CHAPTER IV

PURIFICATION AND CHARACTERIZATION OF SHEEP PLATELET

CYCLO-OXYGENASE: Acetylation by aspirin prevents haemin binding to the enzyme
Purification and Characterization of Sheep Platelet Cyclo-oxygenase

(Acetylation by aspirin prevents haemin binding to the enzyme)

IV.1. Summary

Arachidonate cyclo-oxygenase (prostaglandin synthetase; prostaglandin endoperoxide synthetase; EC 1.14.99.1) was purified from sheep platelets. The purification procedure involved hydrophobic column chromatography using either ibuprofen-Sepharose, Phenyl-Sepharose or arachidic acid-Sepharose as the first step followed by metal-chelate Sepharose and haemin-Sepharose affinity chromatography. The purified enzyme ($M_r \sim 65000$) was homogeneous as observed by SDS/polyacrylamide-gel electrophoresis and silver staining. The enzyme was a glycoprotein with mannose as the neutral sugar. Haemin or haemoglobin was essential for activity. The purified enzyme could bind haemin exhibiting a characteristic absorption maximum at 410 nm. The enzyme after metal-chelate column chromatography could undergo acetylation by (acetyl-$^3$H)aspirin. The labelled acetylated enzyme could not bind to haemin-Sepharose, presumably due to acetylation of a serine residue involved in the binding to haemin. The acetylated enzyme also failed to show its characteristic absorption maximum at 410 nm when allowed to bind haemin.
IV.2. INTRODUCTION

Cyclo-oxygenase, also known as prostaglandin endoperoxide synthetase, catalyses the conversion of arachidonic acid to a prostaglandin endoperoxide. Two well-known sources of the cyclo-oxygenase are seminal vesicles and platelets (215, 218, 219, 222, 228, 229, 321, 322, 323). The enzyme from bovine seminal vesicles has been purified and characterized. It is a membrane-bound glycoprotein enzyme (218, 219, 240). The highly insoluble nature of the enzyme and its relative instability were impediments in its purification (219, 222). Although platelets are known to be active in the synthesis of prostaglandins and other arachidonate metabolites (222, 321, 324, 325, 326) reports on the purification and characterization of the platelet cyclo-oxygenase are scanty. The one published work made use of electrofocusing, Sephadex G-200 gel filtration and hydrophobic chromatography on ethyelagarose to achieve a 4% yield of the enzyme from human platelets. The purity of the enzyme was not commented upon (228).

Aspirin, the widely used antiinflammatory drug, affects platelet function and this is believed to be due to its inhibitory effect on the platelet cyclo-oxygenase enzyme (215, 321, 323). The inhibition by aspirin was correlated with its ability to acetylate an internal serine residue on the cyclo-oxygenase polypeptide (229, 257, 258, 259, 327). During the search for possible acetylated substrates for the enzyme
aryl acylamidase (70, Chapter II) it became necessary to prepare purified platelet cyclo-oxygenase. The purification and some characteristics of the cyclo-oxygenase from sheep blood platelets are reported in this Chapter. Evidence is also presented to indicate that acetylation of the cyclo-oxygenase by aspirin affects its binding to the haem prosthetic group.

IV.3. MATERIALS AND METHODS

**Materials:**

Arachidonic acid, haemoglobin, glutathione, standard sugars, marker proteins, 2-deoxyribose, thiobarbituric acid, epichlorhydrin, NN'-dicyclohexyl carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, phenyl-Sepharose, 2,4,6-trinitrobenzene sulphonate, butane-1,4-diol diglycidyl ether, bromoacetic acid and imidazole were from Sigma Chemical Co. Haemin, 1,6-diaminohexane and Dowex-50 WX-4 were from Fluka AG. ($^3$H)Acetic anhydride was from Amersham and Triton X-100 was from Rohm and Haas Co. Ibuprofen ($\alpha$-methyl-4-(2-methylpropyl)benzeneacetic acid) was from the Boots Co., Bombay, and arachidic acid was from Calbiochem. CNBr was from SISCO Research Laboratories, Bombay.

