SEROTONIN BINDING *IN VITRO* BY RELEASABLE PROTEINS FROM HUMAN BLOOD PLATELETS

A dissertation submitted to Rhodes University

by

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ABBREVIATIONS

5-HT: 5-hydroxytryptamine, serotonin

βTG: β-thromboglobulin
PF4: platelet factor 4

RBC: red blood cells, erythrocytes
PRP: platelet-rich plasma
PPP: platelet-poor plasma

A_{280}: absorbance at 280 nm
λ_{max}: wavelength of maximum absorbance

BSA: bovine serum albumim
cpm: (radioactivity) counts per minute
dpm: (radioactivity) disintegrations per minute; calculated from cpm/efficiency
EDTA: ethylenediaminetetraacetic acid
E.I.: emission intensity (fluorescence)
HNA: heparin neutralizing activity
k: association constant
K_D: dissociation constant
K_m: Michaelis-Menten constant
M_r: molecular mass
n: maximum number of ligands bound per mole protein
pI: isoelectric point
R_f: (thin-layer chromatography) fraction of distance travelled by solvent
RIA: radioimmunoassay
[S]: concentration of the free ligand
SDS: sodium dodecyl sulphate
TAD: tricyclic antidepressant drugs
$\bar{\nu}$: average number of ligands bound per mole protein

ABSTRACT

Among the substances released from human blood platelets are serotonin and various proteins. It was hypothesized that one of these proteins binds serotonin and that serotonin might be important to the protein's function or that the protein might be important to serotonin's function. Two platelet-specific proteins, platelet factor 4 (PF4) and β-thromboglobulin (βTG) were found to bind serotonin *in vitro*. Endogenous PF4 was isolated by serotonin-affinity chromatography and was identified by radioimmunoassay. Purified $[^{125}I]$-PF4 and native PF4 bound to and eluted from a serotonin-affinity column similarly. Ultrafiltration of the homologous protein, βTG, with $[^{14}C]$-serotonin demonstrated binding of about 8 moles serotonin per mole tetrameric βTG with a dissociation constant of about 4×10^{-8} M. Equilibrium dialysis of PF4 with radiolabelled serotonin was attempted, but no binding constant values were obtained because serotonin apparently bound to the dialysis membrane. Since EDTA was one of the two agents that eluted PF4 from the serotonin-affinity gel, calcium binding by PF4 was investigated by equilibrium dialysis. Evidence was obtained for positively cooperative binding of calcium ions by PF4.

It is concluded that PF4 and βTG bind serotonin *in vitro*, that they may also bind *in vivo* when platelets undergo release, and that the functions of serotonin, PF4 and βTG may be mediated in part by serotonin-protein associations.

CHAPTER 1
Introduction and Literature Review

INTRODUCTION

Human blood platelets are known to contain certain physiologically releasable substances that are presumed to perform various functions outside the platelet after release. Many of the releasable substances, such as serotonin (also known as 5-hydroxytryptamine or 5-HT) and the platelet-specific proteins platelet factor 4 (PF4) and β-thromboglobulin (βTG), have functions that have not been fully explicated (Richardson & Withrington, 1977; Pepper, 1980). With an inducer, such as thrombin or collagen, Release I occurs (Holmsen, 1975): serotonin, calcium ions, PF4, βTG and the other contents of their respective granules are released simultaneously (Kaplan et al., 1979; Ryo et al., 1980). Juxtaposition in time and space implies the possibility of interactions among these substances which were previously segregated by storage in separate organelles in the platelet. Interactions among these releasable substances, expecially binding of ligands to proteins, might be crucial for the performance of a function by the protein or by the ligand.

This investigation considers the hypothesis that serotonin is bound by a platelet-specific releasable protein. The binding of serotonin, one of the most enigmatic of all molecules in the body (see this chapter, Section I.C.), to the protein would regulate the concentration of free serotonin in the blood, and might explain the variability in response to serotonin by the vascular walls. Likewise, the association of the protein with serotonin might be important to the protein's physiological function.

The present studies involved, first, a search for a hypothetical platelet protein that might bind serotonin. Next, these studies have attempted to determine the number of binding sites for serotonin on the protein and the binding affinity of serotonin for the protein, because the functions of serotonin and the protein must depend on these factors:

> "We want to know of each molecule or ion which can combine with a protein molecule, 'How many? How tightly? Where? Why?' The answer to the first two questions, and sometimes to the third, can be furnished by the physical chemist, but he will often need to team with an organic chemist to determine the effect of altering specified groups to find if they are reactive. The determination of function is a complicated problem which may be the business of the physiologist or physiological chemist. But the answers to both of the more complicated problems will depend on the answers to the simpler questions, 'How many?' and 'How tightly bound?'" (Scatchard, 1949).

LITERATURE REVIEW

I. Serotonin

A. Storage of serotonin in the platelet dense granules

Platelets are devoid of enzymes for synthesizing serotonin and acquire this biogenic amine entirely by uptake (Douglas, 1970). It is thought that platelet serotonin originates in the enterochromaffin cells of the intestinal mucosa (Paasonen, 1965).

Uptake of serotonin is by two processes: an active transport mechanism that moves serotonin across the platelet membrane at serotonin concentrations less than 5 µg/ml (Hughes & Brodie, 1959) and passive diffusion at higher serotonin concentrations (5-10 µg/ml) (Minter & Crawford, 1974). Serum serotonin concentrations in normal young adult humans are 0,10 to 0,32 µg (Davis, 1959). Plasma serotonin concentrations are less, since practically all of the serotonin in the blood is in the platelets (Udenfriend et al., 1955). Therefore, serotonin uptake by platelets in vivo occurs mostly by active transport and against a considerable concentration gradient, for example, 1000:1 (Pletscher, 1968). The ability of platelets to take up serotonin against a concentration gradient is related to their ATP content (Born et al., 1958).

In one of the earliest papers reporting the ultrastructural localization of serotonin in blood platelets was described a method for electron microscopic visualization of the dense osmiophilic serotonin-containing particles, now known as "dense granules" (Tranzer et al., 1966). In another paper published in 1968, Pletscher described in greater detail the metabolism, transfer and storage of serotonin in blood platelets. Pletscher reported the effects of Rauwolfia alkaloids, benzoquinolizine derivatives, phenylethylamines and imipramine on serotonin storage in the platelets. Whereas Rauwolfia alkaloids, benzoquinolizine derivatives and phenylethylamines decreased the number of dense granules in vitro without inducing alteration of other ultramorphological structures of the platelet, imipramine blocked serotonin uptake without diminishing the number of dense granules. These drug studies were later confirmed by others; it was shown that the decrease of serotonin in dense granules by reserpine was related to the drug's inhibiting transfer of serotonin into the dense granules,

and that imipramine blocked the uptake of serotonin across the platelet plasma membrane (Reimers et al., 1977).

Pletscher et al. (1971) have provided the following account of rabbit platelet serotonin storage: Rabbit platelets contain up to ten dense granules per thin equatorial section of a platelet. These granules are spherical, 100-200 nm in diameter, surrounded by a single membrane, and contain a very dense core that has a diameter of 50-200 nm. Platelets of rabbits treated with reserpine (5 mg/kg) 16 hours before an experiment appear to contain empty vesicles when prepared as usual for electron microscopic examination; platelets incubated at 37 ^{o}C for 2 h with serotonin (1 mg/ml) contain dense granules. Pletscher et al. concluded that the dense granules represent specific storage sites of serotonin in the platelets which are distinct from other subcellular constituents, such as alpha granules.

Besides serotonin, histamine and ATP are concentrated within the dense granules of platelets. In rabbit dense granules these substances occur in a concentration that is about two hundred times their concentration in the intact platelets (Da Prada & Pletscher, 1968).

Calcium is also located within the dense granules, where it occurs at a concentration fifty times higher than in the intact platelets (Pletscher et al., 1971). In the dense granules, calcium is present in such large amounts that they are opaque to electrons (Skaer, 1975). Known as the "secretable calcium pool", calcium associated with the dense granules is released by incubating platelets with thrombin, though calcium stored elsewhere in the platelets is not released (Lages et al., 1977; Costa et al., 1974b) (see Section III in this chapter).

The possibility that serotonin might be stored as a macromolecular complex with ATP and calcium in platelet dense granules was first suggested in 1968 by Da Prada and Pletscher. The following year Berneis reported that serotonin with ATP in aqueous solution in vitro formed micelles of substantially higher apparent molecular mass than tryptamine or histamine in equimolar solutions. Aggregation was enhanced by low temperature and certain concentrations of calcium and magnesium. UV absorption spectra of contents of dense granules and a synthetic mixture of serotonin and ATP in a molar ratio of about two were virtually identical (Berneis et al., 1969b). In another paper the same year Berneis et al. reported that the molecular mass of the micelles depended on the concentrations and molar ratios of serotonin and ATP and that these micelles had apparent molecular masses of

several hundred thousands or more (Berneis et al., 1969a). It was concluded that the micelles were the means of storing serotonin in platelets, since micelle formation would permit high concentrations of serotonin to be stored without concomitant high osmotic pressure (Berneis et al., 1969c; Pletscher et al., 1969). Later it was shown that high calcium ion concentrations inhibited micelle formation and that reserpine also affected phase separation of the in vitro mixtures (Berneis et al., 1970). By UV and nuclear magnetic resonance spectroscopy it was determined that the principal bond in these micelles was ionic and occurred between the side chain amino group of serotonin and the negatively charged phosphate on ATP (Nogrady et al., 1972). The ATP molecules were thought to be stacked vertically, their position being determined by Van der Waal's forces (Berneis et al., 1969a). It has been shown that in vitro tricyclic antidepressant drugs interfered with serotonin storage by competing for ATP in the micelles (Nogrady et al., 1975). From NMR studies it has been determined that serotonin is not essential for the maintenance of a high molecular mass complex inside the dense granules of pig platelets (Costa et al., 1981a). Instead, the important components appear to be the nucleotides and divalent metal ions.

Density gradient studies of human and pig platelets have confirmed that serotonin is concentrated in dense granules along with ATP (Anderson et al., 1974; Salganicoff et al., 1975; Broekman et al., 1975; Kaplan et al., 1979).

More unusual findings were presented by Lewis and Moertel (1978). They compared the uptake and storage of serotonin by human platelets from normal donors and patients with the carcinoid syndrome. Electron microscopic autoradiography of platelets after serotonin uptake had proceeded for 60 min revealed that in platelets from normal donors, most of the label was localized in membranes (52%), alpha granules (27%) and cytoplasm (4%). Only 18% was associated with dense bodies. In platelets of patients with the carcinoid syndrome, 50% of the serotonin was in the membrane, 27% in alpha granules, 10% in cytoplasm and 11% in the dense granules. No explanation for this distribution was given.

Van der Meulen et al. (1983) also reported some serotonin in the fraction which they said was "almost exclusively alpha granules".

Significantly, the concentration of protein in the dense granules is very low (Anderson et al., 1974), which confirms the findings

(above) of the Berneis and Pletscher group that serotonin is in stable micelles, and that protein participation in serotonin storage need not be invoked.

A recent study has shown that transport of serotonin into dense granules may be mediated by a reserpine-sensitive transporter (Fishkes & Rudnick, 1982). The Michaelis-Menten constant for transport (K_m) of serotonin is 1,15 μM. Transport is driven by the electrochemical gradient of protons generated by a membrane-bound ATPase which pumps protons into the granule lumen. The inside of the membrane was found to be acidic and electrically positive. It was proposed that the influx of one serotonin cation was coupled to the efflux of two or more protons.

These findings were supported by another report that the intragranular storage of serotonin in human platelets depends mainly on the proton gradient across the granular membrane (Affolter & Pletscher, 1982). They found that ionophores such as nigericin that caused a collapse of proton gradients induced non-exocytotic release of serotonin from the granules. Exocytosis was ruled out because ATP was released to a different extent from serotonin. Affolter and Pletscher cited various similarities between the platelet dense granule and the adrenal chromaffin granules. Almost simultaneously with the submission of their paper, another group reported the identity of the electron transport chain in the platelet granule membrane with that in the adrenal chromaffin granule membrane (Johnson & Scarpa, 1981). The physiological role of the respiratory chain is not known for either granule.

A pH gradient across the dense granule membrane had been reported previously, and evidence had been presented in the same report that accumulation of serotonin in these granules was coupled to the pH gradient (Rudnick et al., 1980). Rudnick also found that in the presence of ATP at low concentrations of Na^+ (10 meq/L), the transport of the dense granules functioned, but the plasma membrane transport system required 100 meq/L, or higher, concentrations of Na^+. Platelet release is suppressed at low pH and by increased extracellular osmotic strength (Pollard et al., 1977).

Based on measurement of platelet dense granule constituents that were released by thrombin, some species-related differences have been noted in a comparative study (Meyers et al., 1982).

Clinical disorders associated with defects in platelet serotonin

storage granules have been described (White & Gerrard, 1978; Weiss et al, 1979). Impaired nucleotide storage may be responsible for the disorder known as storage pool deficiency (Lages et al., 1983).

B. Localization of serotonin in other platelet structures

Besides dense granules, there appear to be other pools of serotonin within platelets. As much as 5-10% of platelet serotonin may be in the extragranular cytoplasm (Reimers et al., 1977). Costa has described what he calls anomalous compartmentation of serotonin (Costa et al., 1981b). Costa et al. studied the relationship between the non-releasable and releasable pools of serotonin in intact human platelets by adding [^{3}H]-serotonin to platelet-rich plasma in which dense granules of platelets had been labelled previously with [^{14}C] -serotonin. Formaldehyde fixation enabled abrupt inhibition of serotonin uptake into the platelets. Imipramine effectively blocked the movement of extracellular serotonin into the platelet, but did not prevent the movement of vesicular [^{14}C]-serotonin into a non-releasable compartment. After the serotonin left the dense granules, it entered the extracellular medium. Some serotonin from the dense granules was also non-releasable. However, this serotonin which was non-releasable did not mix with serotonin that became non-releasable following uptake from the extracellular medium. Earlier studies by the same group had shown that it is unlikely that serotonin circulates continually between granule and cytoplasm (Costa et al., 1977). It is not known what roles the releasable and non-releasable serotonin stores have.

Besides dense granules and non-releasable cytoplasmic pools of serotonin, there is also evidence that serotonin can bind to the platelet plasma membrane. Serotonin binding to intact platelets in platelet-rich plasma has been studied by many research groups. Evidence has been presented for at least two sites, termed Site A and Site B, for serotonin on the platelet membrane (Gordon et al., 1978). Site A may be involved in platelet stimulation by serotonin (dissociation constant of about 10^{-8} M). Site B may be the carrier for serotonin transport through the plasma membrane (dissociation constant of about 30×10^{-8} M).

The binding of serotonin at the two sites is inhibited by different drugs. Methysergide, cinanserin and dLSD are inhibitory at

Site A. The tricyclic antidepressants (TAD) chlorimipramine, imipramine and amitriptyline are inhibitory at Site B. In similar studies, some with formaldehyde-fixed platelets, there has been confirmation of two binding sites, the one of higher affinity affected by the drugs which also affect aggregation and the lower affinity site, which is affected by the drugs inhibiting serotonin uptake (von Hahn et al., 1980; Kim et al., 1980; Peters & Grahame-Smith, 1980).

In some of these studies curvature of the Scatchard plot was interpreted as unequivocal evidence for two binding sites and the possibility of negative cooperativity at a single site was ignored. Moreover, the Scatchard plots were incorrectly resolved, giving specious values for the dissociation constant and number of binding sites. These errors have been discussed by Nørby et al. (1980). However, Peters & Grahame-Smith (1980), observing Nørby's cautions, obtained two dissociation constants (K_D) for normal human platelets at 2 ^{o}C: Site A, K_D = 0,5-1 nm; and Site B, K_D = 15-36 nM. They also confirmed that Site B binding is affected by TAD and concluded that Site B has an uptake function and Site A has an aggregation function.

Site B, the TAD-sensitive site on the platelet plasma membrane, has been studied intensively. It has been found that Na^+ and Cl^- are required for serotonin transport into platelets and for specific imipramine uptake by platelets (Talvenheimo et al., 1979). The concentration of Cl^- has been found not to influence the Michaelis-Menten constant for serotonin, only V_{max}, indicating that Cl^- is not necessary for binding of serotonin to the transport carrier, but is essential for translocation of the serotonin-carrier complex across the plasma membrane, and/or for the release of serotonin at the inner surface of the plasma membrane (Lingjaerde, 1979). Removal of up to 30% of the platelet sialic acid, which results in changes in the gel electrophoretic glycoprotein pattern, appeared to accelerate the rate of serotonin uptake; but once 50% of the platelet sialic acid was removed, serotonin uptake decreased (Masters et al., 1980).

Digitonin solubilized the specific imipramine-binding activity, the serotonin transporter, from the platelet plasma membrane and it was shown that the binding of imipramine to the transporter was saturable, Na^+-dependent and reversible by serotonin (Talvenheimo & Rudnick, 1980). In the same study the partially purified imipramine-binding component was found to be "comparable in size to soluble proteins," dependent on Na^+ and Cl^-; inhibited by fluoxetine and doxepin; not

inhibited by cinanserin and methysergide; and to have a dissociation constant of 45 nM. Another detergent, CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, has been used to solubilize the same site (Rehavi et al., 1982), and although this protein was also dependent on Na^+ concentration, its K_D was 1,2 nM. In other studies of serotonin binding at the platelet plasma membrane, serotonin uptake inhibitors were found to be more effective than serotonin receptor antagonists both in displacing bound serotonin and in blocking serotonin uptake (Schick & McKean, 1979). The rank order of these inhibitors (from greatest to least) was chlorimipramine, imipramine, cinanserin and cyproheptadine. Costa (1981) postulated that some of the serotonin assumed by Schick and McKean to be on the platelet surface had actually been internalised.

Most of the serotonin remaining in a platelet population following maximal release by thrombin represents serotonin that was present prior to the release reaction (Costa & Murphy, 1977). There is only a very small amount of serotonin uptake by platelets subsequent to platelet release (ibid.).

Verapamil at 2000 - 10 000 ng/ml inhibits serotonin uptake into platelets (Addonizio et al., 1982). Three possible explanations for this inhibition were suggested by the authors: 1) non-specific membrane alteration; 2) action of the drug on membrane receptors; 3) alteration of Ca^{2+} or Na^+ flux.

The review by Drummond (1976) cites 225 references prior to 1975 which deal with, or relate to, uptake of serotonin at the granule and plasma membranes and serotonin receptors.

Lingjaerde (1981) has postulated that some serotonin is found in, or on, the platelet plasma membrane at the time of release. This serotonin had a half-time too short to be calculated.

The localization of serotonin in the platelet may therefore be summarized as follows:

1) Most serotonin is stored in the dense granules and is releasable.

2) Some serotonin is in non-releasable cytoplasmic stores of unknown function.

3) There are at least two platelet plasma membrane pools of serotonin, at least one concerned with serotonin uptake (Site B), another concerned with aggregation (Site B), and possibly others of unknown function.

C. Putative functions of serotonin

Some of the general effects that have been ascribed to serotonin are contraction of smooth muscle, including blood vessels, with concomitant increase in blood pressure (Rapport et al., 1948; Cooper et al., 1974; Lefort & Vargaftig, 1978); antinociception (Wang, 1977; Messing & Lytle, 1977); and temperature regulation (Székely, 1978; Hellon, 1975).

Serotonin can have either a vasodilator, or vasoconstrictor effect, depending on the conditions, amount administered and the area of the circulation (Hardebo et al., 1978). Even though these effects can be powerful, the function of serotonin in the regulation of circulation is not understood. Occasionally carcinoid tumors composed of chromaffin tissue develop. These secrete large quantities of serotonin and cause mottled areas of vasodilation in the skin, but the very fact that these quantities of serotonin do not drastically disturb the circulation makes it doubtful that serotonin plays a widespread general role in the regulation of circulatory function. However, periodic cyanosis and flushing and pulmonary stenosis are associated with these tumours (Guyton, 1971, p. 296).

Responses to serotonin differ not only between species but also between animals of the same species and even in successive tests in the same individual, because many of the effects of serotonin are reflexly mediated (Rapaport et al., 1977). Tachyphylaxis is common when tests are made at frequent intervals (Douglas, 1970).

> "Confirming previous observations...the responses to serotonin of both the arterial and portal beds were more variable than those to any substance hitherto investigated. This variability in response to serotonin may depend upon the prevailing sympathetic vasoconstrictor tone...and the route by which the amine is administered" (Richardson & Withrington, 1977).

This variability in response is characteristic of the effect of serotonin on body temperature, and prompted Hellon to state that ICV injection of a drug "which causes hyperthermia in one species, may cause hypothermia in another and have no action in a third is a

situation which is perhaps without precedent in pharmacology."

Long term injections of serotonin into the subcutaneous tissue of a rat result in local progressive collagenous and fibrous proliferation within the dermis. Exaggerated responses occurred when the injections were given intradermally to patients with connective tissue disease, as compared with the reaction in normal control subjects (Scherbel, 1961). Inflammation and fibroplasia resulted from injections of either serotonin or histamine. Rats receiving both serotonin and LSD or cyproheptadine showed greatly diminished inflammatory reactions and connective tissue growth was retarded.

Serotonin may have a function that relates to the conversion of benign tumours to malignant tumours. Benign tumours always have an increased number of connective tissue cells containing serotonin (Bobro et al., 1976). On the other hand, malignant tumours are characterised by the accumulation of considerable amounts of substances with green luminescence (histamine, catecholamines and di- and poly-amines) in the tissue of the tumour, and particularly in the reactive zone, as well as the disappearance of serotonin from connective tissue cells (ibid.).

Some miscellaneous cellular and subcellular effects due to serotonin are: activation of adenyl cyclase in Aplysia, Fasciola and human neural tissue (Shimahara & Tauc, 1977; Nathanson & Greengard, 1974); alteration of the phosphorylation of certain proteins within Aplysia nerve cells (Lemos et al., 1982); inhibition of amino acid release and protein degradation in skeletal muscle (Garber, 1977); an enhancing effect on the growth of endothelial cells in tissue culture (Saba & Mason, 1975); increase in membrane permeability to sodium, causing depolarization of neurons in molluscs (Gerschenfeld & Stefani, 1968); and possibly regulation of renin (Epstein & Hamilton, 1977).

Aggregation may be the physiological response to serotonin binding in platelets (Boullin et al., 1977). However, some workers have shown that aggregation is impaired by allowing platelets to absorb serotonin (Baumgartner & Born, 1968). The latter researchers believe aggregation of platelets by serotonin is the result of an association of serotonin with specific receptors on the platelet membrane. These receptors are identical with those that effect aggregation, and therefore, according to this theory, they are unavailable for aggregation while involved in uptake of serotonin.

Increased membrane permeability to Ca^{2+} is associated with serotonin (Douglas, 1968; Woolley & Gommi, 1964; D'Amore, 1978).

Douglas postulated that a passive movement of calcium was caused by the presence of serotonin. Woolley & Gommi hypothesized that Ca^{2+} is normally in combination with ganglioside receptors for serotonin, and that serotonin displaces the Ca^{2+} by an ion exchange mechanism. Furthermore, these ions are actively carried through the normally ion-impermeable cell membrane. D'Amore reported an influx of $^{45}Ca^{2+}$ into cultured endothelial cells within one min after the addition of 10^{-5} M serotonin.

A relationship between Ca^{2+} uptake and cyclic AMP in platelet membrane vesicles has been described (Käser-Glanzmann et al., 1977). These researchers found that the inhibition of many platelet activities by high intracellular cAMP concentrations was directly linked to the stimulation of the removal of Ca^{2+} from the cytoplasm.

Within the platelet, serotonin produced a dose-dependent six- to ten-fold increase in cyclic GMP within one min of serotonin addition (Agarwal & Steiner, 1976; Schoepflin et al., 1977). The role of cGMP in platelets was not elucidated, but may have to do with contractility. Agarwal and Steiner (1976) cite references in which other tissues also showed a marked increase in cGMP when serotonin was administered.

Although the function of serotonin in the lung is not known, there is evidence for transfer of serotonin to the pulmonary circulation in normal lungs (White, M.K. et al., 1975). This uptake of serotonin occurs rapidly, without any observable prolongation of platelet transit time. However, in cases of abnormal pulmonary function, serotonin released from the platelets was not taken up by the pulmonary circulation despite significant platelet entrapment; instead, released serotonin was removed by the circulating platelets (ibid.). Since the platelet release reaction is not induced by a normal endothelium (Steubens, 1966; Stemerman, 1981), it has been proposed that healthy lung endothelium may be able to bind plasma serotonin, but that damaged pulmonary endothelium may not have this ability (White, M.K. et al., 1975). As evidence, the study by Strum and Junod (1972) was cited, in which serotonin uptake by pulmonary endothelial cells was demonstrated by radioautography. It has also been noted that high oxygen tension depressed serotonin uptake by cultured endothelial cells and that uptake remained depressed 48 h after exposure to 95% oxygen ended (Block & Stalcup, 1981).

Others have also noted a localization of serotonin in endothelium: Costa et al. (1974a) found that serotonin accumulated in the blood

vessels after cerebrovascular injury. They believed the platelets were the source of serotonin, since labelled serotonin was associated with the injured area when blood incubated with $[^3H]$-serotonin was reinjected. Also fluorescence due to serotonin was correlated with platelet content of serotonin in different species. They wrote, "It seemed, in some instances, as if 5-hydroxytryptamine was localized in the endothelial lining or muscular coat of the vessels." They suggested that this serotonin might alter the permeability of the vessel walls to protein.

Cultured and freshly isolated endothelial cells from bovine aortas exposed to labelled serotonin took up 125-250 pmoles serotonin/mg protein in 3 h by a mechanism that was inhibited by 10^{-4} M imipramine (Shepro et al., 1975). Shepro et al. proposed that an active transport system may be involved in this transfer, because cold (4 ^{o}C), metabolic inhibitors and ouabain (10^{-5} M) reduced serotonin uptake.

In a related study, it was found that tricyclic antidepressants (TAD) such as amitriptyline at therapeutic concentrations produced a dose-dependent reversible increase in permeability in primate cerebral blood vessels (Preskorn et al., 1982). Water and ethanol were used, but the authors proposed that electrolytes and metabolic substrates might be similarly regulated.

It has been reported that serotonin stimulates glycogen hydrolysis in slices of cerebral cortex (Quach et al., 1982). Its action is said to be mediated by a "novel" class of receptors. TADs were among the best competitive antagonists. The authors concluded, therefore, that serotonin may control the carbohydrate metabolism in the brain. Because the effective concentration of TADs in body fluids of patients undergoing antidepressant treatment are in the micromolar range, it is likely that glycogenolysis in brain may be inhibited during such treatments. This blockade may participate in the antidepressant activity or side effects of these drugs.

Maintaining structural integrity of the microvasculature is yet another function that has been ascribed to serotonin (Sweetman et al., 1981). It was claimed that serotonin could prevent thrombocytopenic petechiae and this effect was partly decreased by imipramine.

D. Serotonin-binding proteins

Proteins that bind serotonin have been investigated only in recent years and by only a few groups of researchers. A list of the principal findings is given in Table 1-1.

Particular mention should be made of the work done in Uvnäs' laboratory on serotonin storage in the rat platelet (Åborg & Uvnäs, 1971; Anderson et al., 1974). Åborg and Uvnäs described a study which indicated that certain basic proteins from rat platelets could bind serotonin and that these proteins were associated with granules (originally thought to be the dense granules). Since no distinction had been made among the various granule-containing fractions and since these were combined after density-gradient centrifugation, it is possible that membrane vesicles were present as well as alpha and dense granules. This solution, in which a sulphomucopolysaccharide was present, also had affinity for sodium ions and histamine. Åborg and Uvnäs deduced that serotonin and protein were stored together in the dense granules in a complex with mucopolysaccharide. This interpretation was corrected in a later paper (Anderson et al, 1974), which reported that no serotonin was found in the alpha granules where the sulphomucopolysaccharide and proteins were located, and that the concentration of protein in the dense granules was very low. However, no explanation was given for the earlier paper's report of association between basic proteins and serotonin.

It is interesting to note that the work in the 1971 paper was initiated by the authors' previous findings with rat mast cells (Uvnäs et al., 1970), that histamine and possibly serotonin were bound to a protein in a heparin-protein complex in the mast cell granule matrix. In this complex a bond between the biogenic amine and the carboxyl groups of a basic protein was proposed. The heparin-protein complex formed 95% of the mast cell granule matrix (ibid.). In view of the discovery of PF4 in human mast cell granules (McLaren et al., 1980) and the binding of serotonin by PF4 in vitro, reported in this dissertation and elsewhere (Heemstra, 1983), it seems likely that the heparin-protein complex described by Uvnäs et al (1970) and isolated from rat mast cell granules could only be a heparin-PF4 complex.

Table 1-1 is a chronological list of research on serotonin-binding proteins, grouped by laboratory. Brief notes on the physicochemical characteristics of the proteins involved are also given.

