fraction may arise from the binding of the soluble extracellular plasma enzyme. It is noteworthy, however, that neither leucocytes nor erythrocytes (Anttinen, 1977; S. Menashi, unpublished work) have any detectable collagen glucosyltransferase activity.

Although the activity of collagen glucosyltransferase in the plasma of a given species was remarkably constant (Table 4), considerable variation was found between species (Table 5). Determinations of the enzyme in platelets showed similar species variation, but, with two exceptions, i.e. rat and human platelets, there was a good correlation between enzyme activities in platelets and plasma. These studies indicated that guinea-pig and rat platelets contain the highest activities of collagen glucosyltransferase. It is of interest that a comparative study of the ability of platelets from various species to aggregate with collagen revealed that guinea-pig and rat platelets have a lower tendency to aggregate than do rabbit or pig platelets (Lewis, 1975).

These latter observations, together with the above data on the subcellular distribution of the enzyme in platelets, the demonstration of a large amount of enzyme in platelet-poor plasma and the findings of the effect of substrate conformation on enzyme activity, all argue against the hypothesis that the enzyme plays a role in platelet-collagen adhesion. Normally this enzyme is found in close association with collagen galactosyltransferase, proline hydroxylase and lysine hydroxylase in cells synthesizing collagenous polypeptides (for review see Grant & Jackson, 1976) and although all four enzymes have been demonstrated in platelets (Bates, 1976; Anttinen *et al.*, 1977) their origin and role remain to be established. Since our studies on various animal species indicate that the enzyme activity in platelets correlates fairly well with that in plasma, but not with

that in bone marrow, these results suggest that the platelet enzyme may be derived from the plasma rather than the megakaryocytes from which platelets themselves are derived.

We are grateful to Dr. D. Taylor of the Department of Biochemistry, University of Birmingham, for providing the subcellular fractions of homogenized platelets and for helpful discussions. The financial support of the M.R.C. is gratefully acknowledged.

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