**Preparation of affinity chromatography media:**

Concanavalin A was prepared from Canavalia gladiata (306) and coupled to Sepharose 4B according to the method of Cuatrecasas & Parikh (305). Ricinus communis agglutinin 120
was purified (328) from Ricinus communis beans by guar-gum affinity chromatography and Sephadex G-100 gel filtration. It was then coupled to Sepharose 4B (329).

**Coupling of 1,6-diaminohexane (as a six-carbon spacer arm) to Sepharose:** The activation of Sepharose 4B and coupling was carried out according to Cuatrecasas (330) and March et al (329). Briefly, 20 ml of washed Sepharose 4B was suspended in 40 ml of 2 M-\(\text{Na}_2\text{CO}_3\) and 4 g of CNBr (in a minimum volume of dimethylformamide) was added and stirred for 10 min in an ice bath. The gel was washed with 1 litre of precooled 0.1 M-\(\text{NaHCO}_3\) followed by 200 ml of cold 0.1 M-sodium borate buffer, pH 9.5. The washed gel was suspended in an equal volume of 0.1 M-borate buffer, pH 9.5, and 4.64 g of 1,6-diaminohexane in a minimum volume of the same buffer was added to the gel. The pH was adjusted to 9.5 with 5 M-HCl and the gel was stirred end-over-end at 4°C for 24 h. The gel was washed with 20 vol. of 1 M-\(\text{NaCl}\) followed by water to remove excess amine. The aminated gel was suspended in an equal volume of water and stored at 4°C.

**Coupling of arachidic acid or haemin to diaminohexane-Sepharose 4B:** The diaminohexane-Sepharose (10 ml) was washed with 500 ml of dioxan and suspended in an equal volume of dioxan. The ligand (either arachidic acid or haemin) and NN'-dicyclohexylcarbodi-imide were added to achieve a final concentration of 0.1 M each. In the case of haemin coupling
the pH was adjusted to 4.5 with HCl while stirring. The suspension was gently shaken end-over-end at 27°C for 16 h. The gel was washed sequentially with 100 ml of dioxan, 80 ml of methanol (to remove the precipitated dicyclohexylurea), 100 ml of dioxan, dioxan/water mixture, water, 0.5 M-NaCl and finally water. To the washed gel 1 M-acetic acid was added to adjust the pH to 4.5 and stirred in the presence of 0.1 M-1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide to block any excess free amino groups. After this the gel was washed with 0.5 M-NaCl and water.

At each stage of coupling of the spacer arm or ligand or blocking the excess amino groups, the 2,4,6-trinitrobenzenesulphonate test was done to ensure the efficiency of the reaction (330).

**Coupling of Ibuprofen to diaminohexane-Sepharose 4B:**

Ibuprofen (200 mg) in 15 ml of dioxan was added to diaminohexane-Sepharose (10 ml) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (0.1 M) and the pH adjusted to 4.5 with 2 M-HCl. After stirring for 16 h at 27°C, the excess amino groups were blocked and the gel washed as described for the arachidic acid- or haemin-Sepharose gel.

**Preparation of metal ion chelate gels:**

**Iminodiacetic acid-Sepharose 4B (IDA-Sepharose):** Epoxy-activated Sepharose 4B was prepared using butane 1,4-diol diglycidyl ether by the method of Porath (331) and
iminodiacetic acid was coupled to it as described by Porath et al (308).

**Tiris(carboxymethyl)ethylenediamine-Sepharose 4B**

(*TED-Sepharose*): Ethylenediamine-Sepharose was first prepared from epichlorohydrin-activated Sepharose (331). TED-Sepharose was then synthesized by adding bromoacetic acid to ethylenediamine-Sepharose according to (308).