Table 1-1. Serotonin-binding proteins

Tissue	Principal Findings	References
rat mast cells	Histamine, and perhaps other biogenic amines, are bound to carboxyl groups of a protein in a heparin-protein complex which formed the bulk of the granule matrix.	Uvnäs et al., 1970
rat platelets	Basic proteins and a sulphomucopolysaccharide may be involved in serotonin binding.	Åborg & Uvnäs, 1971
mini-pig synaptosomes	Serotonin-binding protein also binds LSD	Mehl & Weber, 1974,
rat brain	Serotonin-binding protein also binds LSD. The complex fluoresces at longer wavelengths than does the free protein or free LSD.	Shih et al., 1974 Shih & Rho, 1977
rat brain	Used a photosensitive $[^3H]$-arylazide derivative of serotonin to label proteins that bind serotonin. Three proteins were labelled (M_r 80 000, 49 000 and 38 000).	Cheng & Shih, 1979

human platelets	Albumin and one glycoprotein were both bound and unbound on serotonin-affinity gel. Another soluble glycoprotein was bound, but was not characterized.	Pignatti & Cavalli-Sforza, 1975
rat brain	Serotonin affinity gel bound one protein which was eluted with 20 mM Tris, pH 8,1 containing 1 mM Ca^{2+}. The specific activity of the protein was 1-2 pmole serotonin/mg protein before, and 77,3 pmole/mg protein after affinity chromatography. The protein was not characterized.	Rotman, 1978
rat brain cortex	The serotonin-binding protein was in the cytoplasm of synaptosomes, was sensitive to reagents that block sulphydryl groups, was non-dialyzable, was destroyed by heating to 95 ^{o}C for 5 min or by tryptic digestion, migrated on 9,5% electrophoresis gels. Ca^{2+} inhibited binding.	Tamir & Huang, 1974
rat brain	Distribution of binding protein was similar to that of serotonin, i.e., the activity was highest in the midbrain raphe area, hypothalamus and thalamus, low in the cerebellum and intermediate in other regions. 10 µM imipramine did not inhibit binding.	Tamir & Kuhar, 1975

rat brain	EDTA and nucleotides inhibited serotonin binding. 10^{-4} M Fe^{2+} enhanced binding. Ca^{2+} had no effect. The protein-Fe-serotonin complex did not enter 6,5% polyacrylamide gel when electrophoresed even in the presence of 6 M urea. TAD had no effect on binding, but LSD and reserpine inhibited binding.	Tamir et al., 1976
rat brain	Inhibition by drugs that interact with contractile proteins was shown by serotonin-binding protein. Concluded that the binding protein is "an actin-like contractile protein".	Tamir & Rapport, 1976
rabbit and guinea pig myenteric plexus	Determined dissociation constants for the partially purified protein and concluded that this protein is the same as that found by the same group in rat brain.	Jonakait et al., 1977 (Tamir's group)
rat brain and guinea pig myenteric plexus	5,6- and 5,7-dihydroxytryptamine were potent inhibitors, and this inhibition could be demonstrated both in vivo and in vitro.	Tamir & Rapport, 1978
sheep thyroid	Highest concentrations of serotonin and serotonin-binding protein were found in the rostro-central portion of the thyroid.	Bernd et al., 1979 (Tamir's group)

rat platelets	One glycoprotein (M_r = 204 000) and platelet albumin bound serotonin. The glycoprotein had two dissociation constants (K_D), 4,2 x 10^{-8} and 8,1 x 10^{-7} M. The K_D of albumin was 5,5 x 10^{-7} M. Both proteins showed enhanced binding in the presence of 10^{-4} M Fe^{2+}. The glycoprotein was more heat-stable than was albumin. Chlorimipramine had no effect on either. ATP inhibited serotonin- binding by both. Concluded that neither is a part of the membrane receptor nor a component of the uptake mechanism of platelets, but might be involved in storage of serotonin. (See Chapter 6.)	Tamir et al., 1980a
rat brain	One protein with 2 K_D, 4 x 10^{-10} M & 3,2 x 10^{-8} M showed enhanced binding with Fe^{2+}. β-mercaptoethanol was included in SDS buffer to dissociate the aggregate for electrophoresis. No effect by chlorimipramine on binding.	Tamir et al., 1980a
rat brain	Binding was enhanced by gangliosides in vitro, but no binding was found in the absence of Fe^{2+}.	Tamir et al., 1980b

rat platelets	The glycoprotein described by Tamir et al., 1980a was shown by an immunohistochemical technique to be located in the platelet plasma membrane. It was named "serotonectin" and may be involved in serotonin uptake.	Kupsky et al., 1981 (Tamir's group)
rat brain	Disulphide and sulphydryl groups were essential for binding. Inhibition by ATP was due to its chelating properties only. K^+ enhanced binding. Na^+ and 1 mM Ca^{2+} inhibited binding. Suggest that serotonin-binding protein has a storage function.	Tamir & Liu, 1982
rat platelets	Serotonectin is distinct from fibronectin. Serotonectin did not bind $[^3H]$-imipramine (up to 0,25 µM). Serotonectin was associated with the plasma membrane and could be removed by washing platelets with Krebs solution. Serotonectin was also found in the plasma. Serotonectin may be synthesized in the megakaryocytes.	Tamir et al., 1983

II. Platelet Factor 4 (PF4) and β-Thromboglobulin (βTG)

A. History

Platelet proteins that neutralize heparin were discovered more than thirty years ago and since then they have been the subject of much research (briefly reviewed by Moore et al., 1975b). Although the term "heparin-neutralizing activity" or "HNA" was used interchangeably with "platelet factor 4", these reports are relevant to βTG as well as PF4, since βTG is also one of the heparin-neutralizing proteins in platelets. In 1973 Nath et al., suggested confining the use of the term "PF4" to only the releasable anti-heparin activity derived from platelet granules. This definition, however, would still include two proteins besides PF4: LA-PF4 and βTG (Rucinski et al., 1979; Kaplan, 1980) as well as a peptide that is cleaved from the Glycoprotein G molecule (Lawler et al., 1982).

Since the determination of the amino acid sequences of PF4, βTG and LA-PF4 within the past decade, it has been possible to distinguish these three proteins in studies that involve their isolation. The consequent rapid advances in their respective areas are chronicled in Table 1-2. However, in immunofluorescence studies, no distinction can be made among βTG, its precursor LA-PF4 (low affinity platelet factor 4, not to be confused with PF4, which is immunologically distinct), and platelet basic protein (Niewiarowski et al., 1980; Varma et al., 1982), because all of these proteins have the same antigenic site and therefore react with βTG antibody. Thus, in such studies, the term βTG refers to several similar proteins, but not to PF4.

B. Localization of Platelet Factor 4 and β-Thromboglobulin in platelets

PF4 and βTG are stored in platelets in a subclass of alpha granules. It had been deduced in 1973 that HNA was stored separately from serotonin and ADP because a normal quantity of HNA was present in cases of storage pool disease, a disease in which the platelets are deficient in serotonin and ADP (Weiss & Rogers, 1973). Later, a difference in release of lysosomal enzymes and HNA led Walsh and Gagnatelli (1974) to postulate storage of HNA separate from that of lysosomal enzymes. Confirmation that PF4 and βTG are concentrated in a

Table 1-2. Chronology of Platelet Factor 4 and β-Thromboglobulin research since 1972

Year	Reference	Finding
1972	Barber et al.	Characterized PF4 carrier
1972/3	Käser-Glanzmann et al.	Amino acid analysis of PF4
1975a,b	Moore et al.	Isolation and characterisation of βTG and PF4
1976	Moore & Pepper; Levine & Wohl	Heparin affinity chromatography used to isolate PF4 & βTG
1976	Da Prada et al.	HNA stored in alpha granules
1976	Handin & Cohen	Lysine residues of PF4 bind heparin
1976a,b	Bolton et al.	RIA for PF4 and βTG
1977-8	Deuel et al.; Walz et al.; Hermodson et al., Morgan et al.	Four independent groups determined primary structure of PF4
1978	Hiti-Harper et al.,	PF4 inhibits collagenase
1978	Begg et al.,	Primary structure of βTG
1978	Kurachi	X-ray diffraction of PF4 crystals
1978	Dawes et al.	PF4 and βTG can pass freely across some biological membranes
1980	Busch et al., Rybak et al.	PF4 binds to vascular endothelium
1980	McLaren et al.	PF4, but not βTG, is present in human mast cell granules
1980	Goldberg et al.	Vascular permeation by PF4 after endothelial injury
1980	Niewiarowski et al.	LA-PF4 can be converted to βTG in platelet release medium by incubation at 37 °C
1981	Lawler	Prediction of secondary structures of PF4 and βTG from their amino acid sequences
1982	Hagen et al.	Cross-immunoelectrophoresis indicates PF4 exists in the unreleased platelets in a state different from that observed extracellularly after release
1982	Dawes et al.	Heparin injection induces release of PF4 from vascular endothelium
1982	George & Onofre	PF4 is associated with the platelet plasma membrane by a calcium-dependent mechanism after release
1982	McLaren & Pepper	PF4 and βTG are synthesized in megakaryocytes
1982	Taylor & Folkman	PF4 inhibits the growth of new capillary blood vessels
1983	Senior et al.	βTG and PF4 promote directed fibroblast migration
1983	Heemstra	PF4 and βTG bind serotonin in vitro

subclass of alpha granules that does not contain lysosomal enzymes has come from many laboratories (Broekman et al., 1975; Da Prada et al., 1976; Witte et al., 1978; Ryo et al., 1980; Gogstad et al., 1982; McLaren & Pepper, 1982).

βTG is stored as LA-PF4, which is cleaved to βTG after release from the platelets (Fukami et al., 1979). The proteoglycan carrier for PF4 is stored in the same granules as PF4, probably in a complex with PF4 (Barber et al., 1972). PF4 and βTG (or LA-PF4, since the RIA does not distinguish between them) are synthesized in megakaryocytes before they are "packaged" into alpha granules in circulating platelets (McLaren & Pepper, 1982; Ryo et al., 1983), and this constitutes evidence that PF4 and βTG (or LA-PF4) are truly platelet-specific proteins. βTG appeared to be more abundant than PF4 in alpha granules (McLaren & Pepper, 1982).

More controversial is the finding that small amounts of PF4 are sometimes present on the platelet plasma membrane. Evidence has been obtained -- even by the same research groups -- both for the presence and absence of PF4 on the membrane (Niewiarowski et al., 1968a; Niewiarowski & Thomas, 1969). This contradictory evidence was partially resolved by postulating that the appearance of PF4 on the surface of the platelet is a transitory phenomenon and occurs immediately after the platelet release reaction (O'Brien, 1970). As soon as some aggregation occurs, much less HNA is demonstrable on platelets (O'Brien, 1971). With remarkable foresightedness, O'Brien hypothesised that HNA can exist in three forms: a) hidden and inactive in the platelet; b) active at the platelet surface; or c) in a soluble form after the release reaction (O'Brien et al., 1970). Direct evidence for "b" , that is, active HNA at the platelet surface, did not come for twelve more years. In the meantime, many laboratories reported results that generally did support the hypothesis (Barber & Jamieson, 1970; Barber et al., 1972; Lüscher & Käser-Glanzmann, 1974; Moore et al., 1975b; Da Prada et al., 1976; Gjesdal & Pepper, 1977; Fukami et al., 1979).

Direct evidence for the presence of PF4 on the platelet plasma membrane was obtained by immunofluorescence and reported recently (George & Onofre, 1982). The association of PF4 with the platelet plasma membrane was dependent on exogenous Ca^{2+} and on platelet release. However, some PF4 appeared to be on the plasma membranes of the control platelets (no thrombin had been added to these platelets).

To explain this finding, George and Onofre invoked the theory that the trauma of centrifugation can be sufficient to begin platelet release (ibid. and references therein). If this theory is correct, it might explain earlier contradictory reports concerning the presence of PF4 on the platelet plasma membrane.

Calcium-dependence in the binding of PF4 to the plasma membrane had been suggested previously by results reported by Niewiarowski et al. (1974), in which platelets were aggregated by antibody to PF4, and this aggregation was blocked by EDTA.

Evidence has also been presented for the presence of PF4 in the membrane of the alpha granule (Gogstad et al., 1982).

C. Physicochemical characteristics of Platelet Factor 4 and β-Thromboglobuliin

Pepper has reviewed the literature relating to macromolecules that are released from alpha granules, especially PF4 and βTG, but also "cell growth factor" (CGF), β-lysin and vascular permeability factor (Pepper, 1980). He wrote, "It is quite possible that one or two of the 'activities' will subsequently turn out to be different facets of the same molecule" (ibid.). He noted similarities among PF4, βTG, β-lysin, CGF and vascular permeability factors. These similarities include low molecular mass, basic subunits that have a tendency to aggregate or complex with other proteins, heparin and cation exchangers. Pepper has written of their most salient characteristic:

> "Calculations based on the density of the alpha granules show that they are at least 60% protein and therefore contain no more than 40% water. In view of the subsequent rapid release into dilute aqueous solutions, the physical requirements of these proteins must necessarily be rather 'schizophrenic.' On the one hand they are required to pack in high concentration without inter-molecular interaction, but on the other hand they are required to move rapidly into an aqueous environment. It is interesting to note that the amino acid composition and physical properties of these proteins are distinct from the well studied groups of proteins such as plasma and cell membrane proteins. It may well be that as more of these platelet specific macromolecules are analysed and sequenced, they will show a consistent set of features which constitute a class of granule proteins" (Pepper, 1980).

1. Heparin binding

PF4 is the most potent anti-heparin protein known and is able to bind about twice as much heparin per mole as can protamine sulphate (Nath et al., 1973). However, since endogenous levels of heparin in the plasma are low (Horner, 1974), it is not known whether this is a natural function of PF4 in vivo (Handin & Cohen, 1976; Brown et al., 1978; Denton et al., 1983). PF4 binds $[^{125}I]$-heparin in a rapid, saturable manner with a K_D of 1-5 nM (Rybak et al., 1980). βTG also binds heparin, but more weakly than does PF4 (Moore & Pepper, 1976). Free PF4 and PF4 in a complex with its carrier are equally active in the neutralization of heparin (Lüscher & Käser-Glanzmann, 1974), due to the displacement of the carrier by heparin (Barber et al., 1972). The relative binding capacity of glycosaminoglycans for PF4 is: heparin (100), heparan sulphate (75), chondroitin-4-sulphate from PF4 carrier (50), chondroitin-4-sulphate (50), chondroitin-6-sulphate (50), dermatan sulphate (50), hyaluronic acid (0) (ibid.). Heparan sulphate is the major glycosaminoglycan in endothelial plasma membranes (Busch et al., 1980 and references therein).

By modifying specific amino acid residues on the PF4 molecule, Handin and Cohen (1976) were able to show that binding of PF4 to heparin involves lysine, rather than arginine, residues on PF4. The COOH-terminal tryptic peptide of PF4 in high concentrations partly reversed the prolonged thrombin time induced by heparin, suggesting that heparin binding is localized in the COOH-terminal region (Huang et al., 1982).

Recent studies have provided much information about the kinds of heparin (which are referred to as high affinity heparin and low affinity heparin on the basis of their affinity for antithrombin III) bound by PF4 and the kinds of complexes that are formed (Niewiarowski et al., 1979 and references therein; Bock et al., 1980; Luscombe et al., 1981a and b; Vannuchi et al., 1982; Wohl et al., 1982; Jordan et al., 1982; Denton et al., 1983).

Heparin-affinity chromatography (Levine & Wohl, 1976; Wu et al., 1977), which is based on the affinity of PF4 for heparin, is the most commonly used method for purifying PF4.

Heparin is a potent inhibitor of PF4-mediated platelet agglutination (Phillips & Gartner, 1980, p. 425).

2. Proteoglycan carrier of Platelet Factor 4

PF4 in a complex with its proteoglycan carrier, or with heparin, is soluble at physiological pH and ionic strength (Moore et al., 1975b). Free PF4 is insoluble under these conditions (Käser-Glanzmann et al., 1972/3).

The carrier has been characterized in several laboratories. It is dissociated from PF4 at high ionic strength. In one study 0,75 ionic strength caused dissociation (Käser-Glanzmann et al., 1972). The proteoglycan carrier can also be dissociated from PF4 by displacement by heparin (Moore & Pepper, 1976). The carrier is an oligomeric proteoglycan composed of four glycosaminoglycan chains of chondroitin-4-sulphate (M_r 12 000) in covalent linkage with a single polypeptide (M_r of entire carrier 59 000 or 53 000) (Barber et al., 1972; Huang et al., 1982). Four moles of tetrameric PF4 are associated non-covalently with one mole of carrier and this complex dimerizes to produce a complex with total M_r of about 360 000 (ibid.)

The carrier, like PF4, does not contain methionine (Huang et al., 1982). In all centrifugation analyses 1 M NaCl is needed to prevent self-association of the carrier (ibid.). The proteoglycan after chondroitinase digestion or after desulfation fails to bind PF4. Therefore, the intact glycosaminoglycan chain and sulphate group appear to be required for interaction between the proteoglycan and PF4 (ibid.).

3. The primary and secondary structures of PF4 and βTG

The primary and predicted secondary structures of PF4 and βTG have been reported (references in Table 1-2). PF4 and βTG consist of 70 and 81 amino acid residues per monomer, respectively.

PF4 is characterized by a structure that is markedly cationic at the COOH terminus and markedly anionic at the NH_2 terminus. At the COOH terminus there are four lysine residues, arranged in two pairs, alternating with one pair of leucine residues and one pair of isoleucine residues. The seven amino acid residues closest to the NH_2 terminus consist of five acidic residues, one glycine and one alanine

residue. Two intramolecular disulphide bonds are present (Moore et al., 1975b; Handin & Cohen, 1976; Hermodson et al., 1977), but the positions of these bonds have not been determined.

Heparin binding by PF4 involves the lysine residues near the COOH terminus (Handin & Cohen, 1976; Huang et al., 1982). Variations in structure between PF4 and βTG in this part of their respective molecules may account for their significantly different heparin affinities (Lawler, 1981).

There are eight lysine residues and three arginine residues per PF4 monomer, but most of them are adjacent to each other or next to negatively charged amino acids or proline, and are therefore not sensitive to cleavage by trypsin (Haschemeyer & Haschemeyer, 1973, p. 81). Lysine at positions 14, 31, and 46 are susceptible to trypsin attack. Antiheparin activity and antigenicity are eliminated by the action of trypsin on purified PF4 (Nath et al., 1973 and 1975), but different results have been obtained when trypsin has been added to solutions of native protein. Farbizewski et al. (1966) reported that only 11% or 13% of PF4 activity was blocked by 8 h incubation with trypsin or chymotrypsin A, respectively. The difference might have been due to protection of the trypsin-sensitive sites by the native protein's conformation or the presence of an ion or molecule blocking the bonds (see below).

Moore and Pepper's group isolated HNA from platelets which had undergone release (Moore et al., 1975b). They found that HNA was coincident with protein and phosphorus in the sucrose density gradient fraction which had the density of membrane vesicles. This HNA was treated with trypsin, followed by soya bean trypsin inhibitor, but was neither destroyed nor solubilized under these conditions. In view of later findings which demonstrate that PF4 is on the platelet plasma membrane immediately after release (George & Onofre, 1982), the results of Moore et al. suggest that the trypsin-sensitive bonds were either buried within the PF4 molecule or otherwise protected. In this connection, it is interesting to note that when LA-PF4 is converted to βTG, selective cleavage by plasmin and trypsin at lysine-4 occurs as if the other 9 lysine residues are relatively inaccessible (Holt & Niewiarowski, 1980). Holt & Niewiarowski have proposed that a protease capable of selectively cleaving LA-PF4 is actively secreted by platelets.

The clustering of negative charges at the NH_2 terminus of PF4 has

been noted (Hermodson et al., 1977). The amino acid sequence resembles calcium binding loops in other proteins (see Chapter 5). The binding of cations to a protein can produce cationic behaviour in an otherwise neutral protein (Bull, 1964, p. 136). Endogenous HNA (PF4) has been described as cationic or basic (Niewiarowski et al., 1968a; Lüscher & Käser-Glanzmann, 1974). The vascular permeability factor has been called cationic (Nachman et al., 1972). However, the isoelectric point of purified PF4 is 7,6 (Handin & Cohen, 1976), and the isoelectric point of βTG and LA-PF4 are 6,8-7,5 and 7,9-8,2, respectively (Holt & Niewiarowski, 1980). The name βTG was coined by Moore et al. (1975a) because of the electrophoretic migration of βTG to the β-globulin region.

The clustering of opposite charges at the opposite termini of the PF4 molecule initiated the idea that PF4 might have lectin activity (Phillips & Gartner, 1980).

PF4 appears to be very heat-stable, retaining its heparin-neutralizing activity even after being subjected to 100 ^{o}C for 10-30 min (Nath et al., 1973; Levine & Wohl, 1976).

Many physicochemical parameters of βTG have been determined and described in the initial report of βTG (Moore et al., 1975a). βTG, like PF4, lacks interchain disulphide bonds and is not readily denatured or aggregated (Moore & Pepper, 1976). Rucinski et al (1979) have studied βTG and antigenically similar proteins, described means for distinguishing them, and measured their relative contribution to the total HNA of platelets. Kaplan has reviewed the literature concerning βTG research (Kaplan, 1980, 96 references).

The determination of M_r of PF4 has been fraught with problems, such as PF4's ability to bind to Sephadex and consequent anomalous elution (Nath et al., 1973; Handin & Cohen, 1976) (also see Chapter 6) and so it is not surprising that many different values have been obtained by various workers in this field. M_r for PF4 monomers has been reported to be 6900-21 000 (Lüscher & Käser-Glanzmann, 1974; Handin & Cohen, 1976; Moore et al., 1975b; Levine & Wohl, 1976; Nath et al., 1973). The correct value, as determined from amino acid determinations, is 7780 (Hermodson et al., 1977). The corresponding M_r values for βTG and LA-PF4 are 8851 and 9070, respectively (Begg et al., 1978; Rucinski et al., 1979).

It was known as early as 1975 that both PF4 and βTG are composed of tetramers at physiological pH and that both dissociate at low pH to

the monomeric form (Moore et al., 1975a,b; Moore & Pepper, 1976). Recently the same group reported dissociation of βTG tetramers (but not of PF4 tetramers) due to an increase in ionic strength of the medium or a decrease in concentration of the protein at pH 7,5 (Bock et al., 1982). These results imply that βTG might exist in the monomeric form in normal plasma and LA-PF4 might exist in the tetrameric form in the alpha granules. It is interesting that the dimeric form of these proteins or of PF4 has not been reported.

D. Functions of PF4 and βTG

1. Platelet Factor 4

The following functions have been attributed to PF4:

i Heparin neutralization
ii Inhibition of collagenase
iii Inhibition of binding of low density lipoprotein to its cell surface receptor
iv Participation in the induction of platelet agglutination
v Vascular permeability
vi Chemotaxis
vii Inhibition of angiogenesis

i) Heparin binding by PF4 is well known and has been described in the previous section. However, the stoichiometry of heparin-PF4 complexes in vivo and their role in the neutralization of the anticoagulant activity of heparin is still uncertain (Denton et al., 1983). After platelet release, PF4 can be found associated with the vascular endothelium (Dawes et al., 1982). Specific detachment of 50% of cell surface-associated heparan sulphate by platelet heparitinase decreases PF4 binding to the endothelial cells by 50%, thus indicating that heparan sulphate may be involved as a binding site for PF4 (Busch et al., 1980). These studies suggest that heparin-binding and heparan sulphate-binding might be related to a vascular permeability function. In addition, PF4 is released from its binding site on the endothelium by intravenous or subcutaneous injection of heparin (Dawes et al., 1982), and recent studies show an association between heparin and tissues that are subject to environmental influences both in the foetus

and in the adult (Nader et al., 1982). Thus, heparin, and perhaps PF4, may be involved in a defense mechanism.

ii) Collagenases from human skin and human granulocytes have been shown to be inhibited by human PF4 (Hiti-Harper et al., 1978). The concentrations of PF4 used in the study were within the physiological range and no effect in the inhibition pattern was noted on the addition of heparin. In the same study, it was reported that PF4 neither inhibits plasma lipoprotein lipase nor affects various coagulation factors.

iii) Inhibition of the binding of low-density lipoprotein to its cell surface receptor in human fibroblasts has been attributed to PF4 as well as to certain histones and protamine (Brown et al., 1978). Concentrations of PF4 greater than those obtained with platelet release are required for this inhibition, and thus modulation of this interaction in vivo remains speculative.

iv) PF4 agglutinates washed platelets and formaldehyde-fixed washed platelets, as well as formaldehyde-fixed trypsin-treated bovine erythrocytes (Phillips & Gartner, 1980). Heparin is a potent inhibitor of the PF4-mediated platelet agglutination (ibid.), and not only inhibits PF4 agglutination of platelets, but also causes previously formed agglutinates to dissociate. Arginine (60 mM final concentration) also inhibits PF4-mediated platelet agglutination. Lysine is less effective. The amino sugars galactosamine, glucosamine and mannosamine only slightly inhibit PF4-mediated platelet agglutination (ibid.). Arginine residues may play a critical role in the ability of PF4 to crosslink platelets (ibid.). The platelet agglutinating activity of PF4 is not destroyed by trypsin (ibid.).

v) A vascular permeability factor from human blood platelets has been described as a small stable cationic protein (Nachman et al., 1972). This factor has been shown by immunofluorescence to be PF4 (Goldberg et al., 1980). In Goldberg's study, PF4 permeated the blood vessel wall within 10 min of endothelial injury, was still present 30 min after injury, but had disappeared by 240 min after injury. Vascular permeability, though often, is not always, biphasic (Nachman & Weksler, 1972; Anderson & Scotti, 1976, p. 77). When vascular permeability is biphasic, the acute phase occurs within 10 min of injury and is characterized by edema and vasodilation (Nachman et al., 1972). The

delayed phase (at about 3 h) is characterized by leukocytic infiltration of the damaged tissue (Nachman & Weksler, 1972; Anderson & Scotti, 1976, p. 77); Goldberg's results indicate that PF4 is involved in the acute phase of vascular permeability, although its role, once inside the vessel wall, has not been determined. PF4 also enters cultured venous endothelial cells, as has been demonstrated in another study by immuno-fluorescence (Dawes et al., 1982), and permeates several biological membranes (Dawes et al., 1978).

vi) The participation in chemotaxis, the attraction of cells, especially leukocytes, which causes their migration to sites of inflammation, is another recently discovered role for PF4. This is the second phase of vascular permeation (Nachman & Weksler, 1972; Anderson & Scotti, 1976, p. 77). The carboxyl-terminal tridecapeptide of PF4 (including the putative heparin binding site) is a potent chemotactic agent for monocytes and polymorphonuclear leukocytes (Osterman et al., 1982). It has been hypothesized that release of this peptide by proteolysis in the tissues which have been permeated could provide a potent local chemotactic agent for inflammatory cells (Senior et al., 1983). It has also been shown in one study that the addition of the PF4 carrier with PF4 in the chemotactic assay did not reduce the chemotactic activity of PF4 (Huang et al., 1982).

vii) Recent studies have shown that PF4 inhibits angiogenesis, which is the growth of new capillary blood vessels (Taylor & Folkman, 1982). Angiogenesis is important in development, wound healing, chronic inflammation and neoplasia. Taylor and Folkman found that heparin increased the migration of capillary endothelial cells towards implanted tumours, but heparin alone could not initiate angiogenesis. The combination of heparin and a small quantity of an angiogenesis promoter isolated from a tumour had the same effect as four times as much promoter in the absence of heparin. Anticoagulation was not important in promoting angiogenesis, because the non-anticoagulant fraction of heparin was equally effective in this action. Stoichiometric concentrations of protamine which neutralized heparin eliminated the angiogenesis enhancing effect of heparin. Greater concentrations, about twice the stoichiometric concentration, prevented all angiogenesis induced by the tumour extract. This effect of protamine was shown to be neither cytostatic nor cytotoxic. PF4 inhibited angiogenesis at approximately one-half the concentration required for inhibition by protamine. The only other effective

compound in this respect was the major basic protein from eosinophils. It has been hypothesized that heparin modulates the rate of migration of capillary endothelium and that the specific binding of protamine (or PF4) to heparin blocks the linear migration of capillary endothelial cells, thereby interfering with a crucial step in capillary formation.

2. β-Thromboglobulin

The following functions have been attributed to βTG:

i Inhibition of prostacyclin (PGI_2) formation in arterial endothelial cells

ii Stimulation of cell growth in tissue culture

iii Chemotaxis

iv Matrix or packing protein in the alpha granules

i) The inhibition of the production of PGI_2 has been ascribed to βTG (Hope et al., 1979). PF4 is without any such effect. The concentrations of βTG used in the study are those that are possible in localized vascular segments during the platelet release reaction in vivo. It is possible that a major product of the platelet release reaction might contribute to thrombus formation by limiting the amount of prostacyclin formed during adherence of the thrombus to the vascular wall (Hope et al., 1979).

ii) Mitogenic activity (stimulation of cell growth) in tissue culture is associated with highly cationic low molecular weight peptides, which have been incompletely characterized (Kaplan, 1980, and references therein).

iii) βTG has been found to be even more active in chemotaxis than is PF4, especially in causing directed fibroblast migration (Senior et al., 1983). In view of the fact that PF4's carboxyl-terminal tridecapeptide has chemotactic activity (see previous section), and in view of βTG's very different structure at the carboxy-terminus, these findings require explanation.

iv) βTG was originally thought to function as a matrix or packing protein in the alpha granules (Moore & Pepper, 1976), since it constituted the largest single component of the alpha granule. This role, however, may be that of LA-PF4, which is in the alpha granules, and therefore an alternative role must be found for βTG, after it is formed from LA-PF4 outside the platelet (Fukami et al., 1979).

III. Calcium in platelets

The role of calcium in platelets is very complex. This complexity is apparent from the fact that extracellular calcium is required for some, but not all, platelet functions. Calcium is found in several platelet structures and it is likely that each calcium pool serves a different function. Some, but not all, calcium is released rapidly when platelets undergo release. The involvement of calcium in platelet responses to stimulation encompasses nearly every aspect of the cellular response (Ardlie, 1982). The calcium content of platelets is higher than that of most other tissues in the body (Steiner & Tateishi, 1974; Rubin, 1974, p. 5).

Studies of platelet calcium have been reviewed by Rubin (1970 and 1974), Massini (1977), Feinstein (1978a and b) and Ardlie (1982).

In this section only those studies which are directly relevant to the present investigation will be considered.

A. Calcium localization in platelets

Calcium has been found associated with many platelet structures. Calcium is mostly stored in the dense granules (Skaer et al., 1974), but is also present in the alpha granules, cytoplasm and membranes.

In the dense granules Ca^{2+} exists in micelles with serotonin and ATP (Mürer & Holme, 1970; Lages et al., 1977; Holmsen et al., 1973). It is thought that calcium is also stored in human dense granules in a non-ionised form (Skaer et al., 1974).

In one study, half of the calcium in pig platelets was located in granules distinct from the dense granules (Salganicoff et al., 1975). Steiner and Tateishi (1974) reported that 98% of platelet calcium is bound to protein. This implies localization of calcium in the cytoplasm or alpha granules or other organelles that contain protein, since dense granules contain very little protein. Calcium is stored to a small extent in the alpha granules where PF4 and LA-PF4 are stored, and this pool of calcium disappears within 10 sec of thrombin-induced stimulation of platelets (Sato et al., 1975). It was proposed by Sato et al. that this rapid disappearance might be indicative of a possible role for the alpha granule pool of Ca^{2+} in the "stimulus-response mechanism." But Ardlie (1982) suggested that the alpha granules that contain Ca^{2+} might represent an early stage in the formation of dense

bodies, since "Bull's eye" structures were seen in some of the alpha granules.

In the cytoplasm probably the Ca^{2+} is associated with calmodulin (White et al., 1981). In the resting state the cytoplasmic calcium concentration is very low (Massini, 1977; Brass & Shattil, 1982). During the first minute after stimulation, the Ca^{2+} concentration of the cytoplasm increases (Massini et al., 1978) as a result of the granular release of Ca^{2+} to the cytoplasm. Dense granules do not release their contents to the cytoplasm, though, but rather, into the extracellular medium (Heptinstall & Taylor, 1979, and references therein).

On the platelet plasma membrane, calcium-dependent binding of PF4 (George & Onofre, 1982) and thrombospondin (Phillips et al., 1980) occurs after platelet release. EDTA can prevent this binding, although serotonin release induced by thrombin was the same in the presence of 4 mM EDTA (George & Onofre, 1982). Distribution of calcium on the platelet membrane has been studied by Skaer et al. (1974), Feinstein (1978b), Born (1972), Brass & Shattil (1982), and Le Breton et al. (1976).

B. Calcium requirements for platelet secretion and aggregation

Extracellular calcium is required for aggregation, but not for secretion (Feinman & Detwiler, 1975). This, however, should not be interpreted to imply that calcium is not required for secretion, because the data suggest that there is a direct involvement of Ca^{2+} in platelet dense granule secretion (Knight & Scrutton, 1980). Endogenous calcium stores may be sufficient to support secretion. Thus, according to Rubin (1970),

> "Calcium appears to be necessary for the release of serotonin induced by thrombin. The mere omission of calcium from the incubation medium is not always sufficient to depress significantly serotonin release from isolated platelets; however, with the use of chelating agents the secretory response to thrombin is blocked and the blockade is readily reversed by calcium...The need for calcium-chelating agents to manifest the effects of calcium deprivation on intact platelets suggests either that only minute amounts of calcium are necessary to sustain release or that an intracellular or firmly bound pool of calcium is important to sustain release."

Sometimes chelating agents do not block release: thrombin and collagen produced a release reaction even in the presence of EDTA and EGTA in two studies (Lüscher, 1978; Mürer, 1972). Also platelets incubated in EDTA and EGTA long enough to irreversibly lose aggregability (5-7 min at 37 ^{o}C) still released serotonin after addition of sufficient $CaCl_2$ to combine with the chelator (Zucker & Grant, 1978).

EDTA induces the efflux of βTG and serotonin by a process that is temperature-dependent (Grant & Zucker, 1979; Tunbridge et al., 1982). This effect by EDTA implies that the small amount of calcium that is needed for the secretion of βTG and serotonin might be released intracellularly by a temperature-dependent process. Such a calcium pool is not exposed to the plasma, because incubation for longer than 0,25 min with 5 mM EDTA is necessary to change aggregability and surface charge (Grant & Zucker, 1979).

The concentration of free Ca^{2+} in the cytoplasm of unstimulated platelets is 0,1 μM or less (Brass & Shattil, 1982). Half-maximal secretion of serotonin occurs in the presence of 1,9 μM Ca^{2+}, and full release of serotonin is obtained at 10 μM Ca^{2+} (Knight & Scrutton, 1980). The cytoplasmic Ca^{2+} concentration is about 15 μM during platelet activation (Owen et al., 1980).

C. Calcium-induced lysis of platelet granules

In isolated dense granule preparations, calcium enhances the spontaneous release of serotonin and ATP in the absence of thrombin or any other stimulating agent (Pletscher et al., 1968). A similar study with alpha granules has shown that lysis may be induced by 10-120 μM Ca^{2+} (Van der Meulen & Grinstein, 1982). These authors suggested that Ca^{2+} increases the ionic permeability of the alpha granule membrane leading to solute influx followed by osmotically coupled water uptake. Furthermore, they proposed that during platelet activation, Ca^{2+}-induced granule swelling could provide the driving force for membrane fusion required for exocytosis.

D. Calcium as an intracellular messenger

Feinstein (1978a) proposed that Ca^{2+} is required for activation of

platelet release by acting as the primary intracellular messenger or transmitter of information from the platelet plasma membrane to the interior of the platelet. This theory is generally accepted, because thrombin does not affect membrane permeability to calcium (Robblee et al. 1973b) and so endogenous ions of some sort must be mobilized as a result of binding of a platelet stimulant to the plasma membrane receptor. Moreover, this messenger must move intracellularly to begin platelet release. Feinstein (1980) supported this theory with direct evidence that the release of intracellular Ca^{2+} in platelets occurs with a time course consistent with its proposed role in activation-secretion coupling. He reported that Ca^{2+} release (to the interior of the platelet) was induced by thrombin or trypsin and began 0,75-1,8 sec after thrombin stimulation; secretion started 4-8 sec later.

A Ca^{2+}-ATPase is located in the plasma membrane, open canalicular system and dense tubular system (Statland et al., 1969; Dean & Sullivan, 1982). This protein is structurally and immunochemically related to that of skeletal muscle (M_r 100 000) (Dean & Sullivan, 1982). The apparent K_m for Ca^{2+} activation of ATPase is 0,1 µM (ibid.). Calcium is rapidly transported by this "relaxing factor" through the plasma membrane or through dialysis membrane (Statland et al., 1969; Robblee et al., 1973; White & Krivit, 1967; Behnke, 1968). Thus, the relaxing factor may provide the source of calcium necessary to initiate the platelet release reaction. The relaxing factor may be activated by calmodulin (White, G.C. et al., 1981).

Rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase has been found to self-associate to a dimeric form (Silva & Verjovski-Almeida, 1983).

E. Platelet Calmodulin

The ubiquitous Ca^{2+}-binding protein, calmodulin, has been identified in platelets (White et al., 1981). Inhibitors of calmodulin have been shown to inhibit platelet aggregation and release (White & Raynor, 1980). Recent studies (White & Raynor, 1982) indicate that this inhibition may involve the regulation of the Ca^{2+}-ATPase described above. Ardlie (1982) has reviewed other functions of calmodulin in platelets.

Calmodulin from several tissues has been well-characterized. Its

M_r is 17 000 and its primary structure has been determined (Cheung, 1982; Sasagawa et al., 1982). Calmodulin is heat-stable and is not easily denatured by low pH (Cheung, 1982). A conformational change that involves the peptide backbone and secondary structure of calmodulin occurs in the presence of calcium or trifluoperazine. Trifluoperazine is a drug that binds to calmodulin in a calcium-dependent way (Krebs & Carafoli, 1982; Seaton et al., 1983). Calmodulin has been shown to have four putative calcium-binding sites on each molecule (Tufty & Kretzinger, 1975). Glutamate and aspartate residues are concentrated in the sites. Calmodulin can be freed from its bound Ca^{2+} by utilizing its inability to chelate cations at low pH (Haiech et al., 1981).

Some simple peptides, hormones and neurotransmitters, such as adrenocorticotropic hormone, β-endorphin, glucagon and substance P undergo calcium-dependent binding by calmodulin (Malencik & Anderson, 1982). Sequence comparisons show that the peptides that bind calmodulin well contain regions that are structurally similar. The sequence of PF4 does not correspond at any point with the structurally homologous sequence of the calmodulin binding proteins, and therefore calmodulin binding of PF4 would not be anticipated.

F. Vascular permeability

The manner in which calcium controls the permeability of many membranes to cations, water and macromolecules has been reviewed by Rubin (1974).

IV. Platelet Release

Holmsen et al. have reviewed the platelet release reaction (1969, over 150 references), and listed the following characteristics of this process:

1. The substances released are located in the dense and alpha granules.
2. Substances which are located in either the cytoplasm or the mitochondria are retained.

3. Maximal release is reached within 60 sec at 37 ^{o}C.

4. The process depends on energy derived from both glycolysis and oxidative phosphorylation.

5. After release, the platelet functions more or less normally.

6. Extracellular Ca^{2+} promotes many aspects of release.

Most of the known inducers of platelet activity, such as thrombin, collagen and ADP are unable to penetrate the platelet plasma membrane (Lüscher, 1978). They react in some way with receptors on the platelet surface and induce a rapid intracellular mobilization of Ca^{2+} ions. This probably involves the Ca^{2+}-ATPase, known as the relaxing factor (see section III in this chapter).

The course of the release is so rapid that techniques for its precise measurement have become available only in recent years. It is interesting to note that with greater sophistication in technology, the platelet release reaction has appeared to become more rapid. For instance Ca^{2+} release after thrombin stimulation occurred "within 60 sec" in 1969 (Holmsen et al.), "within 10 sec" in 1975 (Sato et al.) and commenced in 5-10 sec in 1980 (Feinstein). By means of quenched-flow aggregometry, serotonin secretion has been found to begin within 1 sec of stimulation and is nearly complete by 4 sec (Gear & Burke, 1982). Hydrolysis of metabolic ATP begins within 1 sec of platelet stimulation (Mills, 1973).

Dense granule secretion probably occurs simultaneously with non-lysosomal alpha granule secretion in a process known as Release I (Pepper, 1980; Kaplan et al., 1979; Ryo et al., 1980). The energy requirements for the two release processes are not the same (Akkerman et al., 1982 & 1983). Lysosomal enzymes from other alpha granules are released later, in Release II (Walsh & Gagnatelli, 1974).

Recent transmission electron microscopy studies by Kawaguchi (1982a,b) have indicated that the contents of the dense granules leave the platelet by a route different from that utilized by the contents of the alpha granules. Moreover, the disappearance of the dense granules precedes the disappearance of the alpha granules. Kawaguchi noted a difference in platelets exposed to thrombin for 15 and 30 sec. At 15 sec the alpha granules were retained, but the contents of the dense granules had already disappeared by way of the dense tubular system. Round empty vesicles were evident simultaneously with the disappearance of dense granules. At 30 sec the alpha granules had decreased in number and the open canalicular system showed conspicuous dilation.

Amorphous materials and electron-dense strands of fibrils could be seen within the open canalicular system. In thrombin-stimulated platelets a connection between the alpha granules and the open canalicular system was confirmed by the same author using the freeze-etching technique. The fibrils mentioned above were thought to have been a product of fibrinogen, previously shown to be present in alpha granules (Day & Solum, 1973; Van der Meulen et al., 1983). The release of alpha granules was initiated by particles of collagen and latex only after the particles were visible in the open canalicular system.

Had there been no connection between the membrane systems, Kawaguchi's results would imply that the contents of the dense granules are released faster than the contents of the alpha granules. However, in platelets, a specialized membrane complex, formed by interaction of channels from the open canalicular system and the dense tubular system has been observed (White, 1972). This complex was observed in electron microscopic studies. Thus, contents of the dense granules and alpha granules may become associated via this complex before they leave the platelet.

When the released proteins emerge on the platelet surface, they may contribute to the "coated membranes" described by Morgenstern (1982). Changes in structure occur concomitantly with the released proteins' new location. PF4 has a more uniform charge distribution than it had when it was within the alpha granule (Hagen et al., 1982). In this form the PF4 does not bind to thrombin, though the original PF4 form did (ibid.). LA-PF4 is converted to βTG by the proteolytic cleavage of its terminal tetrapeptide, probably by a protease that is simultaneously released by the platelet (Holt & Niewiarowski, 1980; Niewiarowski et al., 1980). βTG is the most abundant protein present in the thrombin release products, with the possible exception of platelet albumin (Moore et al., 1975a).

A small temporary pool of serotonin is associated with the platelet membrane immediately after release (Lingjaerde, 1981, p. 164). At this stage it is possibly bound to the newly released macromolecules. Calcium taken up by the platelet during release is partly bound to newly created binding sites (Massini et al., 1978), and also possibly bound to newly released macromolecules. Although extracellular calcium is required for PF4 association with the membrane immediately after release (George & Onofre, 1982), PF4 and βTG are secreted both in the presence and absence of extracellular Ca^{2+}

(Phillips et al., 1980; George & Onofre, 1982; Grant & Zucker, 1979). Two other alpha granule proteins that are also only associated with the platelet plasma membrane in the presence of extracellular Ca^{2+} after release are fibrinogen and thrombospondin (Phillips et al., 1980).

V. Drugs affecting platelet function

Drugs affecting platelet function, though especially used to provide information about serotonin uptake, are also used to investigate other aspects of platelet function. de Gaetano et al. (1975) have reviewed about 150 publications concerning drug effects on platelet function.

The effects of tricyclic antidepressant drugs (TAD), such as imipramine, chlorimipramine and amitriptyline, have been studied by many groups. 1 μM imipramine inhibits serotonin uptake by platelets (Massini & Lüscher,1972). Even greater sensitivity to chlorimipramine and imipramine was reported by Drummond (1976), who found that inhibition could be measured at 0,2-0,7 μM concentrations. Gordon et al. (1978), have reported a K_i value for chlorimipramine inhibition of 0,3 μM or less. Chlorimipramine is the most active of the TAD in inhibiting serotonin uptake by platelets, imipramine and amitriptyline occupy intermediate positions, and maprotiline is much less active (Waldmeier et al., 1976). Inhibition by TAD at the serotonin binding site on the platelet plasma membrane is competitive (Lingjaerde, 1979), but chlorimipramine has an additional inhibitory effect which is thought to be due to an interference with Cl^- in the transport process (ibid.). This latter effect can be seen at nM concentrations of chlorimipramine and has been described as noncompetitive or uncompetitive (ibid.).

The TAD have effects on platelets other than those upon serotonin uptake at the platelet plasma membrane. A shift in serotonin location in the platelet has been noted when platelets have been treated with imipramine (Hussein et al., 1978). Low concentrations of imipramine have been shown to release serotonin from platelets without an appreciable effect on adenine nucleotide metabolism and release (von Pusch & Wesemann, 1979). Imipramine has also been shown to displace serotonin from its ATP complex in vitro (Nogrady et al., 1975), and low concentrations of imipramine caused aecumulation of the drug in the

dense granules when intact platelets were incubated with this drug (Reimers et al., 1977).

The human platelet-collagen adhesion reaction was inhibited by imipramine and amitriptyline, both at a concentration of 2×10^{-4} M (Mohammad & Mason, 1974). 1 µM imipramine affected serotonin re-uptake but not PF4 release when the platelets were induced to release by collagen (Walsh & Gagnatelli, 1974). Chlorimipramine blocked uptake of serotonin to the transitory pool existing on the platelet plasma membrane just after release (Lingjaerde, 1981). Endothelial cells in culture took up $[^{14}C]$-serotonin by a process that was sensitive to cold temperatures (4 ^{o}C) and to the presence of 10^{-4} M imipramine (Shepro et al., 1975).

Langer et al. have reviewed work on high-affinity binding of $[^{3}H]$-imipramine in brain and platelets and its relevance to depression and antidepressant drugs (Langer et al., 1982, 28 references).

Acetylsalicylic acid inhibits the release reaction of platelets (Dougherty, 1976). An interesting effect of aspirin on PF4 is that release depends to some extent on the agent inducing platelet release. In one study aspirin inhibited release induced by ADP and collagen, but not release induced by adrenaline and thrombin (Youssef & Barkhan, 1969). The in vivo effect in normal persons lasted at least three days. In another study PF4 and serotonin release was inhibited by aspirin when the release was induced by ADP and epinephrine, but only partly so when release was induced by collagen and thrombin (Ryo et al., 1980). In yet another study 2 g orally administered acetylsalicylic acid inhibited PF4 secretion when induced by ADP, adrenaline and collagen two hours after the drug was administered (Akman, 1980). 10 mg dipyridamole administered parenterally had the same effect. In normal human subjects, the release of βTG from PRP that was induced by stirring, was reduced by ingestion of aspirin (Santos et al., 1982).

Some membrane-active drugs, such as procaine and the anti-malarial drugs chloroquine, hydroxychloroquine, camoquine and quinacrine (mepacrine) prevent the alpha granule release reaction (Prowse et al., 1982).

Uptake of serotonin by platelets is competitively inhibited by chlorpheniramine maleate and release is enhanced by the drug (Cusimano & Sankar, 1977).

CHAPTER 2

The Isolation of Platelet Factor 4 (PF4) by Serotonin-Affinity Chromatography of Human Blood Platelet Extracts and Identification of PF4 by Radioimmunoassay

Introduction

Two techniques are available to find the hypothesized serotonin-binding protein in human platelets: 1) The solubilized platelet proteins can be incubated with radiolabelled serotonin and then filtered on a gel that separates the protein-radiolabelled serotonin complex from free radiolabelled serotonin on the basis of molecular mass; or 2) The solubilized platelet proteins can be chromatographed on an affinity gel that selectively binds molecules that bind serotonin.

The first technique can be used to demonstrate an association of radiolabelled serotonin with a macromolecule and indicate whether protein is present in the fractions that contain the macromolecule-radiolabelled serotonin complex. This technique also separates endogenous serotonin from the macromolecule, thereby increasing the binding of radiolabelled serotonin to the macromolecule. A disadvantage of this technique, however, is its lack of specificity -- that is, a number of macromolecules could elute in the same fractions with the macromolecule-radiolabelled serotonin complex. Thus, it might be difficult to separate the particular molecule that binds serotonin from other macromolecules.

The second technique is more specific, since only molecules that bind serotonin are retained by a serotonin-affinity gel. This technique also has disadvantages. These are: a) endogenous serotonin can occupy some binding sites on the binding molecule. This would preclude maximal binding of serotonin-binding molecules to the affinity gel; b) the alpha amino group of serotonin covalently bound to the gel is unavailable for binding to the macromolecule. Therefore, if the alpha amino group of serotonin were required by the serotonin-binding protein, no binding would occur to the affinity gel.

For either technique, the protein has to be soluble -- released from the granule in which it is stored, or solubilized from a membrane with which it is associated. A decision has to be taken whether to first release the protein from the platelet in order to separate it

immediately from unreleasable proteins. Subsequent isolation from unreleasable proteins might prove difficult. On the other hand, if the released protein became associated with the platelet membrane, however briefly, as some platelet releasable proteins do (O'Brien et al., 1970; O'Brien, 1971), changes in the protein's ability to bind serotonin might take place due to proteolysis or binding of ions. The protein could also be entrapped within the sticky coating of the platelet surface formed by all release inducers (Holmsen et al., 1969). These considerations imply that disruption of the unreleased platelet could result in a greater yield of serotonin-binding protein.

I. ISOLATION OF PF4 BY SEROTONIN-AFFINITY CHROMATOGRAPHY OF PLATELET EXTRACTS.

Section A. Preparation of the platelet pellet

The procedure followed to prepare the platelet pellet is shown in Fig. 2-1.

Normal human blood was obtained from the Grahamstown Blood Transfusion Service in Fenwal Blood Packs. The blood which varied from 16 hours to 22 days old had been refrigerated since it was collected. The Fenwal Blood Packs are anticoagulated with citric acid and sodium citrate in phosphate buffer. The blood packs were carried on ice to the laboratory where the volume of blood was measured in a plastic cylinder. The blood was centrifuged to precipitate red blood cells (RBC) in 250 ml polyethylene centrifuge bottles in an MSE High Speed 18 Centrifuge, or in a Sorvall Superspeed Centrifuge, at 160-360 g for 10-15 min at 4 ^{o}C (Colombani et al., 1971). The present study showed that greatest platelet yields were obtained when speed and time of centrifugation were carefully controlled (Haslam, 1964). Platelet yields were also greater with fresher blood, as predicted by the fact that human platelets have a lifetime in the peripheral circulation of about 7-14 days (Ebbe, 1971).

The platelet-rich plasma (PRP) was aspirated from above the red blood cell precipitate with a 6-8 cm length of plastic tubing fitted to a rubber teat. The intact platelets were not permitted to come into contact with glass until ultrasonication. Subsequent centrifugation

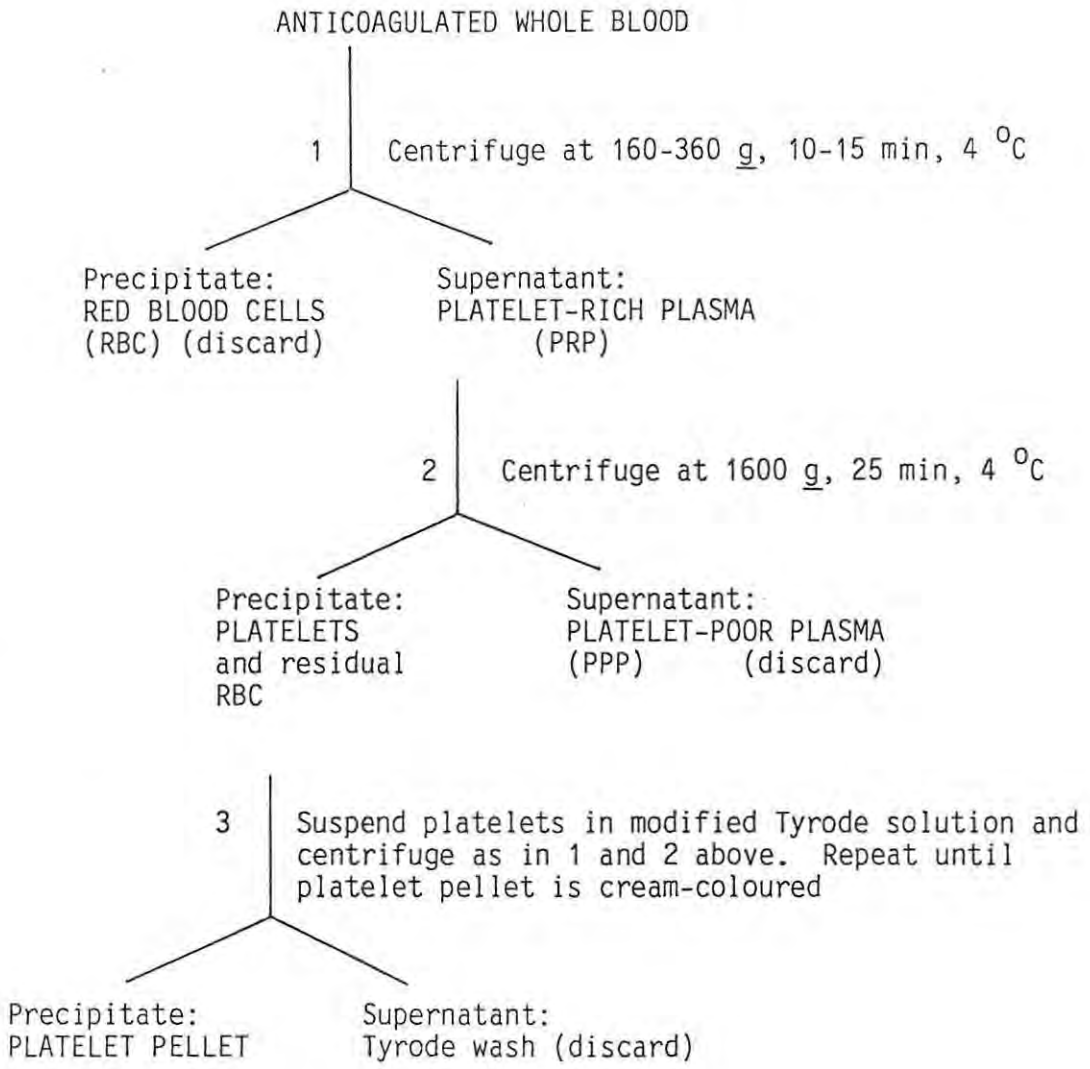

Fig. 2-1. Steps in platelet pellet preparation

depended on the colour of the PRP. If it was straw-coloured, the PRP was centrifuged at 1600-1700 g for 25 min at 4 °C to produce a platelet pellet (Colombani et al., 1971). If the PRP was reddish, it was centrifuged again under the initial RBC-precipitating conditions and then the supernatant was centrifuged to produce a platelet pellet. After the platelet pellet had been precipitated, the supernatant (platelet-poor plasma, PPP) was decanted.

The precipitated platelets were gently resuspended in modified Tyrode solution (Da Prada & Pletscher, 1968), which contained the following components (in g/l): NaCl - 7,60; KCl - 0,42; EDTA - 0,80; NaH_2PO_4-H_2O - 0,14; $NaHCO_3$ - 2,10; glucose - 2,00; and sucrose - 4,50. Freshly prepared modified Tyrode solution has a pH of 7,3, but the pH rises to 8,5 or higher when the solution has been refrigerated or frozen (-20 °C). Therefore, the pH of modified Tyrode solution was checked immediately before suspending platelets in it, and the pH was adjusted if necessary with 0,1 N HCl.

The resuspended platelets were recentrifuged at 120-270 g for 10 min at 4 °C, and aspirated before being reprecipitated at 1600-1700 g. Additional washes with modified Tyrode solution were performed until the final platelet pellet appeared cream-coloured or white. Electron micrographs of several of these platelet pellets demonstrated that the pellets were nearly homogeneous and the platelet membranes were intact (Fig. 2-2A).

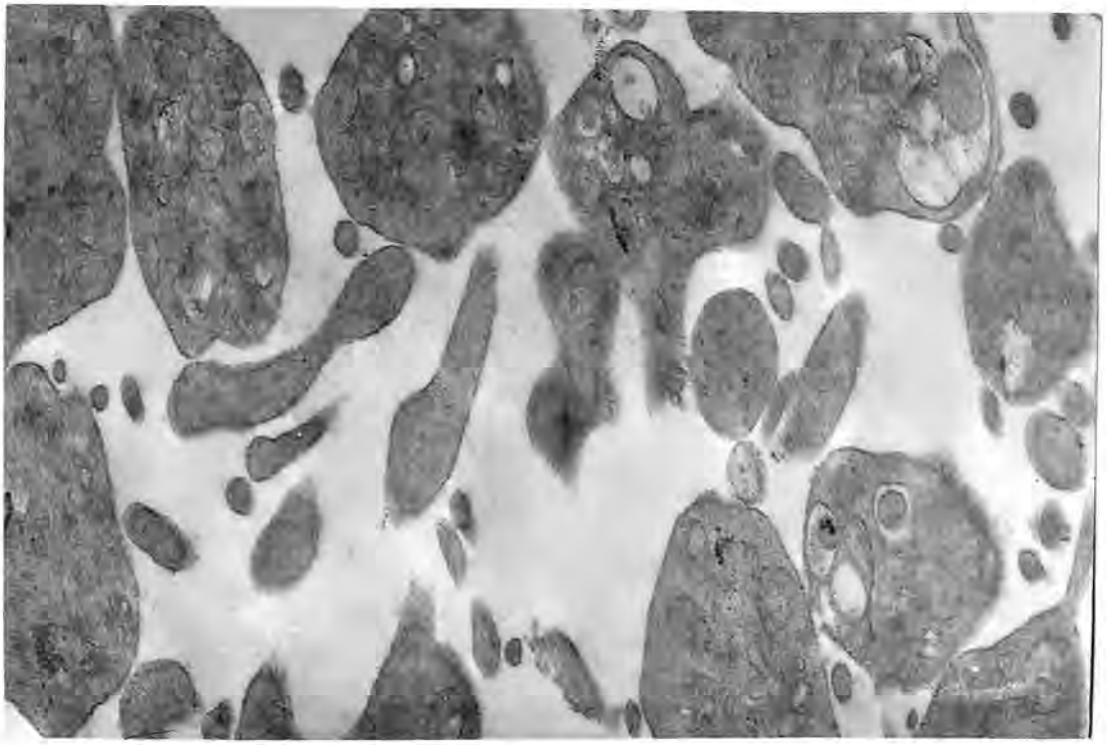

Fig. 2-2A. Electron micrograph of a platelet pellet. (Magnification: 29 750 x)

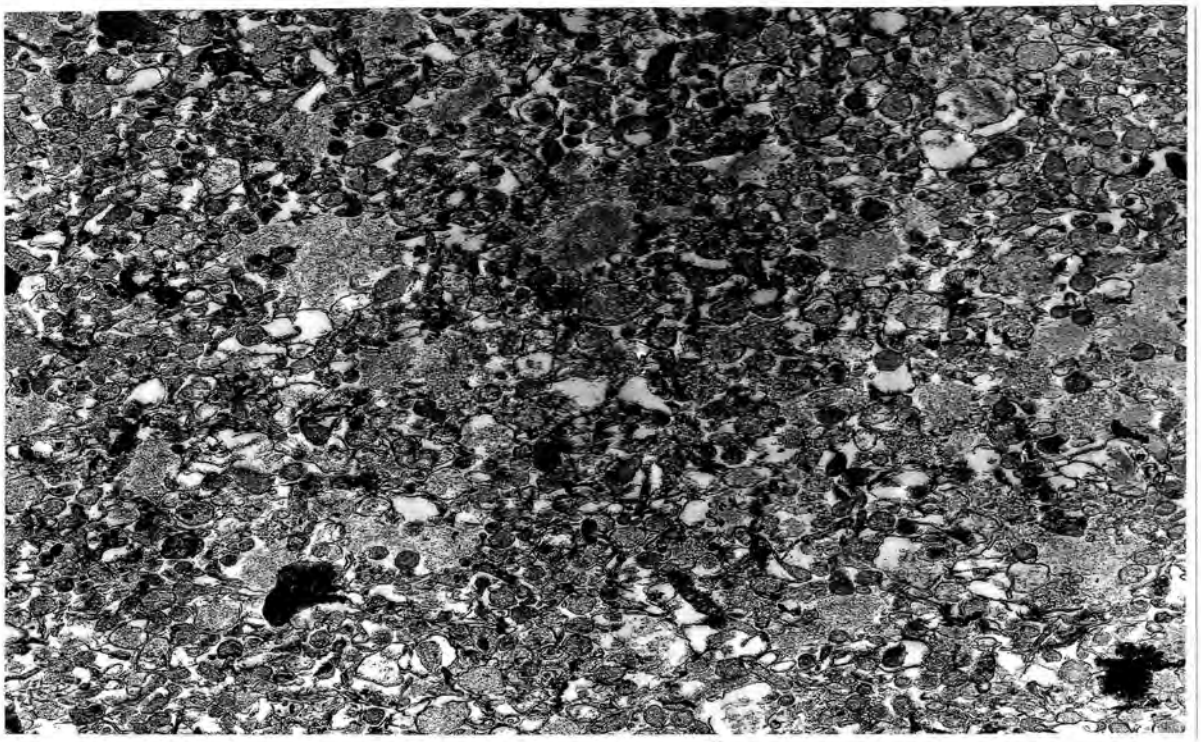

Fig. 2-2B. Electron micrograph of platelets that have been subjected to hypotonic lysis. (Magnification: 42 500 x)

Section B. Evidence that platelets contain a serotonin-binding macromolecule

Gel filtration of a solution of radiolabelled serotonin and platelet proteins (the first technique in this chapter's introduction) provided the evidence that human platelets contain a serotonin-binding macromolecule.