The IDA- and TED-Sepharose gels were packed in columns (12 cms x 0.7 cm) and charged with Zn\(^{2+}\) ions by passing 100 ml of zinc acetate solution in water (3 mg/ml). Unbound Zn\(^{2+}\) was washed off with 50 ml of water. The columns were equilibrated with the equilibrating buffer before introduction of the sample. After each experiment, columns were regenerated by stripping of the Zn\(^{2+}\) ions with 0.05 M-EDTA (pH 7.2)/0.5 M NaCl followed by washing and recharging with Zn\(^{2+}\) ions.

**Determination of enzyme activity**:

Cyclo-oxygenase activity was measured by the thio-
barbituric acid colour reaction of malonaldehyde formed (249,257,332). PGG\(_2\) and PGH\(_2\), the products of the reaction from arachidonic acid (220,321,325) give rise to malonaldehyde in the presence of haem and reduced glutathione (249, 321). The assay mixture contained 100 mM-Tiris/HCl, pH 8.0, 5 mM-GSH, 5 μM-haemoglobin, 0.5 mM-arachidonic acid and the enzyme (100-1000 μg) in a total volume of 1.0 ml.
The reaction was started by the addition of arachidonic acid and terminated after 1 min of incubation at 27°C by the addition of 0.2 ml of 100% (w/v) trichloroacetic acid in 1M-HCl. After thorough mixing, 0.2 ml of 1% (w/v) thio-barbituric acid solution was added and the mixture was heated in a boiling-water bath for 20 min. After cooling to room temperature and brief centrifugation the absorbance of the clear supernatant was measured in an LKB Ultrospec spectrophotometer at 532 nm. In the control reaction mixture the cofactor haemoglobin was omitted at the time of incubation and added after the addition of trichloroacetic acid. Alternative controls where arachidonic acid or enzyme was added at the end of incubation were also run. The amount of malonaldehyde formed was calculated from a standard curve of malonaldehyde obtained by periodate oxidation of 2-deoxy-ribose (333). One unit of enzyme activity was defined as 1 nmol of malonaldehyde formed/ min.

Neutral sugar analysis:

The purified enzyme (70 μg of protein) was precipitated and freed from the detergent with 4 vol. of acetone and dried in vacuo. The dried protein was hydrolyzed in 2 M-HCl at 100°C for 4 h in a sealed tube under N₂ and the neutral sugar fractions were isolated by Dowex-50 column chromatography (334). The sugars were converted to their alditol acetates with myo-inositol as an internal standard and estimated by g.l.c. in a Pye-Unicam model 204 gas chromatographic unit (335).
SDS/Polyacrylamide-gel electrophoresis and Mr determination:

SDS slab gel electrophoresis in a 10% gel was carried out according to Laemmli (276). Protein samples in 10% (v/v) glycerol, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 0.0025% (w/v) Erromphenol Blue were heated in a boiling-water bath for 3–5 min and then subjected to electrophoresis. Standard marker proteins used to determine Mr values were: bovine serum albumin (Mr 68000), γ-globulin heavy chain (Mr 50000), ovalbumin (Mr 45000), γ-globulin light chain (Mr 23500) and myoglobin (Mr 17800). The gels were stained with silver nitrate in an alkaline medium in the presence of formic acid and washed in 3% acetic acid (310).

Protein was determined by the method of Lowry et al (272) with bovine serum albumin as standard. Wherever the protein samples contained Triton X-100, appropriate blanks containing Triton were taken.

Acetylation of protein with (acetyl-³H)aspirin:

(Acetyl-³H)Aspirin was prepared from (³H)acetic anhydride and salicylic acid according to (336). To the labelled aspirin (625 μCi) in ethanol dried under N₂ was added 150 mg of the enzyme preparation previously dialyzed against 20 mM-potassium phosphate buffer, pH 7.0. The aspirin concentration was 100 μM and incubation at 37°C was conducted for 15 min or 60 min. The acetylated protein was dialyzed against the same buffer for 6 h at 4°C to remove free aspirin.
The labelled protein concentrated under vacuum was subjected to SDS slab gel electrophoresis according to Laemmli (276). The gel was processed for fluorography according to Skinner and Griswold (337) and the dried gel was kept in contact with X-ray film at -70°C for 15 days.