Platelets were prepared as described in Section A and the platelet pellet was covered with hypotonic phosphate buffer, pH 7,65 (see Fig. 2-2B, an electron micrograph of the platelet contents after exposure to the hypotonic solution). The platelets were then frozen in this solution at -20 ^{o}C, thawed and repeatedly frozen (a total of 5 times). The platelets were then subjected to maximum intensity ultrasonication, the vessel containing the platelet suspension being immersed in an icebath. Two-min cooling was allowed between 45 sec bursts of ultrasonication. After the third burst, the solution was centrifuged at 37 000 - 39 000 g for 25-30 min at 4 ^{o}C. Intact dense granules of platelets are known to be precipitated by centrifugation at 37 000 g for 20 min (Da Prada & Pletscher, 1968). This precipitate was discarded. The protein concentration of the supernatant was determined by a method described in Section C (below).

0,5 ml of the supernatant, containing 0,200 mg protein (Lowry) was incubated with 16,9 mg $[^{14}C]$-serotonin creatinine sulphate and 50 µl 170 µM ferrous ammonium sulphate at 37 ^{o}C for 15 min. The solution was then fractionated on a K9/15 Sephadex G-25 column which had been equilibrated with 66 mM KH_2PO_4-Na_2HPO_4 buffer (Sorensen's phosphate, hereafter referred to as "phosphate buffer", Diem, 1962), pH 7,65, in a cold room (2 ^{o}C) (Fig. 2-3). The peak appearing at the void volume contained about two-thirds (0,125 mg) of the protein applied to the column. The specific activity was 212 pmoles of $[^{14}C]$ -serotonin/mg protein, as compared with the specific activity determined for serotonin-binding protein in rat brain: 1-2 pmole/mg before and 77 pmole/mg protein after purification, respectively (Rotman, 1978).

These results indicate an association between $[^{14}C]$- serotonin and a macromolecule, because free $[^{14}C]$ -serotonin creatinine sulphate (M_r=407) has to be associated with a macromolecule in order to appear at the void volume of Sephadex G-25 (molecular exclusion limit M_r=5000). The experiment, however, does not identify the serotonin-binding macromolecule.

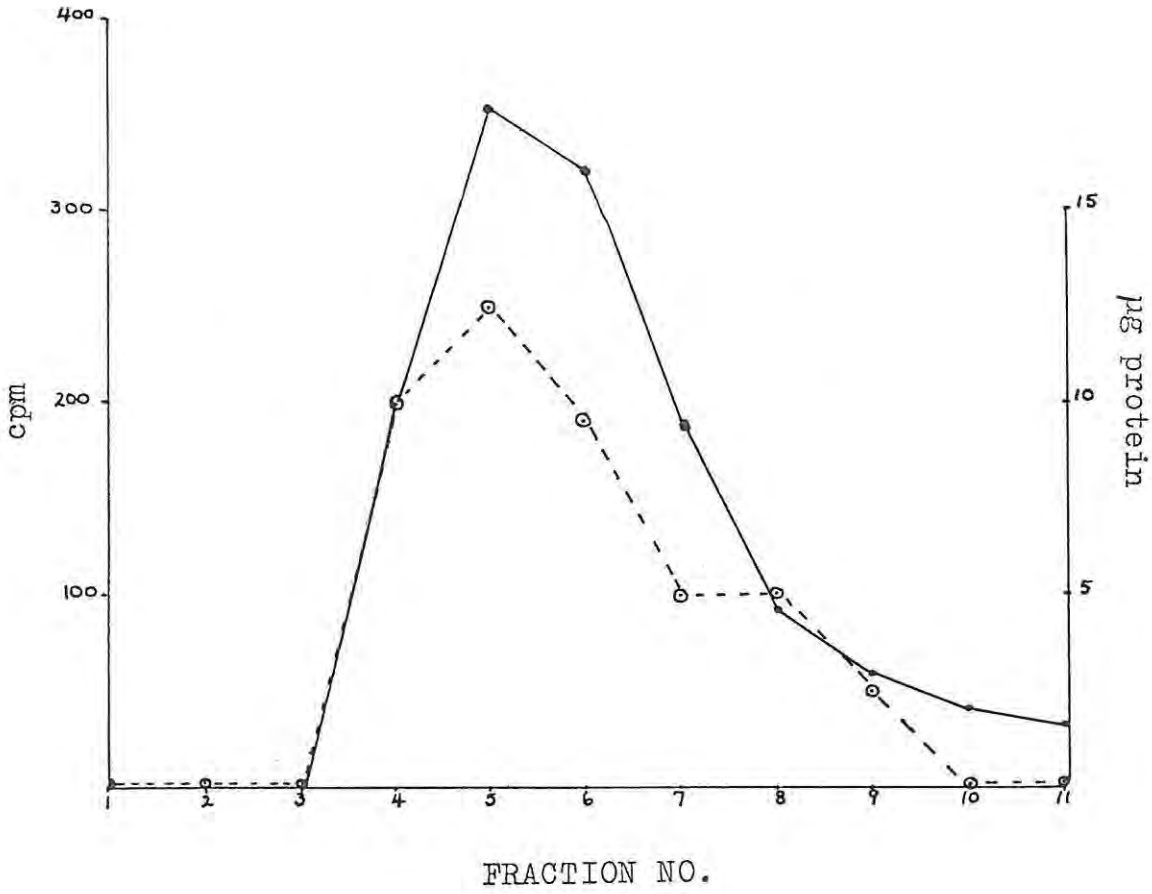

Fig. 2-3. Fractionation on Sephadex G-25 of 200 µg platelet protein after incubation with [14C]-serotonin. Void volume in fraction 4. See text for further explanation. Specific activity was 212 pmol serotonin/mg protein.
Solid line: Radioactivity in cpm/0,1 ml
Broken line: Protein (Lowry) in µg/0,1 ml

Section C. Choice of method of purification and of protein determination

The serotonin-binding molecule shown by the experiment described in Section B (above) to be present might be located in one of the following structures, if it is the hypothetical releasable protein:

1) In the dense granules. It is well known that the serotonin-ATP-Ca micelles in osmiophilic granules are non-diffusible and not hypertonic (Nogrady et al., 1972 and 1975). If this were the case, there would only be a very small amount of serotonin-binding protein, because it has been shown that the protein content of these granules is very low (Anderson et al., 1974).

2) In the alpha granules that undergo Release I, that is, release their contents simultaneously with dense granule release.

3) Associated with the platelet plasma membrane after release. It is known that heparin neutralizing activity (PF4) from the alpha granules (Da Prada et al., 1976), and perhaps other releasable proteins, might be associated briefly with the platelet plasma membrane after release (O'Brien et al., 1970; O'Brien, 1971). Three receptors for serotonin on rat blood platelet membranes are believed to be present under conditions that would cause platelet release (Drummond & Gordon, 1975).

Three possible methods are available to purify the serotonin-binding macromolecule: 1) Use of a release-inducer, followed by centrifugation to remove platelets and subsequent purification of the serotonin-binding macromolecule from the supernatant; 2) Sucrose gradient centrifugation to provide fractions containing specific granules or membrane vesicles, determination of the specific activity of each fraction, and subsequent purification of the serotonin-binding macromolecule from the appropriate fraction; or 3) Solubilization of all possible platelet proteins by means of the techniques used in Section B (above) followed by serotonin-affinity chromatography of the solution. This last method makes an assumption, later justified by empirical evidence, that the presence of many other solubilized substances does not hamper purification of the serotonin-binding protein, the hypothetical releasable protein.

Affinity chromatography is a highly selective technique that has been used to isolate serotonin-binding proteins from brain (Shih et al., 1974; Rotman, 1978). With this method, selective eluants can be

used, though the selection of a specific eluant is still largely a matter of trial and error, because the forces that maintain a ligand-binding protein complex on an affinity column include electrostatic interactions, hydrophobic effects and hydrogen bonding. Usually a change in pH or an increase in ionic strength brings about desorption of bound protein, although with stronger binding, chaotropic salts may be necessary to elute the bound materials. Selective eluants are more effective when binding affinities are relatively low (K_D 10^{-4} - 10^{-6} M) (Pharmacia, 1979). The eluant can be applied in a continuous gradient or step-wise. When substances are very tightly bound to the adsorbent, the flow may be stopped after applying eluant, allowing the bound materials to remain in the presence of the eluting agent before commencing elution; this allows dissociation to take place prior to elution.

Endogenous serotonin competes with the covalently bound serotonin for the serotonin-binding site on the macromolecule. Thus, gel filtration, by removing endogenous serotonin might increase the yield on the affinity column. For this reason gel filtration on Sephadex G-25 was retained as a purification step prior to affinity chromatography.

Sephadex G-25 Fine (Pharmacia) gel filtration was carried out in a column about 1 m long with an internal diameter of 2 cm (bed volume = 300 ml). The void volume was 103 ml, the void time 60 min. The gel was supported by a sintered glass base. The effluent was collected directly as it left the column. A nylon net was used to protect the top of the bed during sample application. A separatory funnel was used as a reservoir and was connected to the top of the column with a rubber stopper. The gel was swollen and prepared according to the manufacturer's instructions. The 66 mM phosphate buffer, pH 7,65, contained 0,02 per cent NaN_3 (sodium azide) as bacteriostat. The stock solutions of sodium and potassium phosphate were refrigerated. The pH was checked on a E350B Metrohm Herisau meter calibrated with Merck Titrasol buffer, pH 6,0 $\pm$0,02. Although the flow rate (1,7-1,8 ml/min) was high, good resolution was not necessary as only the void volume peak was collected. The flow rate remained constant over several months and no clogging of the sintered glass base was experienced. Chromatography was carried out at ambient temperature (16-25 ^{o}C). Periodic filtration of a 0,2 per cent blue dextran solution revealed no irregularities in the gel bed in spite of the temperature

fluctuations. After 15 experiments, this column was moved to a 2 ^{o}C room and repacked with the same G-25 gel. Although the void volume was still 103 ml, the void time increased to 93-100 min. But working at 2 ^{o}C as opposed to ambient temperature produced no differences in chromatographic results. Free serotonin was in fractions collected after more than three void volumes. Three min fractions were collected during the void volume peak.

Samples applied to the Sephadex G-25 column were 3,9-10,4 ml, in accordance with the manufacturers recommendations that the sample volume be within 1-5 per cent of the bed volume. The applied sample contained as much as 23 mg protein. 60-88 per cent of protein applied to the column was recovered in the void volume peak. The void volume peak was sharp and the absorbance at 280 nm of void volume peak fractions occasionally exceeded 1. Fig. 2-4 represents a typical fractionation on Sephadex G-25.

Fluorescence, a technique that has been recommended because of its sensitivity and specificity (Udenfriend, 1969, p. 249), was used to detect the presence of native proteins in the void volume peak of the effluent from the column. With an excitation wavelength of 295 nm and analyzer wavelength of 335 nm, an emission intensity (E.I. 295/335) of 110 was obtained for fractions with A_{280} as low as 0,16; the emission intensity of fractions on either side of the peak was 4 or less (see Fig. 2-4). Fluorescence readings were made on a Perkin Elmer Fluorescence Spectrophotometer 203 with a Perkin Elmer 150 Xenon Power Supply using Hellma quartz cells with a 1-cm path length and five optical windows. Although the sources of fluorescence were not determined, most fluorescence was probably due to intrinsic fluorescent chromophores in proteins, that is, the aromatic amino acids tryptophan and tyrosine. Tryptophan, in particular, has its maximum emission at 348 nm in neutral aqueous solution (Udenfriend, 1969, p. 229). Although free serotonin would not be found in the fractions eluting just after the void volume of a G-25 column, bound serotonin could possibly contribute fluorescence, since serotonin in aqueous solution activated at 300 nm emits fluorescence at 338 nm (García-Castiñeiras et al., 1977). The phosphate buffer used on the G-25 column had E.I. 295/335 = 0 and A_{280} = 0,005.

The fractions constituting the void volume peak were refrigerated until subsequent dialysis for affinity chromatography. It was necessary to dialyse the solution because the starting buffer for

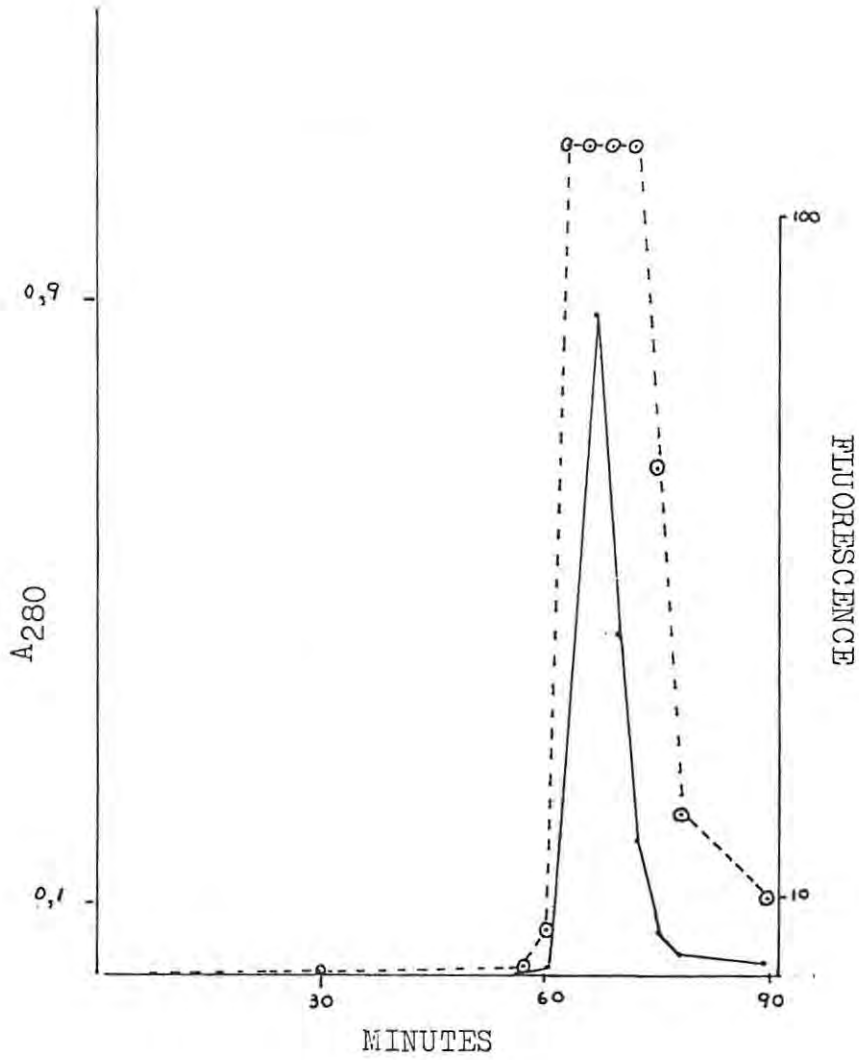

Fig. 2-4. Void volume peak of Sephadex G-25 fractionation of 39 000 g supernatant containing 11,7 mg protein/3,9 ml. The column was equilibrated with 66 mM phosphate buffer, pH 7,65, containing 0,02% NaN_3. 9,99 mg protein (85% of the applied protein) was eluted in the void volume peak.
Solid line: A_{280}
Broken line: Fluorescence at 335 nm in arbitrary units. Excitation at 295 nm.

affinity chromatography usually differed in pH and ionic strength from the buffer used on the G-25 column. Moreover, these fractions were in buffer containing sodium azide. Sodium azide forms a precipitate with the ferrous sulphate constituent of two starting buffers used. Thus, the sample was dialysed first against starting buffer lacking ferrous sulphate if affinity chromatography was to be carried out with starting buffer that contained ferrous sulphate. Dialysis was carried out overnight at 4-8 ^{o}C in small diameter dialysis tubing against a volume ten to fifty times that of the sample.

Three other methods of protein determination were used in the course of this study in addition to absorbance at 280 nm: 1) Lowry et al., 1951; 2) Plummer, 1971; and 3) Hartree, 1972. (Standard curves for all three methods are shown in Fig. 2-5). The Plummer and Hartree methods are modifications of the Lowry method. Unfortunately, all three methods are destructive.

Initial protein measurements were made according to the Plummer method, which uses Folin-Ciocalteau reagent diluted 1:1 with water before use (instead of dilution to 1 N based on titration, as in the Lowry method). The Lowry method, which can measure accurately as little as 12,5 µg protein, was used later when greater sensitivity was required.

The specifically bound protein from affinity chromatography is consistently associated with carbohydrate material, and because the Hartree method is designed to determine accurately concentrations of protein associated with carbohydrate, in particular, glycosaminoglycans, it is the preferred method. In fact, the Hartree method was used exclusively after the association of carbohydrate material with the protein was noted. A disadvantage of the Hartree method is that the samples must be heated twice during the course of a determination; the Lowry and Plummer methods do not require heating.

The Folin-Ciocalteau reagent must be added rapidly in all three methods. A Whirlimixer (Fisons, England) was used to ensure rapid mixing.

Neither the Plummer nor the Lowry methods was strictly linear over the specified range of concentrations (see Fig. 2-5A and B). The Hartree method did, however, give a linear photometric response from 20-140 µg (Fig. 2-5C). These standard curves were obtained using commercial bovine serum albumin. A standard was determined with every group of unknowns. Hartree determinations were reproducible even at

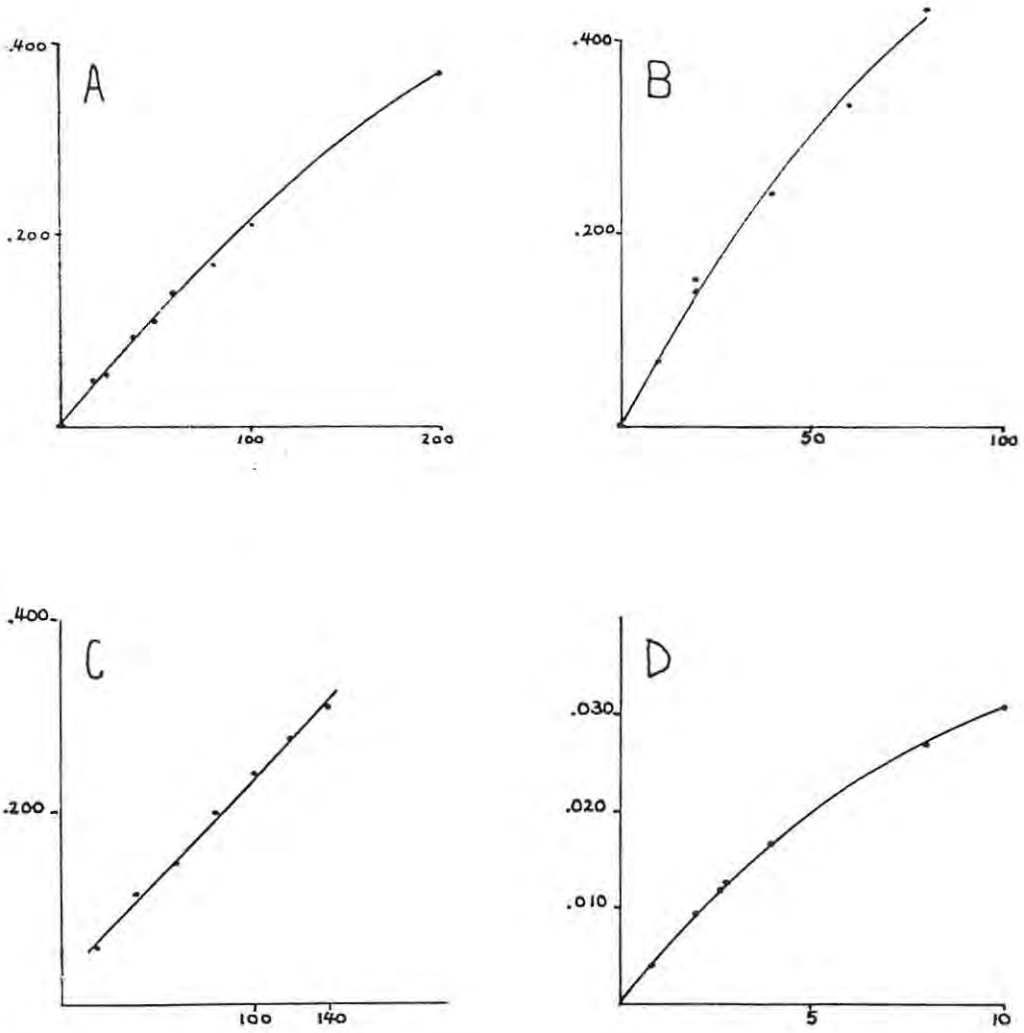

Fig. 2-5. Standard curves for three methods of protein determination.

Ordinate, A_{750}. Abscissa, µg protein.

A. Plummer. B. Lowry C. & D. Hartree.

protein concentrations as low as 0-10 µg, although over this concentration range a linear response was not obtained (Fig. 2-5D). Spectrophotometric readings for all three methods were made on a Beckman DB or Model 25 Spectrophotometer at 750 nm.

Many eluting buffers contain compounds (such as serotonin, Tris, EDTA, urea, dithiothreitol and ammonium sulphate) which react with the Folin-Ciocalteau reagent. Although not an unusual problem, it makes it necessary to subtract the contribution of these substances from the absorbance in the colorimetric test to obtain the absorbance due to the sample protein. Simultaneous analyses of the eluting buffer were used to measure interference from these sources.

The protein content of the 37 000 - 39 000 g supernatants depended on platelet concentration and the volume of hypotonic buffer added to the platelet pellet. Protein content varied from 0,15 to 5,7 mg/ml.

Section D. Serotonin-affinity chromatography

Section D.1. Preparation and regeneration of gels; buffers; application of samples; methods for eluting bound protein.

In serotonin-affinity chromatography, serotonin is covalently coupled to a Sepharose gel. Thus, when a sample is applied to the affinity column, substances which bind serotonin will be selectively bound to the gel, while other substances are eluted. Substances that have been bound to the gel are removed by changing pH, ionic strength, or incorporating a competitor in the eluting buffer. Affinity chromatography gives high purification factors.

Serotonin hydrochloride was used for coupling. Serotonin was coupled covalently to the Activated CH-Sepharose 4B gel (obtained from Pharmacia) according to the manufacturer's instructions. Serotonin becomes linked to the Sepharose by its amino group. Activated CH-Sepharose 4B consists of the N-hydroxysuccinimide ester of the carboxyl group of CH-Sepharose 4B. When the ester is attacked by the unprotonated form of serotonin, the N-hydroxysuccinimide group leaves the gel and a stable serotonin amide linkage is formed. Thus, the amino group of serotonin is unavailable for binding to a serotonin-binding macromolecule. Formate or acetate buffer at pH 4 was used for storing the coupled gel since both Sepharose and serotonin are most

stable at pH 2-6 and storage conditions for the prepared gel depend on the properties of the ligand (Pharmacia, 1979, p. 27).

Activated CH-Sepharose 4B has the capacity to couple 5-7 µmol glycylleucine, or alpha amino groups, per ml swollen gel, and, according to instructions in the Pharmacia handbooks, the ligand should be at a concentration several fold in excess of the concentration of active ester groups. Initial experiments in this investigation were thus done with a gel that had been prepared in the presence of 33 µmol serotonin/ml gel. Columns 1,2 and 5 were packed with this gel, and up to 84 per cent of applied protein was bound.

In subsequent experiments, low concentration serotonin-affinity gel was prepared by use of 2,4 µmol serotonin per ml gel[1]. This gel was used for packing columns 4 and 6. On these columns 34 per cent or less of applied protein was bound (see Table 2-1). Later a 2,0 µmol serotonin per ml gel ratio was used for preparing columns 7 and 8 and about the same amount of binding occurred on these columns. Column 3, which was a control column, was prepared with ethanolamine instead of with serotonin.

The prepared affinity gels and columns were wrapped with aluminium foil because serotonin is light-sensitive.

According to the manufacturer's instructions, affinity gels were regenerated after each use with ten column volumes of 0,1 M Tris-HCl buffer containing 0,5 M NaCl, pH 8,5, and then with ten column volumes of 0,1 M sodium acetate or sodium formate buffer containing 0,5 M NaCl, pH 4,5, before re-equilibrating the column with starting buffer.

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[1] Pharmacia's 1979 handbook, which arrived several months after the early experiments, cautions against high concentrations of ligand: "A high concentration of coupled ligand is likely to increase the strength of binding, steric hindrance and nonspecific binding....For most adsorbents 1-20 µmol ligand per ml gel is suitable. Two µmol per ml gel is most widely used" (Pharmacia, 1979, p. 84).

Table 2-1. Experiments 1-18 on serotonin-affinity gels*

EXP. NO.	SAMPLE PROTEIN MG.	SAMPLE VOL. ML.	COLUMN NO.	COLUMN GEL	STARTING BUFFER Fe	STARTING BUFFER COMP	STARTING BUFFER pH	% PROTEIN BOUND	REMARKS
1	3,7	4,2	2	33	+	Phos	7,3	79	
2	1,1	3,7	2	33	+	Phos	7,3	84	Same sample as in Exp. 5
3	1,9	4,7	1	33	+	Phos	7,3	70	
4	2,2	3,7	2	33	-	Phos	7,3	49	
5	1,3	4,9	2	33	-	Phos	7,3	68	Same sample as in Exp. 2
6	1,4	3,9	1	33	-	Phos	7,3	17	Concentrated in Minicon B15
7	3,0	6,4	2	33	+	Phos	7,3	70	52% ammonium sulphate ppt.
8	1,4	1,4	2	33	+	Phos	7,3	78	" " " "
9	8,1	4,9	4	2,4	-	Phos	7,25	0	See text
10	7,2	7,6	5	33	-	Phos	7,25	32	Pooled Exp. 9 fractions
11	8,6	4,5	6	2,4	-	Phos	7,25	17	
12	3,4	4,4	6	2,4	+	Phos	7,3	23	
13	5,6	7,7	6	2,4	-	Phos	7,25	14	45 min dialysis
14	4,7	7,0	6	2,4	-	Phos	7,25	34	5 h dialysis
15	0,54	0,1	7	2,0	+	Cac	6,5	54	
16	1,11	0,2	8	2,0	-	Cac	6,5	21	
17	0,96	0,2	8	2,0	-	Cac	6,2	17	
18	0,53	0,2	8	2,0	-	Phos	6,9	34	No NaCl in starting buffer

*Abbreviations: EXP. NO., experiment number; GEL, μmol serotonin covalently bound per ml gel; Fe, ferrous ion; COMP, composition; Phos, phosphate buffer; Cac, cacodylate buffer.

The protein which binds to the immobilized ligand is dissolved in two different solutions in the course of an affinity chromatographic experiment. The first solution enhances binding to the ligand and the second desorbs the substance from the ligand. The first solution is termed the "starting buffer", and the second, the "eluting buffer". Both buffers must be compatible with the chemical stability of the gel, the ligand, and adsorbed substances. Since the starting buffer enhances binding of serotonin to the relevant proteins, starting buffer cannot be used with Sephadex G-25 chromatography to separate the protein from endogenous serotonin.

The first starting buffer used in the present study consisted of 0,02 M phosphate buffer, pH 7,3, 10^{-4} M $FeSO_4$, with the addition of 0,5 M NaCl as recommended by Pharmacia for use on affinity columns. This starting buffer was used because Tamir et al. (1976) reported that 10^{-4} M Fe^{2+} enhanced binding of serotonin by a protein from rat brain synaptosomes. After three experiments in which bound protein could not be desorbed from the affinity columns despite the application of several eluting buffers, a phosphate starting buffer, also of pH 7,3, that did not contain Fe^{2+} was used. On affinity gels containing 2 µmol serotonin per ml gel a starting buffer consisting of 0,02 M cacodylic acid, 10^{-4} M $FeSO_4$, 0,5 M NaCl, pH 6,2-6,9, was used. This buffer was superior in two respects to the phosphate-Fe^{2+} buffer used previously: Whereas the A_{280} of the earlier starting buffer was 0,45-0,47, this buffer had a lower value of A_{280}, 0,28. This facilitated spectrophotometric monitoring of the elution of unbound protein. The cacodylate buffer also remained clear under all conditions, whereas the earlier buffer needed on several occasions to be filtered.

Before either the application of samples or the elution of bound protein, the affinity columns were washed with buffer for several hours, until the effluent stabilized at the A_{280} of the starting buffer. In spite of washing the prepared gel by the procedure recommended by Pharmacia, some serotonin was detected in the effluent the first time a gel was used. Washing was continued until serotonin could not be detected in the effluent from the column. Serotonin can be detected by its A_{275} (Chen, R.F., 1968) and fluorometrically at the same excitation and analyzer wavelengths as in the case of proteins (i.e., excitation 270-295 nm, analyzer 330-350 nm) in neutral solution (ibid.) or at 550 nm in 3 N HCl (Bogdanski et al., 1956).

Affinity chromatography was performed in K9/15 (Pharmacia) columns

with the column half-filled (bed volume about 4,5 ml). An R9 Pharmacia reservoir (capacity 40 ml) held the starting or eluting buffer and was used as a Mariotte flask to maintain a constant flow rate of about 24 ml/h during sample application and elution of unbound protein and 6 ml/h during elution of bound protein. An LUV monitor recorded A_{280} of the effluent.

After sample application an A_{280} peak of unbound proteins was eluted. Then gradually the A_{280} returned to the starting buffer value. Starting buffer was continuously applied to the column after sample application until at least ten column-volumes had passed through to carry off unbound substances. The fractions representing unbound material were analyzed for protein concentration. The amount of bound protein was determined by subtracting the unbound protein from protein initially present in the sample. Unbound protein was usually eluted in a peak which began 5-8 ml after sample application and ended about 9 ml later. The results of affinity chromatographic experiments are presented in Table 2-1.

An indication that the protein was strongly bound was that volume of sample applied to the affinity columns bore no relationship to the amount of protein which bound even in volumes greatly exceeding 5 per cent of the bed volume (Pharmacia, 1979) (see Fig. 2-6). Weakly bound substances may be co-eluted with unbound substances when applied in volumes greater than 5 per cent of the bed volume (0,25 ml in these experiments) (ibid.).

The amount of protein bound also bore no relationship to the time elapsing between elution of the sample from the Sephadex G-25 column and its application to the affinity column (Fig. 2-7). Although after ultrasonication and centrifugation, the 37 000 - 39 000 *g* supernatant showed signs of precipitation (strands that appeared to be denatured protein) within minutes after removal from the centrifuge even when the supernatant was kept on ice, the Sephadex G-25 void volume fractions could be kept for days in the refrigerator with no sign of denaturation. This observation supports a report that proteinases in the platelet frozen-thawed supernatant cause proteolytic denaturation of the extract (Nachman, 1975). Proteases capable of acting at neutral pH on endogenous human platelet proteins have been reported (Tsujinaka *et al.*, 1982). Stabilization of the platelet extract was not achieved by dialyzing the 37 000 - 39 000 *g* supernatant against the same buffer and obvious signs of protein precipitation within the

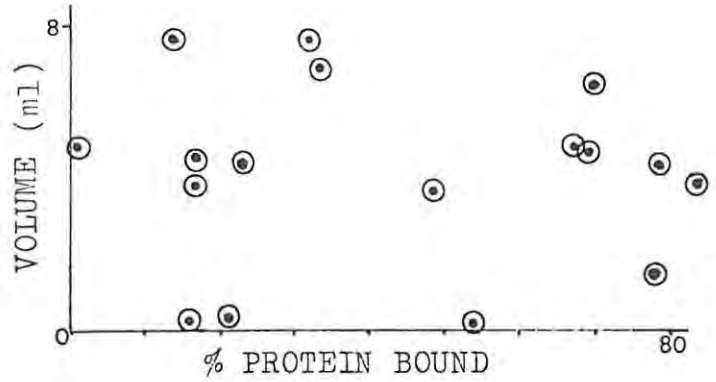


Fig. 2-6. Sample volume applied to affinity columns plotted against protein bound. The bed volume of the columns was 5 ml or less (5% of the bed volume = 0,25 ml or less).

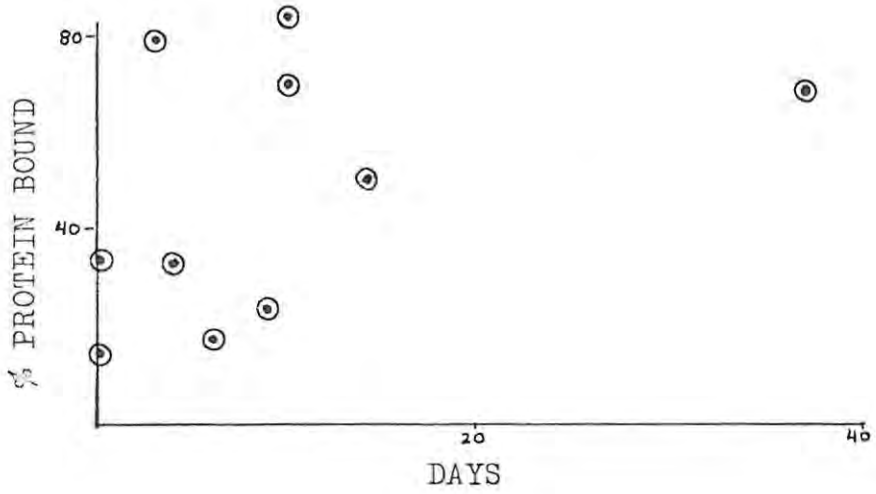


Fig. 2-7. Time since the affinity chromatography sample had been eluted from a Sephadex G-25 column plotted against per cent of protein bound to affinity columns. See text for explanation.

dialysis membrane occurred rapidly. Thus, stabilization brought about by filtration on Sephadex G-25 may have been due to separation of an essential cofactor from the proteinases. The results thus indicated that the serotonin-binding protein retained its binding properties for more than a week at 4-8 ^{o}C in 66 mM phosphate buffer containing 0,02 per cent NaN_3 at pH 7,65.

The two factors that do affect the percentage of binding obtained on the affinity columns are: 1) µmol serotonin/ml gel and 2) presence of Fe^{2+} in the starting buffer. These effects are shown in Fig. 2-8 and Table 2-2.

In order to elute the protein from the serotonin-affinity column, a continuous gradient of 0,02 M Tris-HCl, 1 M NaCl, pH 8,1 containing 0,02 per cent NaN_3 was tried. However, this eluant removed less than 2 per cent of bound protein. The same column with its bound protein was then subjected to elution by a buffer which had been used successfully by Rotman for eluting rat brain synaptosomal serotonin-binding protein (Rotman, 1978) consisting of 0,02 M Tris-HCl, 10^{-3} M $CaCl_2$, pH 8,1. In the present investigation Rotman's buffer also did not remove serotonin-binding protein. A buffer consisting of 0,02 M phosphate buffer, 0,4 M NaCl, 3 x 10^{-5}M serotonin, pH 7,35, suggested by Titeler (personal communication) was tried. Neither this buffer nor several further attempts with Rotman's buffer were successful in removing bound platelet protein from the affinity columns. Further experiments were carried out with a starting buffer not containing Fe^{2+}. An alternative technique to find an effective eluting buffer without having to resort to column elution was adopted. After experiment 5 in which 900 µg protein bound to the column (see Table 2-1), the gel was removed from the column and divided into 15 aliquots. These aliquots were placed in small test-tubes and shaken gently with 2 ml of the different eluants. The buffers used in this experiment are listed in Table 2-3 marked with the symbol ⊠. The tubes were then refrigerated for 18 h, shaken again, and the gel allowed to settle. The supernatants were aspirated from each tube without disturbing the gel, and the solutions were dialysed for four hours against dilute phosphate buffer at pH 7,7. Lowry determinations were done on the dialysed samples. The Lowry method showed no desorption of bound protein by any of these eluants. Some of these buffers were also used for gradient elution on the affinity columns. The Lowry method was also used to test for the presence of protein in the eluate. All of the

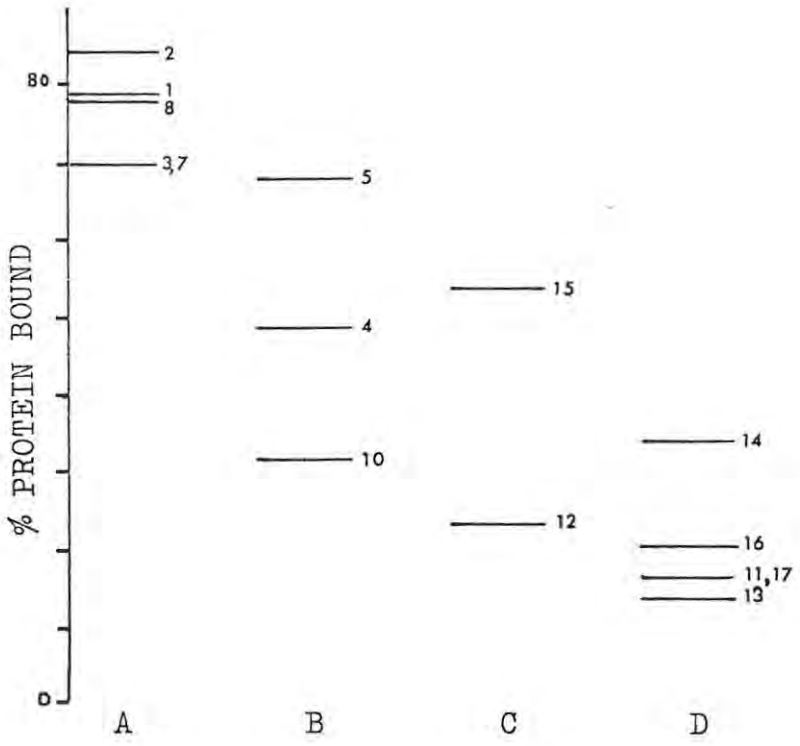


Fig. 2-8. The dependence of platelet protein binding to gel on the presence of 10^{-4} M Fe^{2+} and the concentration of serotonin which is covalently bound to the affinity gel

A - 33 μmol serotonin/ml gel, Fe^{2+} present

B - 33 μmol serotonin/ml gel, Fe^{2+} absent

C - 2 or 2,4 μmol serotonin/ml gel, Fe^{2+} present

D - 2 or 2,4 μmol serotonin/ml gel, Fe^{2+} absent

The affinity chromatographic experiment numbers are written to the right of each result.

Table 2-2. Effect of 10^{-4} M Fe^{2+} on binding to affinity columns

% Protein Bound with Fe	% Protein Bound without Fe	Remarks
84(2)*	68(5)	The only difference between these two experiments was the inclusion of Fe^{2+} in the starting buffer.
23(12)	17(11) 14(13)	All three experiments were done on the same column packed with the same gel
79(1) 78(8) 70(3,7)	49(4) 32(10)	All six experiments employed 33 µmol/ml gel.
54(15)	21(16)	Both experiments employed 2,0 µmol/ml gel.

* The experiment number is in parentheses.

Table 2-3. Eluting Buffers

Composition		pH
0,02 M Tris-HCl, 1 M NaCl, 0,02% NaN_3	⊠	8,1
0,02 M Tris-HCl, 10^{-3} M $CaCl_2$		8,1
0,02 M Tris-HCl, 10^{-3} M $CaCl_2$, 1 M NaCl		8,1
0,1 M Tris-HCl, 10^{-3} M $CaCl_2$, 10^{-4} M ATP	⊠	8,0
0,1 M Tris-HCl, 10^{-2} M $CaCl_2$	⊠	7,95
0,1 M Tris-HCl, 10^{-3} M $CaCl_2$	⊠	7,95
0,1 M Tris-HCl, 10^{-4} M $CaCl_2$	⊠	7,95
0,1 M Tris-HCl, 10^{-4} M ATP	⊠	7,9
0,1 M Tris-HCl, 10^{-4} M ADP, 3 x 10^{-4} M ATP, 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, 10^{-7} M serotonin	⊠	7,9
0,06 M phosphate, 10^{-6} M dithiothreitol, 10^{-7} M serotonin	⊠	7,5
0,06 M phosphate, 10^{-6} M dithiothreitol, 10^{-3} M $CaCl_2$, 2 x 10^{-4} M ATP	⊠	7,45
0,06 M phosphate, 10^{-3} M $CaCl_2$, 10^{-4} M ADP	⊠	7,45
0,06 M phosphate, 10^{-3} M $CaCl_2$, 10^{-4} M ATP	⊠	7,45
0,06 M phosphate, 10^{-4} M ADP, 3 x 10^{-4} M ATP, 10^{-4} M $CaCl_2$, 10^{-4} M $MgCl_2$, 10^{-7} M 5-HT	⊠	7,45
0,06 M phosphate, 10^{-4} M ATP	⊠	7,45
Hanks solution (Merchant et al., 1964)		7,45
0,02 M phosphate, 0,38 M NaCl, 3 x 10^{-5} M 5-HT·HCl	⊠	7,35
0,06 M phosphate, 1,5 x 10^{-3} M $CaCl_2$	⊠	7,35
0,06 M phosphate, 10^{-4} M $CaCl_2$		7,35
0,06 M phosphate, 10^{-3} M $CaCl_2$		7,35
0,02 M phosphate, 0,38 M NaCl, 10^{-4} M reserpine		7,3
8 M urea		7,1
20% saturated ammonium sulphate in phosphate buffer		7,0
3 M KI neutralized with 20% trichloroacetic acid		7,0
0,02 M phosphate, 0,38 M NaCl, 10^{-2} M 5-HT creatinine sulphate		6,45
0,15 M KI, 0,5 M NaCl, 0,02 M trisodium citrate		5,4
0,1 M glycine-HCl		2,6
1 M acetic acid		2,3
5% EDTA		4,5

⊠ see text for explanation

buffers tried failed to desorb bound protein. Eluting buffers that were tried included 10^{-4} - 10^{-2} M Ca^{2+} in neutral phosphate or Tris-HCl buffers with and without ATP and ADP.

The procedure for removing the bound protein from the affinity columns is discussed in Section D.2 below.

Affinity chromatography was also carried out on the precipitates from ammonium sulphate precipitation (52 per cent saturated) of the supernatants resulting from 37 000 - 39 000 g centrifugation, followed by dialysis of the precipitate (without molecular exclusion chromatography). The results of these experiments were similar to those obtained with samples from the void volume of G-25 columns (Table 2-4). Tamir's group (1980a) had used ammonium sulphate precipitation to collect rat platelet serotonin-binding proteins. In their study, the fractions precipitating between 40 and 75 per cent saturation contained the proteins of interest. In the present study it was noted that some of the ammonium sulphate precipitate did not dissolve when dialysed against water, or dilute phosphate buffer, or starting buffer with Fe^{2+} even though dialysis was continued for more than 30 h. The supernatant (which was used for affinity chromatography experiment number 7) could be easily decanted because the precipitate was gel-like, sticky and attached to the wall of the centrifuge tube. This precipitate was later salted back into solution by the dropwise addition of neutralized saturated ammonium sulphate, and was dissolved at about 10 per cent saturation. It was then dialyzed against starting buffer containing 0,3 x 10^{-4} M Fe^{2+} and chromatographed on the same affinity column (experiment number 8); 78 per cent of the applied protein bound to the column.

A control gel with ethanolamine instead of serotonin was prepared from Activated CH-Sepharose 4B (Pharmacia) by the usual procedure. All the active groups on the gel were blocked by treating with ethanolamine (1 M, pH 9) for 1 h, following the procedure recommended by Pharmacia (1979, p. 27). Column 3, the control column, was packed with this gel and equilibrated with starting buffer containing Fe^{2+}. The sample of platelet extract applied to this column had been previously precipitated by ammonium sulphate, redissolved and dialysed in starting buffer with Fe^{2+}. It contained 480 µg protein (Lowry method) of which 356 µg (74 per cent) was not bound by the affinity gel but was still emerging when the column was turned off after fraction 13. Some of the same solution, also dialysed in starting buffer with Fe^{2+}, and

Table 2-4. Comparison of binding on affinity columns* by samples resulting from ammonium sulphate precipitation or G-25 chromatography.

Experiment Number	Sample	% Protein Bound
1	Void volume of G-25	79
2	"	84
3	"	70
7	Ammonium sulphate ppt.	70
8	"	78

* Affinity chromatography on 33 µmol serotonin/ml gel columns with 0,02 M phosphate starting buffer containing 10^{-4} M Fe^{2+}, 1 M NaCl, pH 7,3.

Table 2-5. Serotonin-affinity chromatography of bovine serum albumin (Sigma) on 33 µmol serotonin/ml gel columns.

	% Protein Bound
Starting Buffer without Fe^{2+}	10
Starting buffer with Fe^{2+}	0

containing 600 µg protein (Lowry method), was applied to a 33 µmol/ml gel serotonin-affinity column; less than 1 per cent of this protein eluted as unbound protein. The flow rate of the serotonin-affinity column decreased to less than 4,0 ml/h with application of the sample. The flow rate of the control column did not change from 18 ml/h.

The binding of purified bovine serum albumin (Sigma) to serotonin-affinity gel was examined in two starting buffers. In each case 2,4 mg in 4,0 ml was chromatographed in phosphate buffer at pH 7,3 on the 33 µmol serotonin/ml gel under the same conditions as in experiments 1-8 (Table 2-1). The results obtained are recorded in Table 2-5. Experiment 9 was carried out using a 2,4 µmol serotonin/ml gel column with a starting buffer containing 13 mM phosphate, 0,5 M NaCl, and 0,02 per cent NaN_3 at pH 7,25. The column was filled about one-third full and was washed overnight with starting buffer. Air bubbles were discovered in the gel near the bottom of the column as the sample was being applied; however, since sample application had begun, fractions were collected in the usual manner. None of the protein bound. Since this column was short, it is possible that the bubbles were an indication of gross disturbances in the gel bed, including channel formation. The protein-containing fractions from experiment 9 were pooled and reapplied to a 33 µmol serotonin/ml gel column with the same starting buffer as in experiment 9. In this case, 32 per cent binding was obtained (experiment 10).

Although protein collected in experiment 9 was exposed to room temperature (above 18 ^{o}C) for at least two hours during fractionation, it still bound to an affinity column the next day. This suggests that the serotonin-binding protein is stable.

Elution of the serotonin-binding protein in experiment 10 was attempted with an eluting buffer containing 0,15 M KI (a chaotrope recommended by Pharmacia for difficult elution problems), 0,5 M NaCl and 0,017 M trisodium citrate, at pH 5,4. After applying the eluting buffer, flow was stopped and the adsorbed protein was allowed to remain in the presence of the eluting buffer for 1 h. Then elution was begun at a very slow flow rate. However, no bound protein was eluted.

A different eluting buffer was used on the same column the following day. This contained 0,1 M glycine-HCl, pH 2,6. One eluted fraction contained about 0,1 mg protein, which represented about 4 per cent of the bound protein. This was also the first bound protein eluted.

On the same column 8 M urea, pH 7,1 and 8 M urea containing 10^{-6} M dithiothreitol were subsequently applied, but these eluants also did not remove bound protein.

The 2,4 µmol/ml gel used in column 4 and which gave anomalous results (experiment 9) was used again in column 6 along with more 2,4 µmol serotonin/ml gel that had been prepared at the same time but not used in column 4. It was found that column 6 consistently bound protein (see Table 2-1). The same starting buffer was used in experiments 9, 10, 11, 13 and 14. Experiments 11-14 were carried out using column 6. The percentage of applied protein bound on column 6 was 14-34 per cent, considerably less than the binding obtained in the experiments using 33 µmol serotonin/ml gel. The difference between percentage binding on the 2,4 and on the 33 µmol serotonin/ml gel columns may represent non-specific binding, even though the starting buffers contained 0,4 M or more NaCl. High concentrations of NaCl usually minimize non-specific adsorption of polyelectrolytes to charged groups (Pharmacia, 1977, p. 55).

To summarize, in this section (D.1) binding of platelet protein(s) to serotonin affinity gels has been described. It has been shown that the percentage of applied protein bound to the gel was a function of both the concentration of serotonin covalently bound per ml gel and the presence of Fe^{2+} in the starting buffer. The percentage of applied protein bound was, however, affected neither by the volume of sample applied to the affinity columns nor by exposure of the G-25 eluant to ambient temperature for several hours nor at 2-6 ^{o}C for several days. Although many buffers of various pH, ionic strength and composition were tried, bound protein failed to elute. Less than 26 per cent of platelet protein that had been precipitated by ammonium sulphate was bound to a control column. Almost all of the same extract solution was bound to a serotonin-affinity column. Bovine serum albumin (Sigma) did not bind at all on a serotonin-affinity column with a starting buffer containing Fe^{2+}, and 10 per cent was bound on a serotonin-affinity column with a starting buffer that did not contain added Fe^{2+}.

Section D.2. Elution of bound protein from affinity gels

The results with glycine-HCl, pH 2,6, eluting buffer, gave the first indication that a low pH buffer might be able to remove bound protein from the serotonin-affinity columns. In experiment 11, 1 M acetic acid, pH 2,3 was used in order to try to elute 1450 µg of bound protein from column 6. The results are recorded in Table 2-6.

Fraction 3 was turbid and A_{280} rapidly increased even as it was being read in the spectrophotometer. Solid Tris was added when the A_{280} reached 0,21, because the turbidity was thought to be due to denaturation of eluted protein at acid pH. In subsequent experiments in which 1 M acetic acid eluted the specifically bound protein, this rapid increase in A_{280} was also noted in non-turbid fractions that contained protein.

Column 5 from experiment 10 had 2300 µg (by calculation) of protein bound to it before elution with glycine-HCl, pH 2,6 (see the previous section). About 100 µg of protein had been removed by that eluting buffer. After the urea solutions were applied, the column was washed with distilled water. 1 M acetic acid was then applied. The fluorescence of the eluted fractions tended to confirm that bound protein was eluted by 1 M acetic acid (see Table 2-7).

The unneutralized fractions from 1 M acetic acid elution of columns 5 and 6 were dialysed overnight at 3-5 ^{o}C against two changes of 66 mM phosphate buffer, pH 7,6, using 100 times the volume of the fractions. After 14 h dialysis, protein determinations by the Lowry method indicated that there was 0,02 mg/ml, or less, protein in all the dialysed fractions. The bound protein that had been eluted by 1 M acetic acid, therefore, appeared to have dialysed out of the bags and been lost (Table 2-8). This loss was confirmed at another time with two other solutions of 1 M acetic acid-eluted material, which had A_{280} of 0,115 and 0,330 prior to dialysis. These solutions were dialysed against neutral phosphate buffer which was 40 times the volume of the fractions. The A_{280} of the solution outside the bags was 0,006 before dialysis and 0,034 after dialysis. Protein determinations (Lowry method) of the solutions inside both bags indicated that there was only 0,01 mg protein/ml after dialysis.

Dialysis prior to affinity chromatography was usually carried out overnight and did not result in the loss of protein. However, dialysis

Table 2-6. Elution of serotonin-binding protein with 1 M acetic acid (experiment 11).

pH[1]	Fraction	A_{280}
7	1	N.D.
7	2	0,02
4	3	0,18-0,21[2]
2	4	0,05
2	5	0 04

[1] pH was determined with Johnson Universal Test Papers, because volume of fractions was too small to be measured using a pH meter.

[2] See previous page.

Table 2-7. Elution of serotonin-binding protein with 1 M acetic acid (experiment 10).

Fraction	A_{280}	Emission Intensity 295/335	295/350
1	0,052	N.D.	N.D.
2	0,045	N.D.	N.D.
3	0,126	N.D.	N.D.
3 + 4*	0,148	N.D.	N.D.
3 + 4 + 5*	0,176	110	110
6	0,170	55	52
7	0,145	44	41

* Fractions were combined to obtain a sufficient volume for the fluorescence spectrophotometer cuvettes.

Table 2-8. Loss of acetic acid-eluted serotonin-binding protein through dialysis membrane.

A_{280}		Protein (Lowry) mg/ml
Before Dialysis	After 14 h Dialysis	After Dialysis
0,170	0,070	0,02
0,145	0,101	0,01

after affinity chromatography resulted in almost total loss of the protein. This characteristic of the protein was explained later by identifying it as platelet factor 4, which is known to monomerize at acid pH to a protomer (M_r = 7800) (references in Chapter 1). Proteins that have M_r < 12 000 are known to permeate dialysis membrane (Haschemeyer & Haschemeyer, 1973, p. 36).

Affinity column 6 was washed overnight with phosphate buffer, pH 7,2, and without applying additional sample, was eluted again with 1 M acetic acid. It was then washed with 40 ml of phosphate buffer again until the pH of the column effluent was 7,2. Once more it was eluted with 1 M acetic acid. Both times, as the pH fell from about 6 to 4, the A_{280} of the eluted fractions increased to about 0,07, and gradually returned to the pre-peak value several fractions later, at pH 2,3. In neither case was the peak fraction turbid. The unneutralized peak fraction from the last elution (A_{280} = 0,068) was applied to a Sephadex G-25 column (void volume, 3,7 ml), and chromatographed in phosphate buffer at pH 7. An A_{280}-absorbing material was eluted 6 ml after the void volume. The A_{280} of the peak fraction was 0,038. It was anomalous that the protein now eluted after the void volume, because prior to affinity chromatography, it had eluted at the void volume. This behaviour, however, was consistent with the protein's later identification as platelet factor 4 (references in Chapter 1).

Experiments 15-18 were carried out in columns 7 and 8, which contained 2,0 µmol serotonin/ml gel. In experiment 15 a starting buffer consisting of 10^{-4} M $FeSO_4$ and 0,5 M NaCl in 0,02 M cacodylate buffer at pH 6,5 was used. Since elution of bound protein had occurred in fractions that had pH between about 6 and 4 when the columns were eluted with 1 M acetic acid, the first buffer was of pH 5,2. This buffer contained 0,1 M potassium hydrogen phthalate, 1,0 M NaCl and 1 mM $CaCl_2$. The Lowry method showed that no protein was eluted by this buffer.

Protein was eluted, however, by using 5% EDTA, pH 4,5 eluting buffer. The exact amount of eluted protein could not be determined because 5% EDTA interferes in the Lowry determination and potassium hydrogen phthalate interferes in the absorbance measurements. Rough calculations using the Lowry method, however, showed that up to 21% of bound protein had been released by EDTA.

Thus, the protein specifically bound to the serotonin-affinity columns could be removed by elution with either 1 M acetic acid (pH

2,3) or by 5% EDTA (pH 4,5).

Thin-layer chromatography was used to obtain more information about the serotonin-binding protein, and to exclude the possibility that the eluted material might be serotonin. The protein-containing fractions from experiment 15 (C2-C7) were dialysed against water, concentrated by evaporation at room temperature and chromatographed on thin-layer silica gel plates, according to a procedure described for the detection of polypeptides (Amin et al., 1954). After the plate was sprayed with ninhydrin, a pink round diffuse spot appeared in most lanes in which the EDTA-eluted samples had been spotted (C3-C7, but not C2). The R_f of this spot was 0,15-0,27. The serotonin standard on this plate produced a yellow-brown spot at R_f 0,55 (on six other occasions, the R_f of serotonin was 0,51-0,58 under similar conditions). In addition, a UV-fluorescent spot was present at the origin in the C2-C7 lanes after development and drying, but before spraying. These spots did not colour after spraying with ninhydrin and then heating. The spots that were pink after spraying became brown when the plate was placed in a tank that contained subliming iodine crystals. The most intense spot at R_f 0,15-0,27 was seen in the lane in which the C5 fraction had been applied. The C5 fraction also had the highest protein concentration as determined by the Lowry method. After exposure to iodine, another brown spot appeared in the C3-C6 lanes, at R_f 0,61-0,72. This spot had not been visible when the plate was sprayed with ninhydrin and heated. Potassium phthalate buffer and 5% EDTA spotted separately and together in other lanes on the same plate gave spots that were different in colour and position from those in the C2-C7 lanes.

A sample of the acetic acid-eluted protein was concentrated in a Minicon B15 concentrator (Amicon) and applied to a thin-layer plate. After development in a solvent consisting of 10:45:30 ammonia:isopropanol:ethyl acetate and drying, a bright UV-fluorescent spot was seen at the origin. This spot did not colour after it had been sprayed with ninhydrin solution and heated, but became dark brown when exposed to subliming iodine. (This spot is discussed further in the next section.) No other spots appeared on the plate after it had been sprayed with ninhydrin solution and heated. It is possible that low molecular mass protein was lost in the Minicon B15 concentrator, which is intended for use with proteins of M_r greater than 15 000. On a second plate run in the same solvent system, acetic acid-eluted

protein that had not been processed in the Minicon concentrator produced a spot at R_f 0,35 which was blue after reaction with ninhydrin.

Another indication that the Minicon B15 concentrator adsorbed the serotonin-binding protein was that when the Minicon B15 was used to concentrate a sample to be applied to the affinity column, about two-thirds of the protein processed in the Minicon could not be recovered. Furthermore, when the recovered protein was applied to the serotonin-affinity column in experiment 6, the percentage of protein that bound on the affinity column amounted to only 17%, the lowest percentage of bound protein that was obtained on 33 µmol serotonin/ml gel (Table 2-1).

The ability of the serotonin-binding protein to traverse a dialysis membrane was also examined using thin-layer chromatography (Amin et al., 1954). An undialysed sample of acetic-acid eluted protein from experiment 18 produced a ninhydrin-positive spot (purple) which extended from the origin to R_f 0,24. A second aliquot of the same sample was then dialysed against ten volumes of distilled water three times in succession. After concentration the following observations after development and reaction with ninhydrin were noted: a 20 min dialysate (diffusate) gave a pink or purple spot extending from above the origin to R_f 0,22; successive dialysates gave smaller pink spots at R_f 0,22; the dialysed solution after the third aliquot gave a pink line at R_f 0,22.