**Purification of cyclo-oxygenase:**

**Isolation of platelets:** Sheep blood was collected from the slaughterhouse at the time of killing in siliconized glass beakers containing 5 mM-EDTA, pH 7.2 (final concentration) as anticoagulant. Platelets were isolated according to Wolfe and Shulman (338) with slight modifications. Briefly, the whole blood was centrifuged at 120 g for 10 min at 20°C to remove the erythrocytes. The supernatant was recentrifuged at 120 g at 20°C for another 10 min. The resultant supernatant was further centrifuged at 3000 g for 20 min at 4°C. The sedimented platelets were suspended in the washing buffer as described by Baenziger et al (339) and recentrifuged at 3000 g for 20 min at 4°C. The final sediment of platelets was suspended in 20 mM-potassium phosphate buffer (pH 7.5)/0.7 mM-phenol/1 mM-2-mercaptoethanol (buffer A) and stored at -20°C.

**Solubilization of the enzyme:** All operations were done at 0-4°C. Deep frozen sheep platelets (4 g wt.) were homogenized in 12 ml of buffer A containing 0.05% (v/v) Triton X-100. The homogenate was centrifuged for 1 h at 50000 g and the
supernatant devoid of enzyme activity was discarded. The pel, having the activity, was rehomogenized in 8 ml of buffer A containing 0.5% (v/v) Triton X-100 and kept for 1 h at 4°C. It was then centrifuged for 90 min at 50000 g. The clear supernatant, called the 'solubilized enzyme', having cyclo-oxygenase activity was collected.

**Hydrophobic chromatography using ibuprofen-, arachidic acid- or phenyl-Sepharose affinity column:** The solubilized enzyme was applied to one of the three (ibuprofen-Sepharose) arachidic acid-Sepharose or phenyl-Sepharose) affinity columns (6.5 cm x 1.5 cm) previously equilibrated with buffer A. The column was washed with buffer A until the effluent was free of protein as observed by absorbance at 280 nm and then eluted with buffer A containing 1% (v/v) Triton X-100 at a flow rate of 2 ml/h. Fractions (2 ml) were collected.

**Metal chelate chromatography:** (a) The active fractions showing cyclo-oxygenase activity were pooled and applied to an IDA/Zn column (12 cm x 0.7 cm) previously equilibrated with buffer A containing 0.1% (v/v) Triton X-100 at a flow rate of 2 ml/h. Washing of the column was continued until the absorbance at 280 nm coincided with that of buffer A containing 0.1% (v/v) Triton X-100. The enzyme was eluted from the column with a gradient of 0-10 mM-imidazole (20 ml each) in buffer A containing 0.1% (v/v) Triton X-100. Fractions (2 ml) were collected at a flow rate of 4 ml/h. The active fractions showing cyclo-oxygenase activity were
pooled and dialyzed extensively against buffer A containing 0.1% (v/v) Triton X-100. (b) The dialyzed enzyme was loaded on a TED/Zn column (12 cm x 0.7 cm) previously equilibrated with buffer A adjusted to pH 7.2 and containing 0.1% (v/v) Triton X-100. The cyclo-oxygenase activity which emerged in the unbound effluent and washings was collected in 2 ml fractions and dialyzed against buffer A containing 0.1% (v/v) Triton X-100.

**Haemin-Sepharose affinity chromatography:** The dialyzed enzyme was applied to the haemin-Sepharose affinity column (6 cm x 0.7 cm) (flow rate 1 ml/h) previously equilibrated with buffer A, washed with the same buffer (50 ml) and eluted with buffer A containing 1% (v/v) Triton X-100. This enzyme preparation was immediately used for activity measurements or dialyzed against buffer A and stored at -20°C.