5% EDTA was applied a second time to column 7 in experiment 15, and 12 more protein-containing fractions were eluted. These were designated ED 1-12. It was calculated that they contained a total of 0,1 mg protein (Lowry). This determination could be made with more certainty than it could for the initial EDTA-eluted fractions from experiment 15, because the initial EDTA-eluted fractions contained a gradient of EDTA (up to 5%), whereas ED 1-12 contained 5% EDTA. The contribution of EDTA was subtracted as described previously (this chapter, Section I.C.). 0,1 ml of 5% EDTA produces A_{750} = 0,32-0,33 in the Lowry determination. Altogether approximately 55% of bound protein was eluted by 5% EDTA in experiment 15. 5% EDTA was used to elute bound protein in experiment 16, as well, and the elution profile of this experiment is shown in Fig. 2-9.

The observation that EDTA removes the serotonin-binding protein from the affinity column could imply that calcium or another divalent

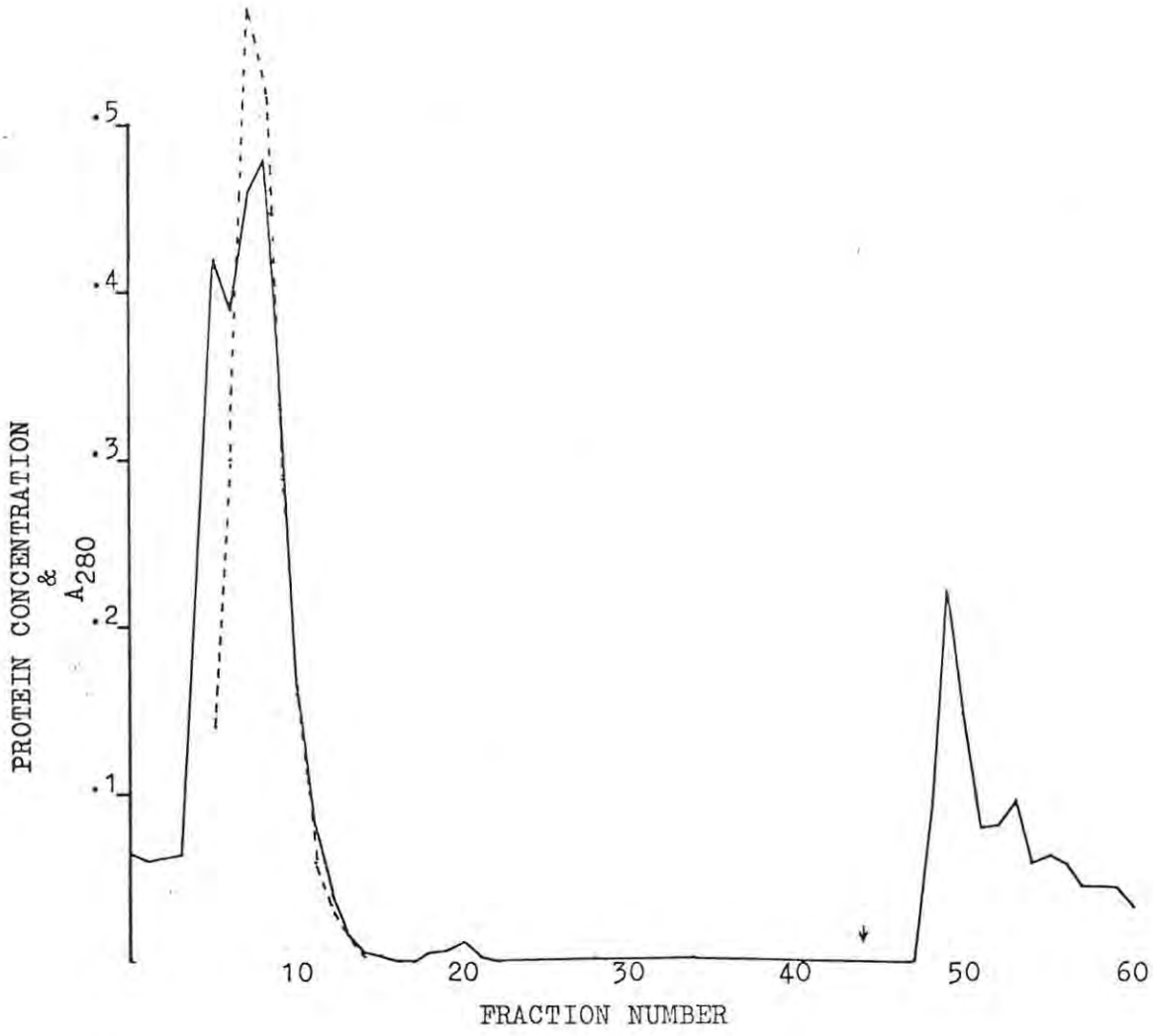


Fig. 2-9. Elution profile of experiment 16.
0,2 ml containing 1,11 mg protein was applied to a serotonin-affinity column equilibrated with cacodylate starting buffer, pH 6,5. Fractions were 0,4 ml. 21% of applied protein was bound by the gel. Elution with 5% EDTA, pH 4,5, was started in fraction 44 (at arrow).

Solid line - A_{280}

Broken line - Protein concentration in mg/ml (Lowry and Hartree)

The scale on the ordinate applies to both A_{280} and protein concentration.

cation is involved in the binding of the serotonin-binding protein to serotonin. This implication suggested that certain avenues would be worth investigating and gave rise to a series of experiments which are described in Chapter 5.

To summarize, evidence has been presented in this section (Section D.2.) to show that 1) The protein(s) which bound to serotonin on the affinity columns could be eluted with 1 M acetic acid or 5% EDTA; 2) Purification of the protein(s) which bind to serotonin-affinity columns by affinity chromatography causes the protein to elute from Sephadex G-25 columns after the void volume. This is in contrast to when these proteins are not subjected to affinity chromatography. In this case, they elute at the void volume. 3) When chromatographed and developed on thin-layer plates (Amin et al., 1954), the protein(s) which bind to serotonin on the affinity columns produce a ninhydrin-positive spot at R_f 0,15-0,27; and 4) The protein(s) which bind serotonin on the affinity columns are able to permeate dialysis membrane, and are at least partially adsorbed by Minicon B15 concentrators.

Section D.3. Spectroscopy of serotonin-binding protein

Ultraviolet absorbance spectra, the ratio of absorbance at 280 and 260 nm, and fluorescence spectroscopy were used to obtain more information about the serotonin-binding protein.

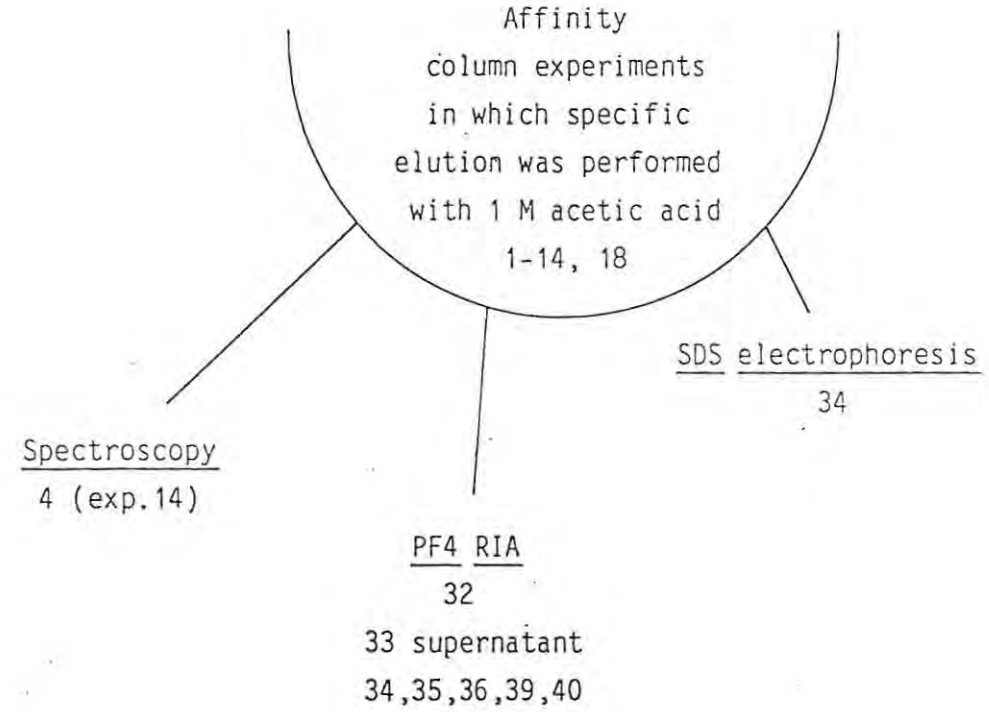
The G-25 void volume samples, after dialysis in starting buffer and before application to affinity columns, had molar extinctions close to 1,0; i.e., A_{280} was very close to the values for the protein concentration determined by the Lowry method. Such absorbance is typical of a mixture of proteins like that present in a platelet extract. However, when the protein concentration was calculated from the absorbance using a molar extinction of 1,0 in the case of the acetic acid-eluted serotonin-binding protein, a value that was 3-5 times as great as the protein concentration as determined by the Lowry method was obtained. For example, fractions 77, 78 and 80 from experiment 18 had A_{280} of 0,128, 0,132 and 0,052 respectively, while protein concentrations determined by the Lowry method were 0,025, 0,025 and 0,010 mg/ml respectively. Since neither the starting nor the eluting buffer contributed to the absorbance (A_{280} = 0,005 and 0,000

respectively), it is possible that either a non-proteinaceous substance which absorbed at 280 nm was eluted with the protein, or the protein did not react completely with the reagents in the Lowry determination due to some interfering agent.

Thin-layer chromatography of fractions containing serotonin-binding protein showed a diphenylamine-reacting spot at the origin, which is possibly a glycosaminoglycan. This spot was seen on plates that had been chromatographed and developed according to the method of Amin et al. (1954) and then sprayed with Stahl's spray number 53 (Stahl, 1965). The presence of this spot confirms the suspicion that the serotonin-binding protein may be PF4, because PF4 is associated with a glycosaminoglycan carrier in vivo. This spot was described in the previous section as a UV-fluorescent spot which appeared on thin-layer plates in lanes where samples concentrated in a Minicon B15 had been applied. This behaviour resembles that of the PF4 carrier, because its M_r is 59 000, and it therefore would not be adsorbed by the Minicon B15 concentrator.

The ratio A_{280}/A_{260} is usually about 1,75 for proteins containing only amino acid residues, 0,80 for muco- and glycoproteins and 0,50 for nucleic acids (White et al., 1973, p. 135). For the samples applied to the affinity columns, this ratio was 1,1, and it was 0,40-0,80 for the fractions of serotonin-binding protein. The absorbance of fractions containing serotonin-binding protein was measured immediately after each fraction was eluted from the columns. It has been noted previously (in Section D.2.) that A_{280} sometimes increased in turbid and non-turbid fractions that had just been eluted from the affinity columns. On many occasions, the A_{280} of such fractions decreased while they were being measured in the spectrophotometer. The drift was not random. The A_{260} value decreased much more than did the A_{280} value, so that the ratio A_{280}/A_{260} always increased with time. The final ratio (10-30 min after elution) usually was 0,70-0,80. Since the spectrophotometer needle did not drift when other samples were read, a dynamic process such as a conformational change of the serotonin-binding protein could have been occurring.

A possible explanation for these results is that a bound nucleotide had been lost from the protein. Indeed, evidence for the presence of a nucleotide in bound fractions is given by the ultraviolet absorbance of the combined fractions ED 1-3 (from experiment 15) (see Fig. 2-10 and 2-11). λ_{max} was at 258-264 nm, which is consistent



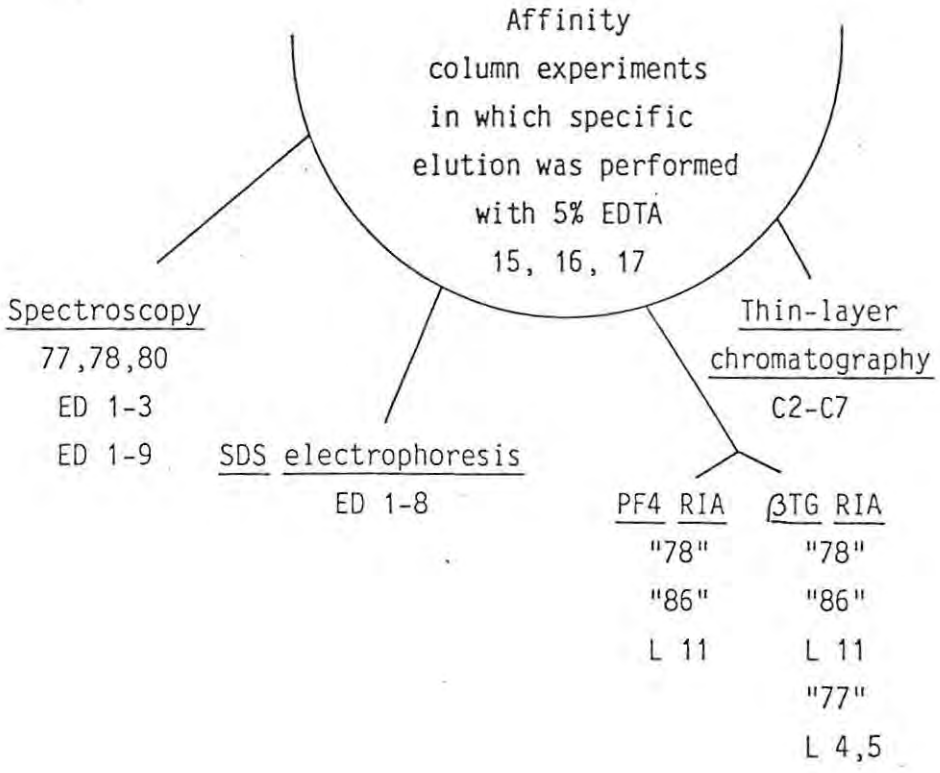


Fig. 2-10. Origin of particular fractions referred to in Chapter 2.

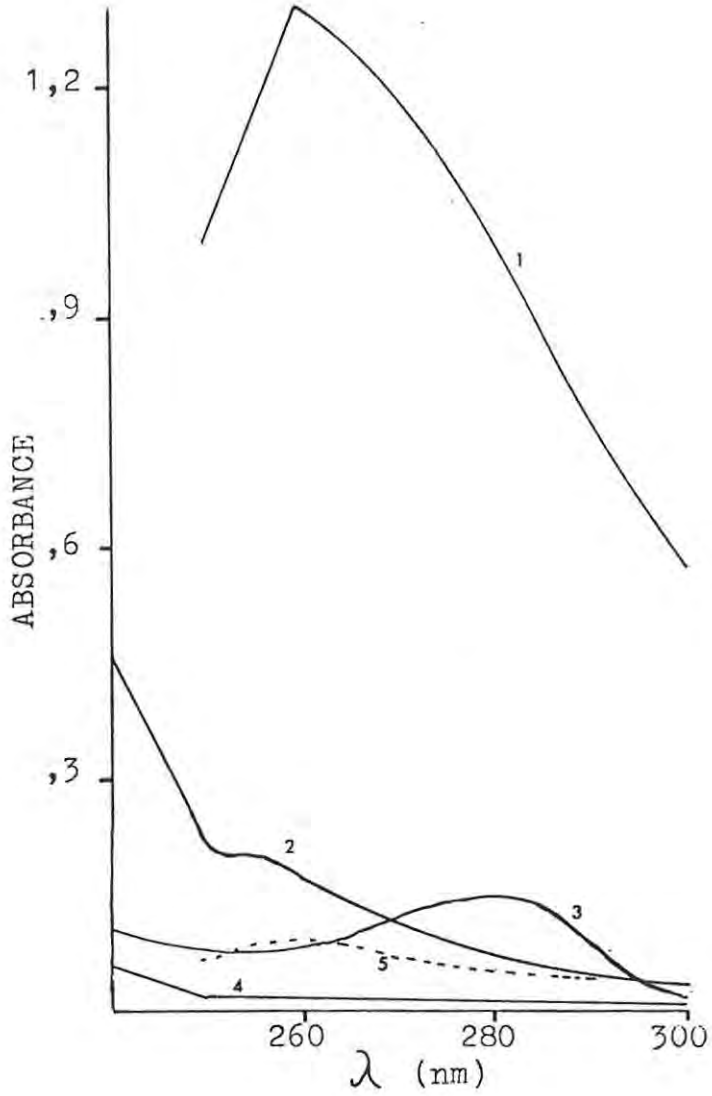


Fig. 2-11. Ultraviolet absorbance scans of serotonin-binding protein, bovine serum albumin and purified PF4.

1: EDTA-eluted serotonin-binding protein (fractions ED 1-3 from experiment 15)

2: acetic acid-eluted serotonin-binding protein

3: bovine serum albumin in 1 M acetic acid

4: purified PF4 (from Pepper) in 1 M acetic acid

5: purified PF4 (from Pepper) in 20 μM $CaCl_2$, 100 mM KCl, 600 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 20 mM Tris, 1 mM K oxalate, pH 7,4.

Absorbance was determined in all cases with the reference cell containing all components of each solution except protein.

with the presence of a nucleotide. Furthermore, no fluorescence could be detected at 308-350 nm when fractions ED 1-9 were activated at 275-295 nm. This is typical of a nucleotide. The serotonin-binding protein fractions from the affinity columns usually did fluoresce under these conditions, irrespective of whether they were eluted by 1 M acetic acid or by 5% EDTA (see Fig. 2-13 and 2-14). The anomalous results obtained with fractions ED 1-9 have no obvious explanation.

The presence of a bound nucleotide associated with the serotonin-binding protein was implied by the results of another experiment. An EDTA-eluted fraction (A_{280} = 0,108) from experiment 17 was chromatographed on a Sephadex G-25 column equilibrated with 0,02 M cacodylate, 0,5 M NaCl at pH 5,9 (Fig. 2-16) with the following results: 1) the protein was eluted after the void volume; 2) the A_{280} of five fractions eluted from the column was higher than that of the applied sample; and 3) the A_{280}/A_{260} ratios of fractions 6, 7 and 8 were 0,75, 0,69 and 0,64 respectively, that is, consistent with carbohydrate-associated proteins. The A_{280}/A_{260} ratio of the applied sample was 0,43. An explanation for this is that the A_{280} of the sample may have been blocked or quenched by bound nucleotide and that this nucleotide was separated by G-25 chromatography.

However, in the same experiment, the same procedure apparently did not separate the glycosaminoglycan from the protein. Protein determinations of fractions eluted from the G-25 column by the Lowry method gave results which were consistent with the presence of a polysaccharide still in association with the protein. For example, for fractions 7 and 8 the A_{280} were 0,262 and 0,325 respectively, and protein content (Lowry method) was only 0,01 and 0,07 mg/ml respectively. Additional evidence for the association of polysaccharide with the protein is provided by Hartree protein determinations of the same fractions. The values obtained for the protein concentrations in these samples by this method indicated that the protein concentrations were up to five times greater than had been obtained by the Lowry method.

For comparison, Fig. 2-11 includes the ultraviolet absorbance scans for acetic acid-eluted protein, bovine serum albumin in 1 M acetic acid and purified PF4 in 1 M acetic acid. It is to be noted that λ_{max} for the serotonin-binding protein and purified PF4 is not at 280 nm. Most proteins contain aromatic residues (tryptophan, tyrosine and phenylalanine) and, like bovine serum albumin, absorb maximally at

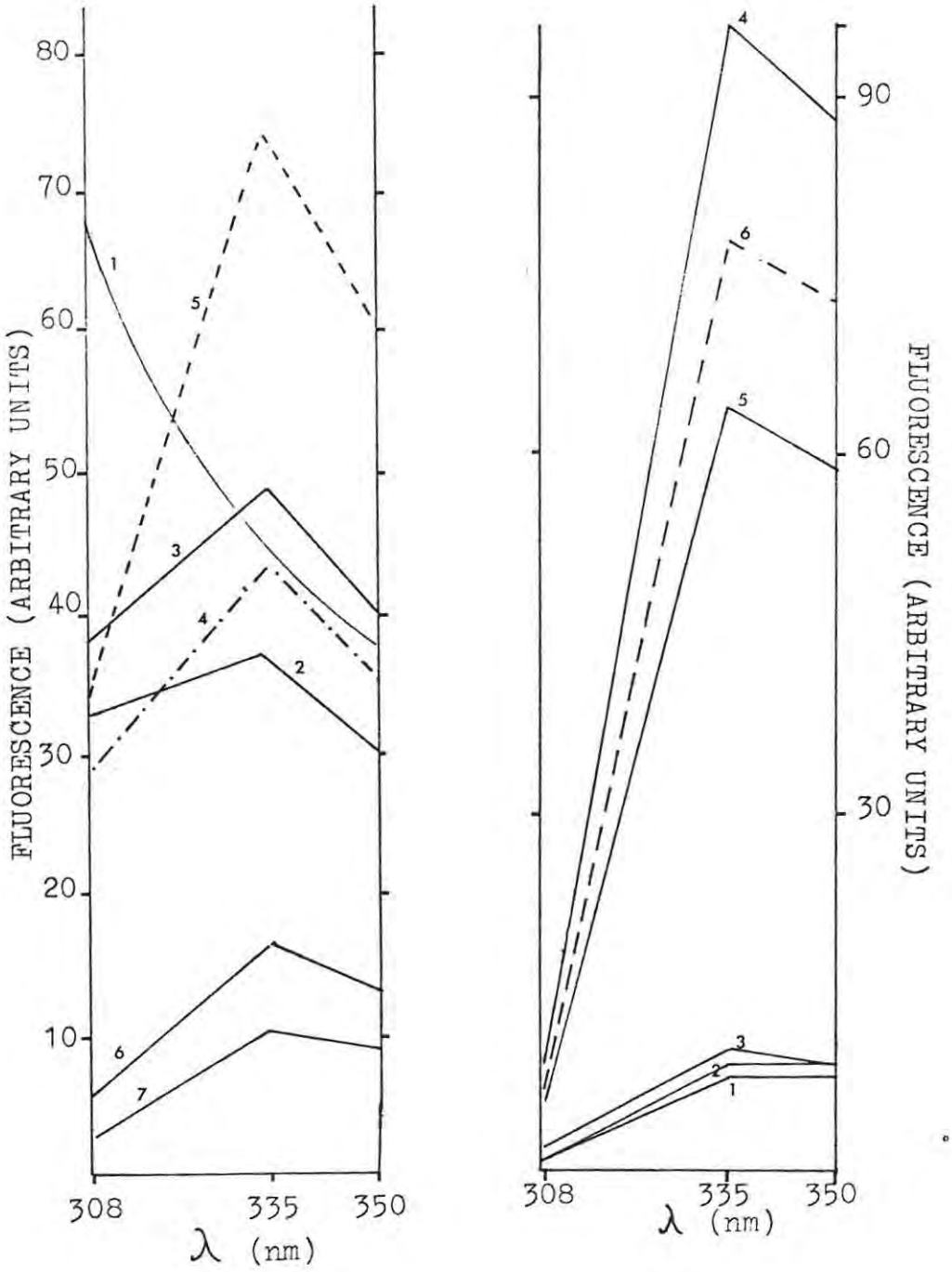


Fig. 2-12 and 2-13. Fluorescence data from experiment 14. Activation at 295 nm for all samples. In Fig. 2-12, the numbers refer to the fraction number of unbound protein. In Fig. 2-13, the numbers refer to the fraction number of bound protein eluted with 1 M acetic acid, except for number 6 (see text).

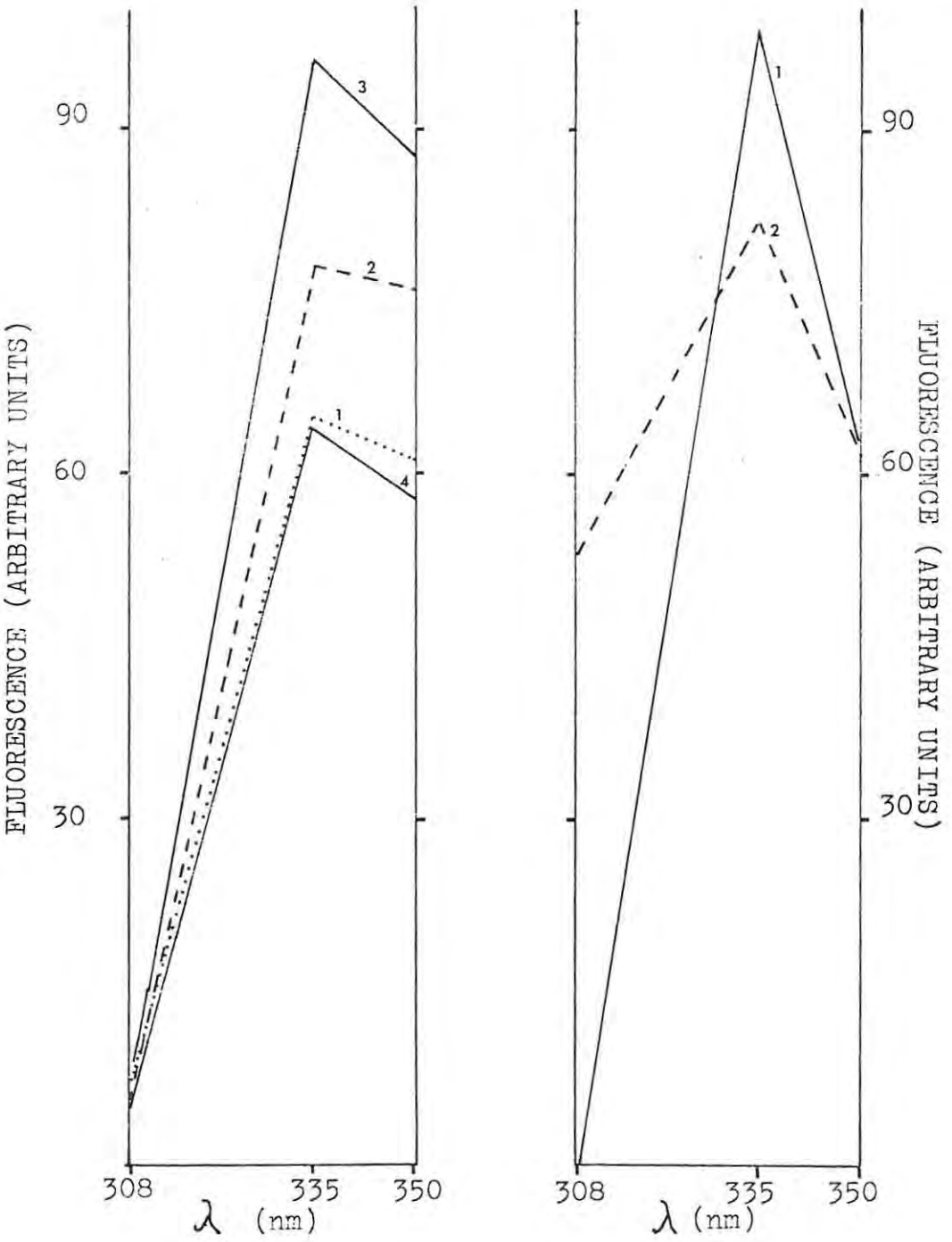


Fig. 2-14. Fluorescence of serotonin-binding protein. 1, acetic acid-eluted protein on column 20 days (2 ^{o}C), read about 1 h after elution; 2, EDTA-eluted protein on column 7 days (2 ^{o}C), read about 30 min after elution; 3 and 4, acetic acid-eluted protein on column about 2 h, fluorescence read within 1 min of elution.

Fig. 2-15. Fluorescence in 1 M acetic acid of serotonin (1) and bovine serum albumin (2).

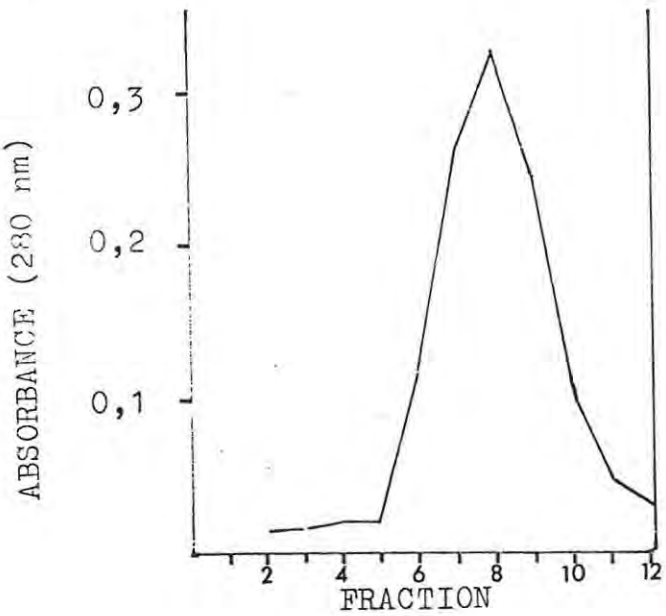


Fig. 2-16. Elution profile of EDTA-eluted serotonin-binding protein on Sephadex G-25 in 0,02 M cacodylate buffer, pH 5,9, containing 0,5 M NaCl. The applied sample had A_{280} of 0,108. See text for additional data.

about 280 nm (Udenfriend, 1962, p. 201). In proteins lacking tryptophan but containing tyrosine, λ_{max} is influenced by the buffer pH because the hydroxyl group on tyrosine can ionize. However, λ_{max} remains between 270-303 nm over the pH range 2-13 (Beaven, 1961).

The fluorescence of the unbound and bound protein in experiment 14 was measured at 308, 335 and 350 nm (excitation wavelength 295 nm) and is shown in Fig. 2-12 and 2-13.

Immediately after recording the fluorescence, fraction 4 (see Fig. 2-13) was applied to a Sephadex G-25 column and eluted with neutral phosphate buffer. The fluorescence of the peak protein fraction from the G-25 column, which was eluted at 2-2,5 times the void volume, is shown in Fig. 2-13 (plot 6).