**IV.4. RESULTS AND DISCUSSION**

**Enzyme assay and peroxidase activity:**

The cyclo-oxygenase activity was measured by haemoglobin-induced malonaldehyde formation and any non-specific malonaldehyde formed was eliminated by the control devoid of haemoglobin. The lipoxygenase activity of bovine platelets does not need any cofactors except molecular oxygen for malonaldehyde formation (241). Moreover, aspirin (0.4 mM) and indomethacin (0.4 mM), which completely inhibited the cyclo-oxygenase activity in crude platelet preparations, are
not known to inhibit lipooxygenase (241,340). Malonaldehyde formation through thromboxane synthetase activity in the cyclo-oxygenase pathway has been claimed (222,341). However imidazole, which inhibits thromboxane synthetase by approx. 80% at 1.5 mM (341,342) caused only about 15% inhibition of the cyclo-oxygenase activity (see Table 14), suggesting that the malonaldehyde arises primarily due to cyclo-oxygenase activity in the purified platelet enzyme.

Peroxidase activity was measured using tetramethyl-phenylenediamine according to (219). Although peroxidase activity was detectable in platelet homogenates, none of the purified preparations from several batches gave any measurable peroxidase activity. This would suggest that the peroxidase activity has been either dissociated or inactivated in the purified platelet cyclo-oxygenase. No specific observations on the association of peroxidase activity with platelet cyclo-oxygenase have been reported.

**Purification of cyclo-oxygenase:**

Of the many methods tried for solubilizing the cyclo-oxygenase the use of Triton X-100 was the most effective. The solubilized enzyme was not stable unless phenol was included in the buffer (239). Thus in all the purification steps buffer containing 0.7 mM-phenol and 1 mM-2-mercapto-ethanol was used. Binding of the enzyme was nearly 100% in the hydrophobic, IDA/Zn and haemin-Sepharose columns. Three
different hydrophobic chromatography columns, namely ibuprofen-Sepharose, arachidic acid-Sepharose and phenyl-Sepharose, were tried as the first step of purification. Of these, the ibuprofen-Sepharose gave a better purification (Table 12,13) and therefore was routinely used. The IDA/Zn and TED/Zn metal chelate chromatographies were developed based on the inhibition by Zn$^{2+}$ of the cyclo-oxygenase activity. The differential behaviour of IDA/Zn and TED/Zn columns has been exploited in the purification of the enzyme. In the IDA/Zn chelate column, the enzyme was bound and eluted with imidazole whereas in the case of the TED/Zn chelate column the enzyme did not bind. The differential interaction of serum proteins with the IDA/Zn and TED/Zn columns is well documented (343). The elution from IDA/Zn column by imidazole suggests an interaction between the immobilized metal ion and exposed histidine residues of the protein (343). This was further confirmed by the observation that histidine at 10 mM could also elute the enzyme from the IDA/Zn column. Haemin, the prosthetic group of the enzyme, was used as a ligand in the final affinity chromatographic step of purification. The dissociation of the enzyme-haemin complex from the column was achieved by increasing the concentration of the detergent Triton X-100 (240). Table 12 gives the details of a typical purification procedure using ibuprofen-Sepharose for hydrophobic chromatography in the initial step. About 144-fold purification of the enzyme with a 7% recovery was achieved. Table 13 gives the comparative purification data
Only a part of the homogenate was used for the purification.

One unit of enzyme activity is defined as one mumol of malonatehydro formed/min.

<table>
<thead>
<tr>
<th>Step</th>
<th>Hematin-Sepharose</th>
<th>TEA/Zn-Sepharose</th>
<th>IDA/Zn-Sepharose</th>
<th>Immotor-Sepharose</th>
<th>Solubilized enzyme</th>
<th>Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>144.0</td>
<td>147.0</td>
<td>142.0</td>
<td>0.3</td>
<td>2.0</td>
<td>12.0</td>
</tr>
<tr>
<td>12</td>
<td>135.0</td>
<td>132.0</td>
<td>74</td>
<td>0.8</td>
<td>14.0</td>
<td>16.0</td>
</tr>
<tr>
<td>30</td>
<td>122.0</td>
<td>120.0</td>
<td>180</td>
<td>1.5</td>
<td>10.0</td>
<td>16.0</td>
</tr>
<tr>
<td>63</td>
<td>99.2</td>
<td>98.8</td>
<td>220.4</td>
<td>4.0</td>
<td>6.0</td>
<td>10.0</td>
</tr>
<tr>
<td>100</td>
<td>2.8</td>
<td>2.73</td>
<td>60.1</td>
<td>0.98</td>
<td>1009</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>Total</th>
<th>Total</th>
<th>Total</th>
<th>Volume</th>
<th>Total</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AS THE INITIAL AFFINITY CHROMATOGRAPHY STEP OF PURIFICATION

PURIFICATION OF CYCLO-OXGENASE BY USING IMMOTOR-SEPHERASE

**Table 12**
<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Specific Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>161.0</td>
<td>141.0</td>
<td>7</td>
</tr>
<tr>
<td>12</td>
<td>119.0</td>
<td>123.0</td>
<td>14</td>
</tr>
<tr>
<td>35</td>
<td>44.0</td>
<td>120.0</td>
<td>41</td>
</tr>
<tr>
<td>58</td>
<td>12.1</td>
<td>59.2</td>
<td>63</td>
</tr>
</tbody>
</table>

Hydrophobic chromatographic step

**Table 13**

A comparison of the purification data using Phenyl-Sepharose, Iodophen-Sepharose and Acidic Acid-Sepharose as the initial hydrophobic affinity chromatographic step.
using the three different hydrophobic columns in the initial step. Even though ibuprofen-Sepharose gave a better purification compared with the other two hydrophobic columns, the final 144 to 153-fold purification and recovery (7-9%) was approximately the same in all the three procedures (Table 13).

**SDS-gel electrophoresis:**

The platelet cyclo-oxygenase purified utilizing any of the three hydrophobic chromatographic procedures as the first step gave a single protein band by SDS slab gel electrophoresis and silver staining (Fig.28). The subunit $M_r$ by SDS-gel electrophoresis under reducing or non-reducing conditions was found to be 65000. Ho et al (228) reported an $M_r$ of 79432 for human platelet cyclo-oxygenase and Van Der Ouderaa et al (219) found an $M_r$ of 72000 for the purified seminal vesicle cyclo-oxygenase. The aspirin-labelled cyclo-oxygenase has been reported to have $M_r$ values of 72000 (323) or 85000 (257) from platelet membranes and a value ranging from 68000 to 85000 from vesicular gland microsomes (215, 229, 257, 322).

**Neutral sugar composition of the purified enzyme:**

Neutral sugar analysis of the purified enzyme by g.l.c. revealed the presence of mannose (3.7 mg/100 mg of protein). There was no detectable glucose or galactose. In the course of this purification studies the binding ability of the enzyme to Con A-Sepharose and Ricinus communis agglutinin 120-Sepharose was also tested. It was found that the enzyme could bind to
Fig. 28. SDS/polyacrylamide-gel electrophoresis and silver staining of protein bands at various steps of purification of cyclo-oxygenase. The hydrophobic chromatography step used was Ibuprofen-Sepharose in (A), phenyl-Sepharose in (B) and arachidic acid-Sepharose in (C). Lane 1 represents standard marker proteins from top to bottom of $M_r$ 68000, 50000, 45000, 23500 and 17800 (1 µg each). In (C) bands corresponding to $M_r$ 50000 and 23500 are not clearly visible. Lane 2, Platelet homogenate; Lane 3, solubilized enzyme; Lane 4, hydrophobic chromatography eluate; Lane 5, IDA/Zn column eluate; Lane 6, TEP/Zn column eluate and Lane 7, Hemin-Sepharose column eluate. Enzyme protein loaded for electrophoresis was 5–15 µg.
Con A-Sepharose but not to Ricinus communis agglutinin 120-Sepharose. This observation further confirmed the presence of mannose and absence of galactose in the enzyme.