Twenty days after experiment 18 was performed, elution of affinity column 8 was recommenced and a few more 1 M acetic acid-eluted fractions were collected. These had A_{280}/A_{260} = 1,12-1,28 but the fluorescence of the eluted protein was like that of other acetic acid-eluted fractions (Fig. 2-14). It is possible that the absorbance ratio changed from its characteristic value of 0,40-0,80 because of instability of a bound nucleotide or carbohydrate to acid. For comparison, Fig. 2-14 includes plots of fluorescence of serotonin-binding protein fractions after various other periods of exposure to 1M acetic acid and EDTA. Fig. 2-15 is a plot of the fluorescence of bovine serum albumin and serotonin in 1 M acetic acid.

The fluorescence measurements of the serotonin-binding protein, when freshly eluted from columns, were erratic. These fractions, even when perfectly clear, caused a jerky or sluggish movement of the fluorescence spectrophotometer needle. This phenomenon has been noted before in connection with ultraviolet absorbance, and confirms that the serotonin-binding protein undergoes some sort of change. Indeed, the fluorescence of the serotonin-binding protein changed under conditions that would affect both bound ligands or polysaccharide (Fig. 2-17 and 2-18). When freshly eluted from the serotonin-affinity column and activated at 295 nm, fluorescence of the serotonin-binding protein at 308, 335 and 350 nm resembles curve 1, Fig. 2-17 (also shown in Fig. 2-13 and 2-14). However, when the serotonin-binding protein had first been dialysed for one day against a serotonin solution and then chromatographed on a G-25 column equilibrated with phosphate buffer at pH 7,6, a different fluorescence curve was obtained. Curve 2 in Fig. 2-17 is the plot of the fluorescence of the void volume fraction of the

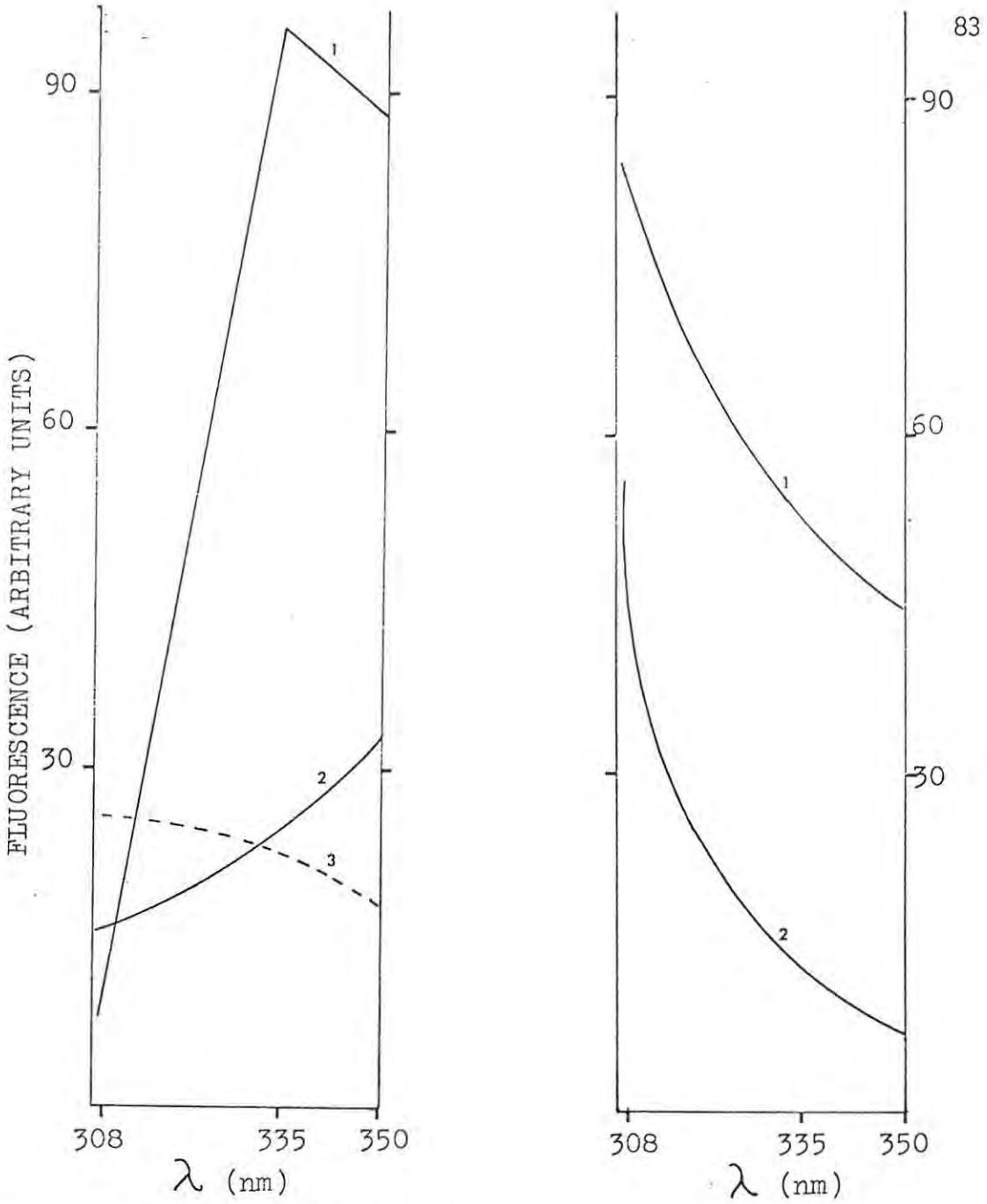


Fig. 2-17 (left) and 2-18 (right). Fluorescence of serotonin-binding protein after various treatments that would affect bound moieties. Activation at 295 nm.

17-1: Immediately after elution from affinity column

17-2: After elution from affinity column, dialysis for 1 day against a serotonin solution and chromatography on G-25, pH 7,6. Void volume fraction.

17-3: After elution from affinity column, chromatography on G-25, and dialysis against buffer that was 80 times sample volume, pH 7,3, for 3 days.

18-1: After elution from affinity column, and dialysis against 100 times sample volume, pH 7,7 for 5 days. Ascorbic acid included in buffer.

18-2: Purified PF4 (from Pepper) in 1 M acetic acid.

G-25 column and curve 3 in Fig. 2-17, that of the serotonin-binding protein after a sample had been chromatographed on Sephadex G-25 and then dialysed against about 80 times its volume of phosphate buffer at pH 7,3 for 3 days.

A sample of serotonin-binding protein that was dialysed against about 100 times its volume of ascorbic acid-phosphate buffer (pH 7,7) for 5 days fluoresced as indicated in Fig. 2-18, curve 1. For comparison, the fluorescence of purified PF4 is also plotted (Fig. 2-18, curve 2). From Fig. 2-17 and 2-18 it appears that contact with serotonin increases fluorescence at the upper analyser wavelengths and that exhaustive dialysis decreases this fluorescence, while at the same time the relative tyrosine fluorescence (at 308 nm) is increased. A possible interpretation of these data is that serotonin could induce a conformational change that buries a tyrosine residue within the protein.

II. IDENTIFICATION OF PLATELET FACTOR 4 (PF4) BY RADIOIMMUNOASSAY

Section A. Introduction

The results obtained from the various experiments and studies which have been described so far indicate that the serotonin-binding protein(s) have certain unique characteristics. λ_{max} does not have the typical value of 280 nm. The protein(s) permeate dialysis membranes in acidic solution and also show anomalous behaviour on Sephadex G-25 (eluting at the void volume prior to serotonin-affinity chromatography and later than the void volume after serotonin-affinity chromatography). Furthermore, there is evidence for association with a polysaccharide or glycosaminoglycan and possibly also a nucleotide.

Two platelet proteins, β-thromboglobulin (βTG) and platelet factor 4 (PF4), are similar to the serotonin-binding protein (Moore & Pepper, 1976; Moore *et al.*, 1975b). Neither of these two proteins, however, has been shown to have an affinity for serotonin. Both proteins are oligomeric and dissociate at low pH into monomers of M_r 8851 and 7780, respectively. (See Chapter 1.) Since neither contains tryptophan and the tyrosine content is low, their ultraviolet

absorbance is weak. βTG and PF4 are not denatured by acidic conditions. βTG and PF4 are major components of the platelet. Although PF4 *in vivo* exists in a complex with a glycosaminoglycan, *in vitro* PF4 can be separated from the complex. When separated, PF4 is soluble in acid but not soluble at neutral pH except at high ionic strength. βTG may also be associated with some glycosaminoglycans *in vivo*, but its affinity for them is not as great as is PF4's. Both βTG and PF4 can permeate biological membranes.

In addition, PF4 binds to Sephadex and therefore elutes from Sephadex columns in an unpredictable manner (Handin & Cohen, 1976; Nath *et al.*, 1973).

Radioimmunoassays were performed to determine if the serotonin-binding protein might be either βTG or PF4. βTG and PF4 have homologous amino acid structures (see Chapter 1), but the radioimmunoassays are, nevertheless, highly specific (Moore & Pepper, 1976). A 10 000-fold excess of βTG is required to give the same results as for PF4 in the PF4 radioimmunoassay, and a 500- to 1000-fold excess of PF4 is required to give the same results as for βTG in the βTG radioimmunoassay (Begg *et al.*, 1978).

Section B. β-thromboglobulin (βTG) Radioimmunoassay (RIA)

The βTG RIA kit was obtained from Amersham International Limited and used within one week of receipt. The expiry date was 8 days after the radioimmunoassays were performed. (The expiry date is determined by the 60-day half-life of [^{125}I]-labelled βTG.) The shape of the standard curve (using standards supplied by Amersham) was similar to that of the example shown in the figure of the kit's instruction booklet. The ratios of mean counts of the 10 and 100 ng standards were 1,71 and 1,89 on the two occasions the RIA was performed. (A standard curve is prepared each time that a group of unknown samples is assayed.) These values are consistent with normal performance of the test. 4,6 min was needed to accumulate 10 000 counts from the 10 ng standard. This is 0,6 min longer than it should have been, and was probably due to the age of the kit.

Both the βTG and the PF4 RIA are competitive assays, which means that the lower values are obtained with the higher concentrations of nonradioactive protein. The radioactivity of the precipitated

protein-antibody complex is measured. The standard curve for βTG is similar in shape to that of PF4 (See Fig. 2-20). The percentage bound is determined for the unknown samples, and the concentration of βTG is obtained from the βTG standard curve in the same manner as the PF4 concentration is obtained from the PF4 standard curve.

The βTG RIA results were obtained by liquid scintillation counting in Bray's scintillation fluid (Bray, 1960). All samples (standards as well as unknowns) had a similar external standard channels ratio, and therefore no correction was necessary for quench. Liquid scintillation is an accepted method for measuring radioactivity produced by ^{125}I, because ^{125}I decays mostly by the emission of Auger and conversion electrons at 12 and 40 keV and these two groups are counted with 76% efficiency in liquid scintillation systems (Horrocks, 1974 and 1976).

βTG RIA determinations were performed on serotonin-binding protein that had been eluted from the serotonin-affinity column by 5% EDTA (Table 2-9). This included several fractions ("77", "78" and "86") which had not been dialysed after they were eluted from the affinity column (in case protein might be lost). Some supernatant of ultrasonicated platelets that had been frozen since centrifugation was diluted with water or with 5% EDTA and assayed to determine whether EDTA interferes in the assay. Although the dilution was not sufficient for the concentration of βTG to fall on the standard curve, it can be seen that the average cpm values were similar for both the 5% EDTA- and water-diluted samples. Therefore, it is a reasonable assumption that the presence of EDTA in the unknown samples does not affect the results.

In addition, the βTG RIA was performed on other serotonin-binding protein samples. These had also been eluted by 5% EDTA from the affinity column, but had been concentrated by the addition of 2,4 g Sephadex G-25 and then centrifuged. The supernatant (6,5 ml) had A_{280} = 0,322 and A_{280}/A_{260} = 0,83. Tris had been added to raise the pH of the solution from 4,5 to 7, and then 3,3 mg serotonin-HCl had been added. The solution was incubated at 38 ^{o}C for 1 h, and then 1 ml was chromatographed on Sephadex G-25. "L4,5" was eluted just after the void volume and its A_{280} was 0,10. "L11" was eluted at 1,6-2,0 void volumes and its A_{280} was 0,12. It can be seen in Table 2-9 that all of the unknowns reacted slightly with the βTG RIA. However, the last column of the table indicates that the specific activity of the samples

Table 2-9. βTG RIA Results

Sample	A_{280}	avg cpm	βTG ng/ml	Protein (Hartree) mg/ml	ng βTG / mg protein
73,76,77: "77"	0,20	826	200-220	0,69	300
78,79,80,83: "78"	0,15	1701	20-29	0,46	53
84,85,86: "86"	0,09	2063	2	N.D.	N.D.
L 11	0,12	1620	34	0,35	100
L 4,5	0,10	1749	28	N.D.	N.D.
US super dil H_2O	N.D.	447	200	N.D.	N.D.
US super dil EDTA	N.D.	388	200	N.D.	N.D.
H_2O blank	N.D.	2328	0		
EDTA blank	N.D.	2094	0		

* See text for explanation of samples assayed. All assays in duplicate.

was low, that is, either βTG is not a major component of the fractions eluted from the affinity column, or its antigenic site is not available.

After the βTG RIA had been performed, some of the [^{125}I]-βTG was left over and this was used to confirm that βTG is completely excluded from Sephadex G-25. A K9/15 (Pharmacia) Sephadex G-25 Fine column was equilibrated with 66 mM phosphate buffer, pH 7,5, at 20,5 ^{o}C. An aliquot of the [^{125}I]- βTG was applied, fractions were collected, and radioactivity measured in a Packard Auto-Gamma Scintillation Spectrometer 5120 Gamma Counter. Although counts were low because the experiment was conducted 34 days after the expiry date of the material, most of the radiolabelled material was eluted at the void volume (Fig. 2-19). The small second peak represents low molecular mass by-products of the ^{125}I-labelling reaction (Pepper, personal communication). These results indicate that βTG is excluded from G-25.

Section C. Platelet factor 4 (PF4) Radioimmunoassay (RIA)

Like the βTG RIA, the PF4 RIA is a competitive reaction in which nonradioactive PF4 in samples competes with a constant amount of [^{125}I]-PF4 for binding sites on a limited amount of PF4 antiserum. Thus, the percentage of radioactive PF4 bound to antiserum is inversely proportional to the concentration of nonradioactive PF4 in the sample. The radioactivity in the ammonium sulphate-precipitated PF4-antibody complex was measured using a gamma counter. A standard curve was prepared as for the βTG RIA, based on the standards supplied in the PF4 RIA kit (Fig. 2-20).

The PF4 RIA kit (Lot 28939 HL) of Abbott was used to measure PF4 in samples that were diluted 10^3 to 10^5 times with dilution buffer supplied in the kit, or, when that was finished, buffer of the same pH (8,45) which contained 0,01 M Tris, 0,15 M NaCl and 0,2% bovine serum albumin. This buffer was of similar composition and produced similar results with standards as did the dilution buffer provided by Abbott.

The PF4 content was measured in duplicate samples of fractions eluted from the serotonin-affinity columns by 1 M acetic acid and 5% EDTA (Table 2-10). One of these fractions (34) had been stored at 4 ^{o}C since its elution by 1 M acetic acid 10 days previously. When it was diluted 10^5 times, it reacted with PF4 antiserum in the RIA, producing

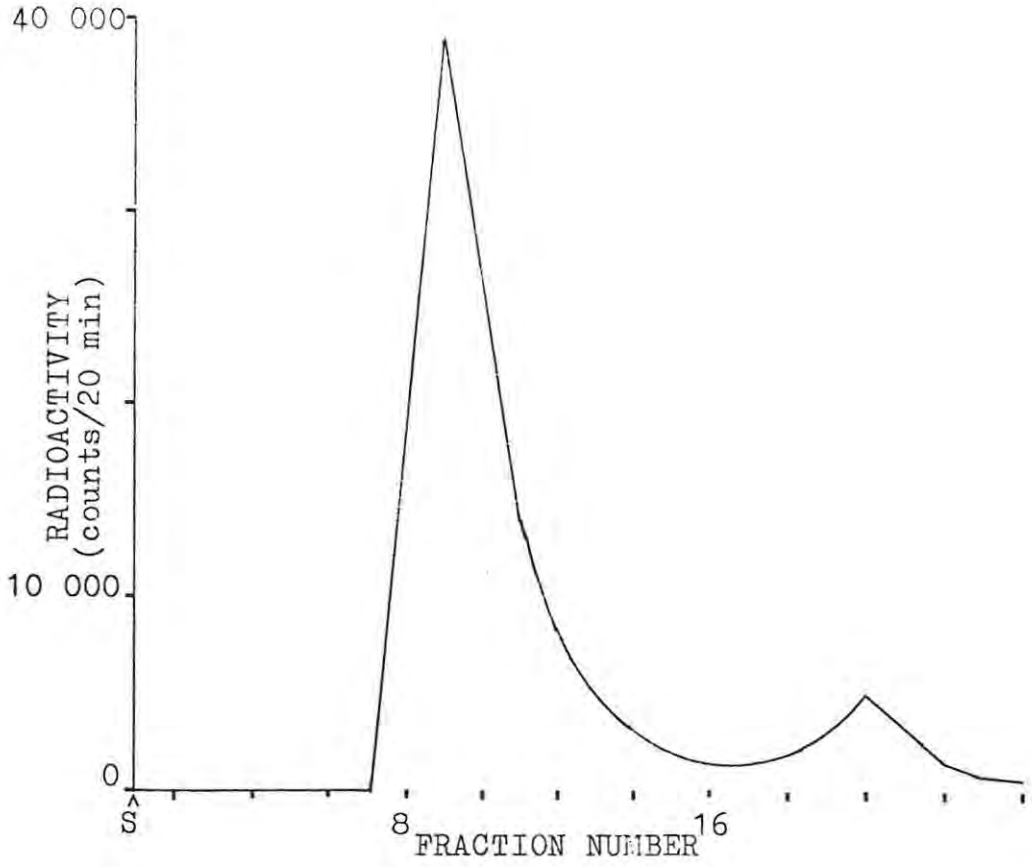


Fig. 2-19. Fractionation of [^{125}I]-βTG on Sephadex G-25 in 66 mM phosphate buffer, pH 7,5, at 20 ^{o}C. Void volume occurred in fraction 8. Sample was applied at S.

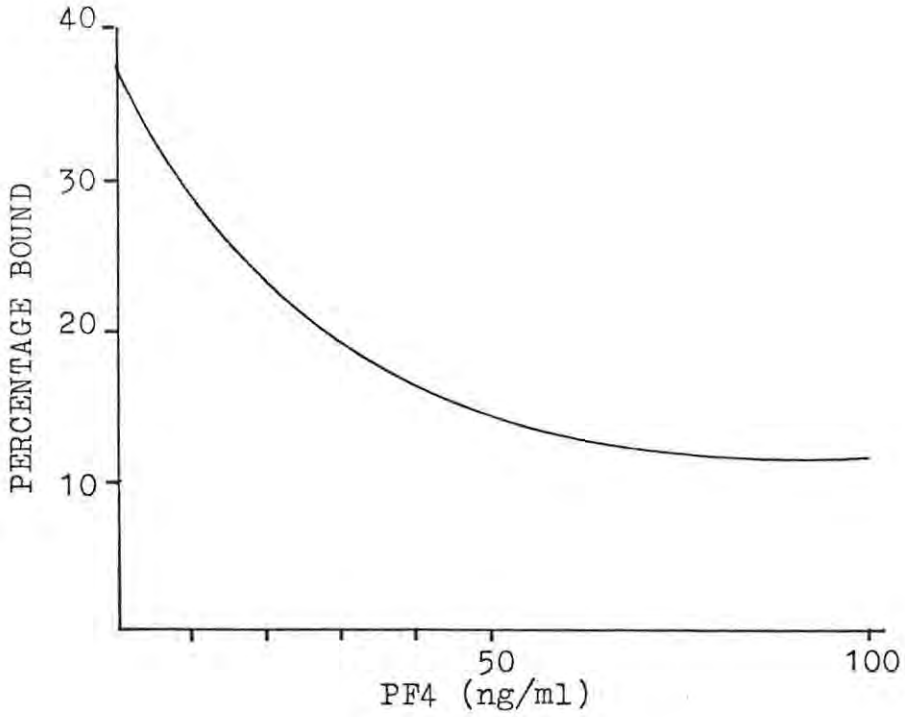


Fig. 2-20. Standard curve for PF4 RIA. Data was obtained with standards from the Abbott PF4 RIA kit. The percentage bound is determined by dividing the cpm of the standard or unknown by the cpm of the "total count tubes" prepared as described in the PF4 kit. The average percentage bound for each PF4 standard is plotted as shown in the figure above. To determine the concentration of PF4 in unknown samples, percentage bound is first determined. Then the corresponding PF4 concentration value is read from the intersection of a vertical line extended to the abscissa from the point on the curve corresponding to the percentage bound.

Table 2-10. PF4 RIA results

Sample	avg PF4 ng/ml	(Hartree) protein ng/ml (dil fract*)	ng PF4 / ng protein
EDTA-eluted "78"	21	460 000	$4{,}56 \times 10^{-5}$
EDTA-eluted "86"	9	N.D.	N.D.
L 11	17	350 000	$4{,}85 \times 10^{-5}$
32	0	0	0
33 Supernatant (dil 10^4)	6	27	0,22
34 (dil 10^5)	14	14	1
34 boiled (dil 10^5)	7	14	0,5
35 (dil 10^4)	10	N.D.	ca. 1
36 (dil 10^4)	8	N.D.	ca. 1
39 (dil 10^4)	6	N.D.	ca. 1
40 (dil 10^4)	10-20	18	ca. 1

* The protein determination was carried out on the undiluted sample.

values for PF4 concentration that were close to the total protein content as determined by the Hartree method. Furthermore, some of the diluted sample retained half of its PF4 antigenicity after boiling. PF4 is known to be heat-stable (Nath et al., 1973).

Fractions 35, 36, 39 and 40 (stored at 4 ^{o}C since elution) also reacted with PF4 antibody after they had been diluted 10^4 times. Fraction 32, whether diluted or undiluted, did not react with PF4 antibody and did not contain protein, according to the Hartree determination.

Fraction 33 had been frozen (at -20 ^{o}C) since elution 10 days previously, and when it was thawed, it contained a white flocculent precipitate that partly dissolved upon the addition of 1 M NaCl. Such dissolution is characteristic of PF4 (Moore et al., 1975b). The resulting solution, diluted 10^4 times, reacted with PF4 antiserum, producing values for PF4 concentration that were about one-fifth the total protein content. These data suggest that freezing at -20 ^{o}C may cause more denaturation of PF4 than does storage at 4 ^{o}C.

Some of the frozen samples ("78", "86" and L11) had been assayed for βTG five months previously (see Table 2-9). Their reaction with βTG antibody had been very slight, 53-300 ng βTG/mg protein. Their reaction with PF4 antibody was also slight (Table 2-10). A possible explanation for this low result could be the temperature at which the samples were stored.

It is interesting to note that after addition of ammonium sulphate in the RIA, the heaviest precipitates were in the tubes for the 100 ng standard (the most concentrated standard) and samples of 34, 35, 36 and 40.

Section D. Binding of [^{125}I]-PF4 on control- and serotonin-affinity columns

The binding of [^{125}I]-PF4 from the RIA kit was measured on serotonin- and control-affinity gels. Control gel was prepared as follows: 1 g freeze-dried Activated CH-Sepharose 4B (Pharmacia) was mixed with 200 ml cold 1 mM HCl and incubated at 2 ^{o}C for 30 min. The mixture was then filtered, and the gel mixed with 100 ml 0,1 M Tris HCl, pH 8,1. The mixture was refrigerated at 4 ^{o}C overnight. This treatment results in the blocking by Tris of all active groups on the

gel (Pharmacia, 1979). The gel was packed into a K9/15 column and washed with formate buffer (0,1 M sodium formate, 0,5 M NaCl, pH 4,0) and then with starting buffer, 66 mM phosphate buffer, pH 7,0 (100 ml), until the column was equilibrated.

0,1 ml of the [^{125}I]-PF4 from the RIA kit was applied to the control column and eluted at a flow rate of 0,20 ml/min. The temperature was 13 ^{o}C. Fractions were collected and counted in the gamma counter. The gel itself was then transferred from the column to a vial and also counted in the gamma counter (Table 2-11). The results indicated that only 6% of the total radioactivity remained bound to the control gel.

Another 0,1 ml of [^{125}I]-PF4 from the RIA kit was applied to a serotonin-affinity column and eluted with starting buffer, 66 mM phosphate buffer, pH 7,0. The flow rate was 0,25 ml/min and the temperature was 12 ^{o}C. As in the previous experiment, fractions were collected and counted in the gamma counter (Table 2-12). The column was washed with 100 ml starting buffer, after which the column was eluted with 1 M acetic acid, and finally with 5% EDTA. The results that show that PF4 binds specifically to serotonin are consistent with those obtained by serotonin-affinity chromatography of platelet extracts. Moreover, since the alpha amino group of serotonin is covalently linked to the Sepharose gel, it is obvious that a free alpha amino group on serotonin is not necessary for binding by PF4.

Section E. PF4 RIA results with heparin, serotonin and drugs

Possible interference in the RIA experiments by heparin, serotonin, tricyclic antidepressants and other drugs was examined. The usual RIA procedure was altered only by substituting 10 µl of the 50 ng/ml PF4 standard that was supplied with the kit plus 40 µl of test solutions for the 50 µl sample of unknown. The results are summarized as averages of duplicates in Table 2-13. All of the compounds tested had some effect on the RIA. This indicates that the PF4-antibody system is sensitive <u>in vitro</u>.

Values of 9265 cpm or higher should have been obtained if a drug had nullified the competitive effect of serotonin. However, the highest value obtained was 9041. This was for chlorpromazine. The lowest value, 7607, was obtained for imipramine. When the results are ranked from lowest to highest cpm, the order of drugs is similar to

Table 2-11. $[^{125}I]$-PF4 binding on control affinity column

	Total cpm	Percentage of total cpm
1) Unbound fractions	10 386	94
2) Bound to control gel	663	6
Sum of 1) and 2)	11 049	100

Table 2-12. $[^{125}I]$-PF4 binding on serotonin-affinity column

	Total cpm	Percentage of total applied	bound
0,1 ml $[^{125}I]$-PF4	11 070	100	
Unbound fractions	3 193	28	
100 ml wash (unbound)	75	0,7	
Bound (by difference)	7 893	71,3	100
Acetic acid-eluted	1 100	10	14
EDTA-eluted	1 090	10	14
Remaining on column (by difference)	5 703	51	72

Table 2-13. PF4 RIA results with serotonin, heparin and drugs which affect serotonin uptake or storage.

	Average cpm
50 µl 10 ng/ml standard (std)	9364
10 µl 50 ng/ml std, 40 µl Abbott's dil buffer	9537
10 µl 50 ng/ml std, 40 µl my dil buffer	9702
10 µl 50 ng/ml std, 40 µl (0,1 µmole) serotonin creatinine sulphate, 6 units heparin	8141
10 µl 50 ng/ml std, 40 µl 6 units heparin	7698
10 µl 50 ng/ml std, 40 µl (0,1 µmole) serotonin creatinine sulphate	7043
Solution A, which consists of 10 µl 50 ng/ml std, 30 µl (0,14 µmol) serotonin·HCl, 10 µl dil buffer	9265
Solution A, but 10 µl chlorpromazine (500 µg) instead of 10 µl dil buffer	9041
Solution A, but 10 µl reserpine (10 µg) instead of 10 µl dil buffer	8996
Solution A, but 10 µl imipramine (28 µM final concentration) instead of 10 µl dil buffer	7607
Solution A, but 10 µl amitriptyline (10 µg) instead of 10 µl dil buffer	8767
Solution A, but 10 µl mianserin (10 µg) instead of 10 µl dil buffer	8806
Solution A, but 10 µl doxepin (12,2 µg) instead of 10 µl dil buffer	8503
Solution A, but 10 µl dothiepin (10 µg) instead of 10 µl dil buffer	8945
Solution A, but 10 µl chlorimipramine (34 µM final concentration) instead of 10 µl dil buffer	8066

that established by two groups of workers investigating inhibition of serotonin binding in human brain membranes and inhibition of serotonin binding on intact human and rat platelets (Paul et al., 1981; de Felipe et al., 1982). In both studies the two most effective drugs were imipramine and chlorimipramine. Chlorpromazine was one of the least effective drugs. Amitriptyline and doxepin occupied intermediate positions in both studies. In the present study, the order from most to least effective was:
imipramine, chlorimipramine, doxepin, amitryptiline and chlorpromazine.

The results must be interpreted with caution because the RIA represents a complicated system in which many equilibrium reactions are taking place. Sufficient evidence exists, however, to indicate that serotonin and drugs may affect the PF4-antibody reaction, and that further research is warranted.

Section F. SDS electrophoresis of [^{125}I]-PF4 and serotonin-binding protein

1. Method

7,5% acrylamide gels containing 3,8% bis-acrylamide, 0,1% SDS, 0,15% ammonium persulphate and 0,025% Temed were prepared in 0,27 M Tris buffer, pH 8,8. The SDS concentration used by other workers varies from 0,1% (Wu et al., 1977) to 0,3% (Moore & Pepper, 1976) and 1,0% (Fairbanks et al., 1971). The overlay solution described by Fairbanks (ibid.), consisting of 0,1% SDS, 0,15% ammonium persulphate and 0,05% Temed was applied immediately after the gels were poured. It was left on the gels for about 2 h, at which time it was replaced with electrophoresis buffer. The gels were allowed to stand at ambient temperature overnight, or for a few days.

The electrophoresis buffer, pH 8,3, consisted of 0,025 M Tris, 0,192 M glycine and 0,1% SDS.