**Cofactor requirement:**

Cyclo-oxygenase from sheep and bovine vesicular glands has been shown to require either haemin or haemoglobin for its activity (218, 219, 240). Fig. 29 shows the activity of platelet cyclo-oxygenase at various concentrations of haemin and haemoglobin. In the case of haemoglobin, maximum activity was reached at 5 μM and thereafter up to 40 μM the activity remained the same. With haemin, the maximum activity was reached at 20 μM. Fe$^{2+}$ or Fe$^{3+}$ up to 0.4 mM did not have any influence on the enzyme activity either alone or with haemoglobin/haemin.

**Haemin binding:**

Fig. 30 shows the haemin-binding ability of the enzyme. The purified enzyme as such did not show any peak of absorbance at 410 nm, indicating that it was devoid of the prosthetic group haemin. However, after binding to haemin, the enzyme showed a peak at 410 nm characteristic of the haemin-bound enzyme. The haemin-binding ability and the absorption maximum of the platelet enzyme is similar to observations made with the seminal vesicle enzyme (240).
Fig. 29. Effect of various concentrations of haemin or haemoglobin on enzyme activity: The assay was carried out as described under methods. Haemin (■) or haemoglobin (○) was pre-incubated for 5 min at 27°C in the assay mixture and the reaction started by the addition of arachidonic acid. In the control reaction mixtures haemin (□) or haemoglobin (○) were added at the end of the reaction after the addition of the addition of trichloroacetic acid in HCl.
Fig. 30. Absorbance spectrum of the haemin-bound cyclooxygenase: The purified enzyme (15 μg) was dialyzed against 20 mM-potassium phosphate buffer, pH 7.5, for 12 h. Haemin (16 μg) in 0.03 ml of ethanol was added to the enzyme in a final volume of 0.4 ml and incubated at 37°C for 30 min. After thorough dialysis against 20 mM-potassium phosphate buffer, pH 7.5, to remove the unbound haemin, the solution was made up to 1 ml in the same buffer and the absorbance was measured at various wavelengths. A control reaction mixture devoid of the enzyme was run under the same conditions and used as blank for the spectrophotometer readings. The unbroken line indicates the spectrum of the haemin-bound enzyme; the broken line indicates the spectrum of the enzyme untreated with haemin.
**Effect of hydrogen donors:**

The formation of PGI₂ and consequently malonaldehyde from arachidonic acid by cyclo-oxygenase is known to be facilitated by the presence of hydrogen donors (239). The effect of various concentrations of hydrogen donors on the platelet cyclo-oxygenase activity was tested in the presence of the cofactor haemoglobin (Table 14). The maximum stimulation was given by phenol at 1 mM, hydroquinone at 0.1 mM and tyrosine and adrenaline at 2.5 mM. All the hydrogen donors showed some stimulatory effect at or below 1 mM. Above 5 mM all the hydrogen donors caused inhibition.

**Effect of metal ions:**

Zn²⁺ and Cd²⁺ at 10 mM gave about 60-75% inhibition of the platelet cyclo-oxygenase activity. Many other metal ions (Ba²⁺, Ca²⁺, Mg²⁺, Mn²⁺) had no effect at 10 mM but were inhibitory at a high concentration of 1.0 M.

**Acetylation of the platelet cyclo-oxygenase by (acetyl-³H)aspirin:**

The purified enzyme after haemin-Sepharose chromatography when subjected to acetylation by (acetyl-³H)aspirin did not become labelled as observed from SDS-gel electrophoresis and fluorography. This was similar to observations made by others (215, 219, 327) who reported the relative ease in acetylating the membrane-bound enzyme and difficulty in
TABLE 14

EFFECT OF HYDROGEN DONORS ON CYCLO-OXYGENASE ACTIVITY

<table>
<thead>
<tr>
<th>Hydrogen donor</th>
<th>Activity (% control) at various concentrations (mM) of the donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.005</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>103</td>
</tr>
<tr>
<td>Imidazole</td>
<td>109</td>
</tr>
<tr>
<td>Histidine</td>
<td>106</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>115</td>
</tr>
<tr>
<td>Serine</td>
<td>106</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>100</td>
</tr>
<tr>
<td>Phenol</td>
<td>100</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>100</td>
</tr>
</tbody>
</table>

Various concentrations of the hydrogen donors were added to the assay mixture, incubated for 5 min and the reaction was started by the addition of arachidonic acid. In the control assay mixture the hydrogen donor was added after arresting the reaction.
acetylated the purified enzyme from seminal vesicles. The platelet enzyme preparation after TED/Zn column chromatography could be acetylated by (acetyl-\(^3\)H)aspirin. However the labelled enzyme and other labelled contaminating proteins (when aspirin labelling was done for 1 h some contaminating proteins were also labelled. 336), when subjected to haemin-Sepharose chromatography did not bind to the column and were recovered in the unbound fractions (Fig.31). There was no radioactivity in the eluted fractions from the haemin-Sepharose column. It is likely that the internal serine residue of the cyclo-oxygenase enzyme which has been demonstrated to undergo acetylation by aspirin (216,219,327) may be involved in the binding to haemin and that acetylation interferes in this binding. An additional experiment was done to confirm this. The enzyme from the TED/Zn column was acetylated by non-radioactive aspirin. The acetylated enzyme and non-acetylated enzyme were allowed to bind haemin and their absorbance at various wavelengths was measured as described in Fig.30. The aspirin-treated enzyme failed to show the peak at 410 nm characteristic of haemin binding, whereas the non-acetylated enzyme showed this peak.

The non-ionic detergent octylglucoside has been shown to dissociate the enzyme-haemin interaction of cyclo-oxygenase (240), similar to the dissociation by Triton X-100 observed in the present studies. This indicates a hydrophobic type of interaction between the apoprotein and the prosthetic group.
Fig. 31. Fluorogram of SDS-gel electrophoretic pattern of fractions from the haemin-Sepharose column after passage of \(^{3}H\)acetylated enzyme: Acetylation by (acetyl-\(^{3}H\))-aspirin of the enzyme preparation obtained from TED/Zn column chromatography was carried out for (a) 1 h using 150 mg of protein and (b) 15 min using 75 mg of protein as described under Materials and Methods. The enzyme was then passed through the haemin-Sepharose column and fractions (1.5 ml in (a) and 1 ml in (b) were collected. SDS-gel electrophoresis of aliquots from each fraction (approx. 15–20 \(\mu\)g of protein) was carried out. Lanes 1–5 represent the unbound fractions from haemin-Sepharose; the arrow indicates the band corresponding to the cyclo-oxygenase enzyme. (acetyl-\(^{3}H\))Aspirin treatment for 1 h results in the labelling of many contaminating proteins in (a) whereas treatment for 15 min labels the cyclo-oxygenase primarily in (b) (ref. 336).
Similar hydrophobic or non-covalent binding between haem and protein has been reported for other haemoproteins also (344,345,346). It is conceivable that acetylation of the serine residue affects the hydrophobic interaction and binding of haemin to the cyclo-oxygenase enzyme.

In conclusion, a procedure involving a combination of different affinity chromatographic techniques for the purification of platelet cyclo-oxygenase was described. This is the first report on the purification to apparent homogeneity of platelet cyclo-oxygenase. It was also demonstrated that acetylation by aspirin affects the binding of the apo-cyclo-oxygenase to the haemin prosthetic group. It is tempting to speculate that this effect of aspirin may be one of the factors responsible for the loss in cyclo-oxygenase activity observed upon acetylation of the enzyme by aspirin.