The sample buffer, pH 8,8, consisted of 0,09 M Tris, 9% glycerol, 9% β-mercaptoethanol and 1,8% SDS. The samples were boiled for 2-3 min. To increase the density of the samples and serve as tracking dye, a few drops of a solution containing sucrose (4 g/10 ml distilled water) and bromphenol blue (4 mg/10 ml) were added to the samples just before the samples were applied to the gels.

After electrophoresis, the gels were reamed with isopropyl alcohol and removed from the tubes. Isopropyl alcohol was used instead of water to prevent possible elution of small bands. The method of Fairbanks et al. (1971) was used for staining the gels. Gels were stained for protein in a solution of 25% isopropyl alcohol, 10% acetic acid and 0,025% Coomassie blue. Destaining was accomplished with 10% isopropyl alcohol, 10% acetic acid and 0,0025% Coomassie blue for 6-9 h, then 10% acetic acid overnight.

Electrophoresis was performed on ISCO (Nebraska, USA) electrophoretic equipment. Constant power at 1 mA/gel initially and then 3 mA/gel was used. Migration took place towards the anode. The cell was water-cooled during electrophoresis, which took 6-8 hours.

2. Results and Discussion

Samples of protein that had been eluted from the serotonin-affinity columns by 1 M acetic acid or 5% EDTA usually produced only one band, Band I, when stained for protein (Fig. 2-21, 1). Band I was very sharp and narrow and occurred at about 90% the distance travelled by the tracker dye. The position of Band I is consistent with that of PF4 monomers (M_r = 7780).

The samples eluted from the affinity columns occasionally also produced a second band when stained for protein, Band II (Fig. 2-21, 2). When stained with toluidine blue for carbohydrates, Band II was initially negatively stained (lighter than the background colour), but became visible (blue) after about 2 weeks. However, another area of the gel immediately stained purple with toluidine blue; this purple area was irregular and extended from about the tracker dye position to Band II (Fig. 2-21, 6). With the carbohydrate stain, Band I was invisible.

The position of Band II (Fig. 2-21, 5) between Band I and the major band produced by commercial bovine serum albumin (BSA) (Fig. 2-21, 4) is consistent with the expected position of the proteoglycan carrier for PF4, since the M_r of the latter is 59 000 and that of BSA is 68 000. Furthermore, the staining characteristics of Band II are consistent with its having been produced by a glycosaminoglycan (i.e., the band is visible when stained for either protein or carbohydrate). The negative staining property of Band II is similar to that described

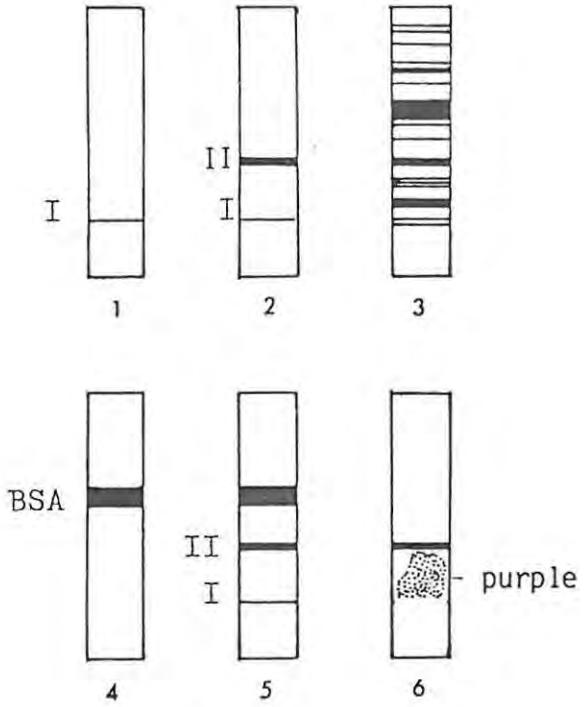


Fig. 2-21

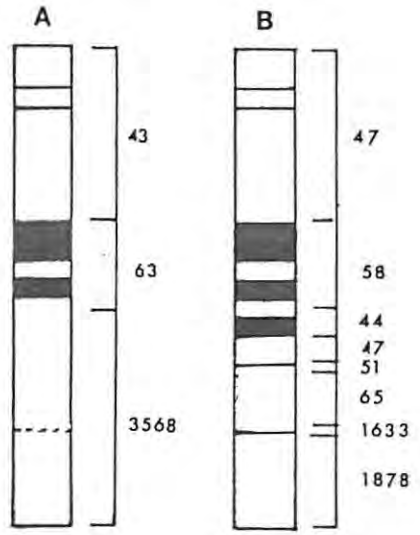


Fig. 2-22

Fig. 2-21 (left) and 2-22 (right). SDS electrophoresis gels. Electrophoresis was in the direction of the anode (bottom of the gel). The gels were stained for protein as described in Section F.1, except for 2-21, 6, which was stained with toluidine blue for carbohydrates.
Fig. 2-22: SDS electrophoresis gels of $[^{125}I]$-PF4 from the PF4 RIA kit. A, $[^{125}I]$-PF4 sample only; B, $[^{125}I]$-PF4 co-electrophoresed with 34. The numbers in Fig. 2-22 refer to the cpm of individual sections as determined in the gamma counter (background was 31 cpm).

for a PF4-heparin complex (Handin & Cohen, 1976).

The results obtained from electrophoresis of 34 (Table 2-10) supported the identification of Band I as monomeric PF4 and of Band II as the proteoglycan carrier for PF4. Evidence from the PF4 RIA and Hartree protein determination suggest that the protein in 34 was pure PF4, since its specific activity was 1 ng PF4/ng protein.

The band seen on gels to which bovine serum albumin was added (Fig. 2-21, 4 and 5) was never present on gels containing serotonin-binding protein from the affinity columns, although the supernatant of the platelet extract produced a heavy band at the same position as that occupied by commercial BSA (Fig. 2-21, 3).

SDS electrophoresis of the $[^{125}I]$-PF4 sample from the RIA kit produced the gel that is depicted in Fig. 2-22, A. The several bands which appeared on the $[^{125}I]$-PF4 gels were caused by presence of BSA and BGG in the Abbott product (private communication, Abbott). Band I on this gel was very faint, due to the low content of PF4 in the sample. This gel was sliced at the points indicated in the diagram and the pieces were counted in a gamma counter. 99% of the radioactivity was located at the anode end of the gel, beyond Band II.

On another gel $[^{125}I]$-PF4 was co-electrophoresed with 34 (Table 2-10) and the stained gel was sliced into sections (Fig. 2-22, B). Again, 99% of the radioactivity was present in the section of the gel anodal to Band II. Band I, which comprised the thinnest section of the gel, had associated with it 45% of the gel's total radioactivity. The remainder of the radioactivity was associated with the anode endpiece, the position occupied by small by-products of radiolabelling. This area of the gel did not stain with Coomassie blue, and therefore did not contain protein.

Section G. Chapter Summary

The results obtained from experiments described in this chapter show that:

1) The protein that binds to the affinity column shows specific affinity for serotonin. A control using an ethanolamine affinity column in place of serotonin, bound less than 26% of the protein, whereas 99% of the protein of the same solution bound to the serotonin-affinity column (Section I.D.1).

2) The serotonin affinity gel displays selectivity. Bovine serum albumin does not bind to the serotonin-affinity gel when applied in a starting buffer containing Fe^{2+}, and in the absence of Fe^{2+}, only 10% of the bovine serum albumin is bound to the gel (Table 2-5, Section I.D.1).

3) The following evidence shows that the protein which binds specifically to the serotonin-affinity column is bound strongly:

a) The volume of sample applied to the affinity columns is independent of the amount of protein bound to the column even when the volumes greatly exceeded 5% of the bed volume (Fig. 2-6, Section I.D.1).

b) Only 5% EDTA, pH 4,5, and 1 M acetic acid, pH 2,3, out of 28 buffers tried (including chaotropes), were effective as eluting buffers (Sections I.D.1 and D.2).

4) The serotonin-binding protein was stable.

a) It retained its binding properties for more than a week at 4-8 ^{o}C in 66 mM phosphate buffer containing 0,02% sodium azide at pH 7,65 (Fig. 2-7, Section I.D.1).

b) Protein from experiment 9 was left at room temperature (above 18 ^{o}C) for at least two hours during fractionation and was still able to bind to a serotonin-affinity column the next day (Section I.D.1).

5) The serotonin-binding protein has a low molecular mass.

a) It permeated dialysis membrane after having been eluted from the affinity column with 1 M acetic acid (Table 2-8, Section I.D.2).

b) It emerged later than the void volume of Sephadex G-25 after elution from the serotonin-affinity column (Section I.D.2).

c) Most of it was lost on a Minicon B15 concentrator, which is intended for use with proteins of M_r of 15 000 or greater and adsorbs proteins of lesser mass (Section I.D.2).

6) The following observations indicate that the serotonin-binding protein is associated with carbohydrate:

a) A_{280} has a value 3-5 times that of the protein concentration as determined by the Lowry method, but of about the same value when the protein concentration is determined by the Hartree method (Section I.D.3).

b) Thin-layer chromatography of the serotonin-binding protein showed that, besides a ninhydrin-reacting protein, a

diphenylamine-reacting substance is present (Section I.D.2).

c) The A_{280}/A_{260} ratio of the serotonin-binding protein fell in the range from 0,70-0,80 (Section I.D.3).

7) The serotonin-binding protein may be associated with nucleotide that is not as strongly bound as is the carbohydrate.

a) The A_{280}/A_{260} ratio of solutions freshly eluted from the affinity column was 0,40-0,70, but this ratio increased with time to a maximum of about 0,80 (Section I.D.3).

b) 5% EDTA eluted a substance with λ_{max} at 258-264 nm that did not fluoresce from the serotonin-affinity column (Section I.D.3).

8) The absence of an absorbance peak at about 280 nm (Fig. 2-11, Section I.D.3) indicates that the serotonin-binding protein has a very low aromatic amino acid content.

The above points show that the serotonin-binding protein might be PF4. This identification is supported by the following evidence:

9) Protein in some fractions eluted from the affinity columns reacted nearly 1:1 with PF4 antibody (Table 2-10, Section II.C.), but only slightly with βTG antibody (Table 2-9, Section II.B.).

10) $[^{125}I]$-PF4 on serotonin-affinity and control columns behaved like the serotonin-binding protein from platelet extracts (Tables 2-11 and 2-12, Section II.D.).

11) The serotonin-binding protein appeared to have a molecular mass similar to that of PF4, because, when the serotonin-binding protein was co-electrophoresed on SDS gels with $[^{125}I]$-PF4 and the gel was stained for protein, about half of the radioactivity was found in association with the very narrow protein band which represented the serotonin-binding protein (Section II.F.).

12) The fluorescence pattern of exhaustively dialysed serotonin-binding protein was similar to that of purified PF4 (Fig. 2-18, Section I.D.3).

All the evidence cited supports the initial hypothesis that a platelet-specific releasable protein does bind serotonin in vitro. Further work attempts to characterize the binding in terms of the number of binding sites for serotonin on PF4 and the binding affinity of serotonin for PF4, because the functions of serotonin and PF4 must depend on these factors.

CHAPTER 3
Investigations into Serotonin Binding by Purified Human PF4 *in vitro*

I. Introduction

Equilibrium dialysis is an accepted method for the determination of binding affinity and the number of binding sites for a ligand on a protein. Repetitive experiments investigating serotonin binding to PF4 *in vitro*, however, showed that equilibrium dialysis was not entirely satisfactory, for reasons which will become apparent.

II. Materials

PF4: PF4 was prepared according to the method of Wu *et al.* (1977), starting with whole blood from normal human volunteers. The blood had been drawn 10 h or less before preparing the platelet pellet. Platelet concentrates are not available locally. See Section III, below, for details of the PF4 preparation. Purified PF4 prepared from platelet concentrates was kindly supplied by Pepper, Edinburgh, and Niewiarowski, Philadelphia.

Serotonin: $[^{14}C]$-serotonin creatinine sulphate (57 mCi/mmol) and $[^{3}H]$-serotonin creatinine sulphate (10 Ci/mmol) were supplied by The Radiochemical Centre, Amersham. $[^{3}H]$-serotonin binoxalate (33,8 Ci/mmol) was obtained from New England Nuclear. Concentration of serotonin is obtained from dpm by the formula $2{,}22 \times 10^{9}$ dpm = mCi, and the specific activity of the radiolabelled serotonin.

Membranes: Union Carbide membrane and Spectra Por 2 and 4 membranes (Spectrum Labware, USA) were used for equilibrium dialysis in cells made by the Chemical Rubber Co. (USA). Membranes (14 cm long) were soaked in distilled water long enough to produce separation of the adhering surfaces, cut along each edge, then heated to 80 ^{o}C in 2 per cent Na_2CO_3 for 10 min. The membranes were cooled and rinsed in several changes of distilled water. Prepared membranes were stored in water in the refrigerator. Sodium azide (0,02%) was added if the prepared membrane was to be stored for more than a few days.

Equilibrium dialysis cells: Equilibrium dialysis experiments were done in multi-cell plastic blocks (Chemical Rubber Co., USA). Five pairs of 1-ml cells are contained in each block. The ports were closed with stoppers.

Other chemicals: Hypoxanthine, bovine serum albumin, mixed chondroitin sulphate and ADP were obtained from Sigma. Sephadex G-25 and Heparin-Sepharose CL-6B were supplied by Pharmacia (Sweden).

Buffers: 66 mM phosphate buffer, pH 7,4, containing 1 M NaCl was used for all experiments described in this chapter except as noted.

III. Methods

a) Isolation of PF4 (Wu et al., 1977): The method was slightly modified. The platelet pellet was prepared and the platelet contents released by the methods described in Chapter 2, Sections I.A. and B. The 23 350 g supernatant was mixed with prepared heparin-sepharose gel that had been washed and swollen according to the manufacturer's instructions. After 2 h at 20 ^{o}C, the mixture was filtered over G3-fritted glass, then suspended in phosphate buffer, pH 7,2-7,4, and poured into a K16 (Pharmacia) column. The settled gel was step-eluted, according to Wu et al., with increasing concentrations of buffered NaCl at 0,25 M, 0,5 M, 1 M and 1,3 M. PF4 was eluted with 1 M NaCl. Less than 5 mg of lyophilized purified PF4 was the yield from 7 l whole blood. Wu et al. reported that they obtained 93 µg PF4/ml platelet concentrate.

b) Equilibrium dialysis: Equilibrium dialysis is a material balance method for measuring the concentrations of free ligand and protein in a system that has achieved equilibrium. In order to demonstrate that a system is at true equilibrium, it is necessary to show that the final concentrations of protein, ligand and complex can be reached from either direction (Williams & Lefkowitz, p. 30, 1978). For convenience in calculating the concentrations of ligand, trace amounts of radiolabelled-serotonin were added to nonradioactive serotonin. The solution containing PF4 was separated from the opposite chamber by a membrane which was permeable to all the components of the system except to the PF4. Radiolabelled serotonin was introduced into the chamber that contained PF4. After equilibration, a sample from the chamber containing PF4 (the P side of the membrane) gave the sum of the concentrations of bound and free serotonin. A sample from the

other side of the membrane (the N side) gave the concentration of free serotonin. Therefore, the concentration of bound serotonin was represented by the difference in concentration between the P and N sides of the membrane. The average number of serotonin molecules bound to each molecule of PF4, $\bar{\nu}$, was calculated by dividing the concentration of bound serotonin by the total concentration of PF4.

$\bar{\nu}$ is calculated at equilibrium to be $\left(\frac{[5\text{-HT}]_P - [5\text{-HT}]_N}{[5\text{-HT}]_P + [5\text{-HT}]_N}\right)\left(\frac{[5\text{-HT}]_{Total}}{[PF4]_{Total}}\right)$

where 5-HT_F is free serotonin; 5-HT_{PF4} is serotonin bound to PF4; and 5-HT_{Total} is the total concentration of serotonin added to the system. Although $5\text{-HT}_P + 5\text{-HT}_N$ should be equal to 5-HT_{Total}, the terms are retained in the equation above because some serotonin may be bound to the membrane in the experiments described in this chapter.

$5\text{-HT}_P + 5\text{-HT}_N$ is the concentration of all soluble serotonin, that is, serotonin that is free and serotonin that is bound in any soluble complex, such as, bound to a soluble protein. As described in Section IV., equilibrium did not appear to be attained in these experiments, and ∇ is used strictly for comparative purposes. To designate this, $\bar{\nu}$ is referred to as "apparent $\bar{\nu}$" in this chapter.

c) Gel filtration: The method was a modification of that used by Tamir et al. (1980a). The constituents of the system were mixed in a small volume of buffer and incubated. Then 0,1 ml of the solution was chromatographed on an equilibrated Sephadex G-25 column. Fractions were monitored for radioactivity. Radioactivity due to free serotonin emerges after three void volumes (see Chapter 2); thus, radioactivity at the void volume represented serotonin that was bound to PF4 or to another macromolecule. The flow rate of the G-25 column was about 0,3 ml/min. 24 fractions were collected, the first at t=0 (the time of sample application) and the last, after the free serotonin peak. The void volume peak occurred in fractions 4-8. "Total radioactivity" was the radioactivity measured in 0,1 ml samples taken from fractions 4-24.

Radioactivity measurements: The external standard channels ratio method was used initially to determine efficiency. Quench curves for ^{14}C and ^{3}H were prepared for each of the two Beckman LS3150T scintillation counters used, so that dpm values for either isotope might be obtained from either instrument. Later experiments were done on a Beckman LS2800, which automatically corrected

for quench by comparison with commercial quenched standards and printed out dpm values.

IV. Equilibrium Dialysis Results and Discussion

The first equilibrium dialysis experiment was carried out using PF4 supplied by Niewiarowski (Fig. 3-1). On the basis of data at 8,75 h, the average number of serotonin molecules bound by one molecule of PF4 ($\bar{\nu}$) was 4,2. At 23,5 h $\bar{\nu}$ was 3,3. This discrepancy makes the equilibrium dialysis results suspect. It is possible that true equilibrium had not been attained. For this reason all values obtained for $\bar{\nu}$ in the group of experiments described in this chapter will be considered as apparent $\bar{\nu}$. In addition, the radioactivity (dpm) of the nonprotein (N) side increased nearly 6 times as much as the radioactivity of the protein (P) side decreased over the same time period. This is anomalous since a decrease on the P side is expected to occur concomitantly with an increase on the N side and ought to be of similar magnitude, that is, $5\text{-HT}_P + 5\text{-HT}_N$ is a constant.

The experiment was repeated using more of the same PF4 solution (refrigerated during the 2-day interim). At 10 h, the apparent $\bar{\nu}$ was 2,5 and at 14 h the apparent $\bar{\nu}$ was 1,2. Between 10 and 14 h, the radioactivity of the N side increased more than 4 times as much as the radioactivity of the P side decreased. A possible explanation is that the increase on the N side was due to serotonin that previously had been attached to the membrane and now was free. The higher total dpm of 5-HT_N plus 5-HT_P at 14 h than at 10 h supports this hypothesis.

Equilibrium dialysis of serotonin and 0,0645 μM PF4, which was prepared in this laboratory following the method of Wu et al (1977), was conducted at 2-4 ^{o}C. These results were totally inconclusive as samples taken at 19,5 h had apparent $\bar{\nu}$ of -2,47, a negative apparent $\bar{\nu}$ value. This implies that more serotonin was on the N side than on the P side of the membrane. However, samples taken at 21 h had an apparent $\bar{\nu}$ of 1,6, as did samples taken 5 h later (after 2 h at ambient temperature). After an additional 19,5 h at ambient temperature, the apparent $\bar{\nu}$ was -1,9. The decrease in radioactivity on the P side during the last time interval was not reflected in an increase on the N side, but rather, the values for the N side also decreased. Since the total cpm of the system decreased as compared with the results from the

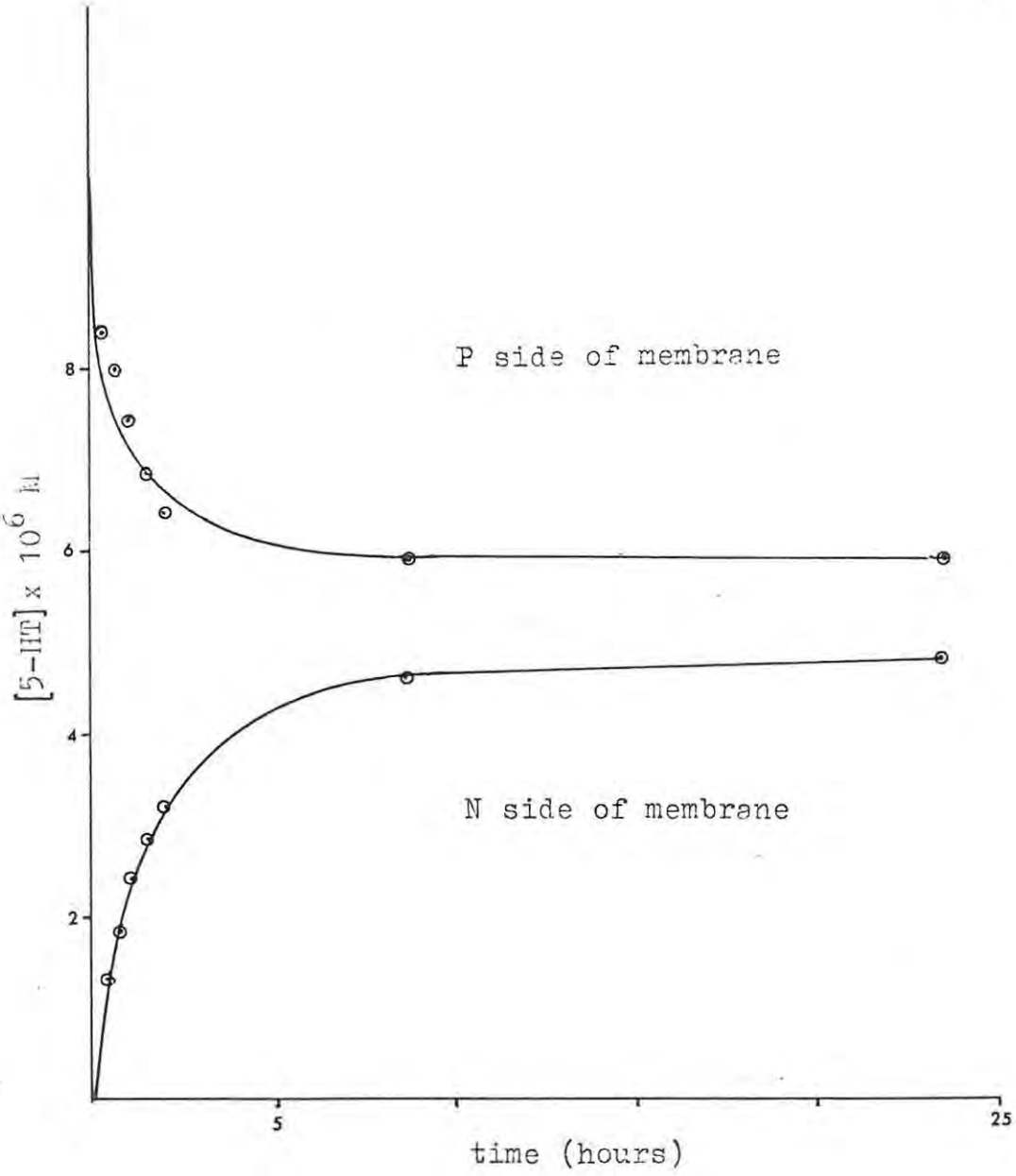


Figure 3-1. Equilibrium dialysis at 20 °C in a Chemical Rubber Company multi-cavity cell of 0,536 µM purified tetrameric PF4 (supplied by Niewiarowski) in 1 M NaCl, 66 mM phosphate buffer, pH 7,4, with 17,5 µM [^{14}C]-serotonin creatinine sulphate added to the P side of the membrane (Union Carbide). Radioactivity was measured on both sides of the membrane. Each point represents the mean of two samples.

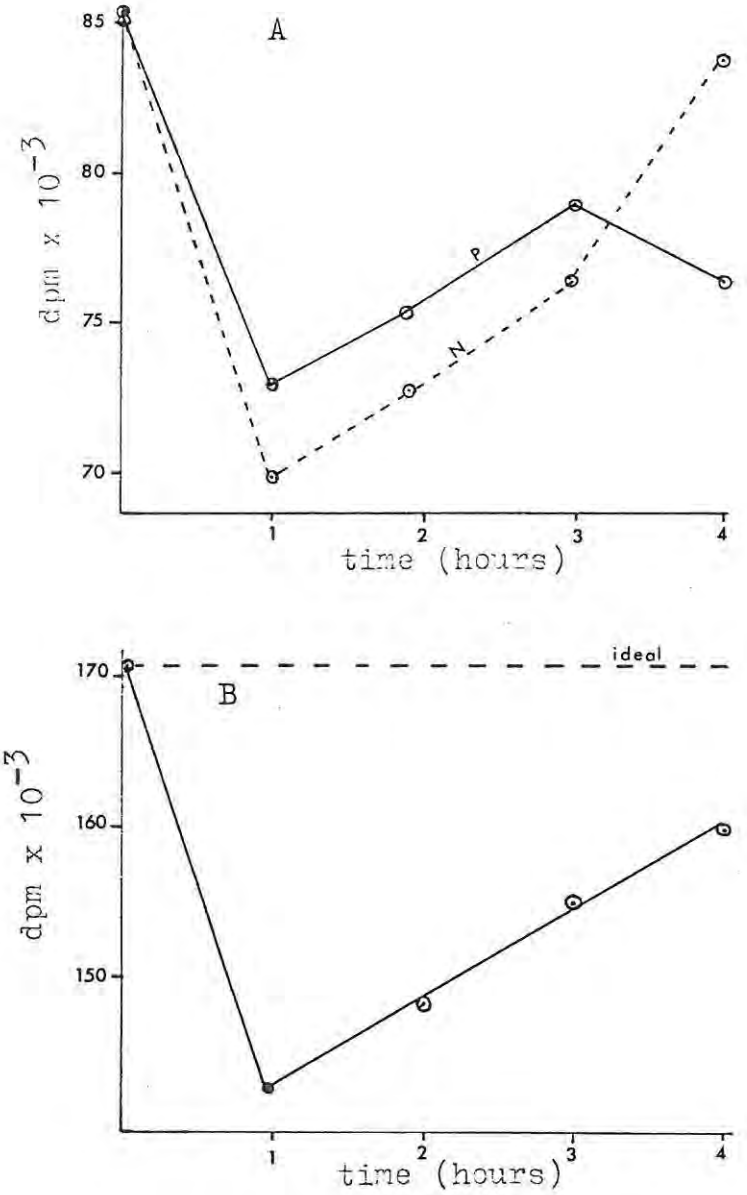
previous samplings, it seemed possible that radioactive serotonin became attached to the membrane during the last 19,5 h period.

In a subsequent experiment, after 9,75 h at 17 ^{o}C, with the same solution of PF4, apparent $\bar{v}$ values were 0,004, 0,97 and 2,03. In this experiment PF4 concentration was 0,025 μM and the [serotonin]/[PF4] ratios were 1,45, 4,8 and 9,7 respectively. Using the same PF4 concentration, but using PF4 supplied by Pepper, and with a serotonin concentration of 2,6 μM, ([serotonin] / [PF4]) = 100 , values of 6,4 and 7,0 for apparent $\bar{v}$ were obtained after incubation at 17 ^{o}C for 8 h. After 24 h values were -2,4 and -1,5. (All equilibrium dialysis measurements were in duplicate.) The radioactivity of the P side decreased by more than twice the increase on the N side between 8 and 24 h.

These results implied that 1) equilibrium was not being attained during equilibrium dialysis (additional experiments confirmed this), and 2) serotonin was binding to the membrane. An equilibrium dialysis cell containing Pepper's PF4 (0,648 μM) and 16 μM [^{14}C]-serotonin was refrigerated for 23 h and then incubated at ambient temperature. Samples were taken immediately after refrigeration and then at hourly intervals for the next four hours. The apparent $\bar{v}$ of the samples immediately after refrigeration was -0,02; apparent $\bar{v}$ values at ambient temperature were 0,54; 0,42; 0,37 and -1,1 for the next 1, 2, 3 and 4 h, respectively. The dpm of the P and N sides of the cell are plotted in Fig. 3-2A. Total dpm of the samples immediately after refrigeration was 170 560; the same calculation for samples taken 1, 2, 3 and 4 h after refrigeration gave values of 142 757; 148 152; 155 400 and 160 417 (see Fig. 3-2B). In an ideal system with no ligand-binding by the membrane, the total dpm of the N and P sides at all sampling times would be the same. Steinhardt and Reynolds (1969) have warned that significant errors in the determination of $\bar{v}$ are likely when there is the "loss" of ligand shown in Fig. 3-2B.

To confirm these results, duplicate pairs of cells with and without PF4 were prepared and all samples were measured by weight, instead of by volume. Also [^{3}H]-serotonin creatinine sulphate was substituted for [^{14}C]-serotonin creatinine sulphate. Samples were taken at 0,066, 2, 2,5 and 19 h. The results are plotted in Fig. 3-3 and suggest that labelled serotonin was bound by the membrane at 2 and 2,5 h and that this binding was enhanced by the presence of PF4.

The ionic strength of the buffer in which the membrane had been



Figures 3-2A and 3-2B. Equilibrium dialysis of 0,65 µM PF4 (supplied by Pepper) with 16 µM [^{14}C]-serotonin creatinine sulphate. Membrane: Union Carbide. The cell was refrigerated for 23 h at which time (0 in figures above) samples were taken. Then the cell was incubated at 16 °C for 4 more hours, and samples were taken hourly. In A, the dpm of the P and N sides are plotted separately. In B, the sum of the dpm of the P and N sides is plotted. A departure from the ideal is indicated, suggesting ligand-binding by the membrane.

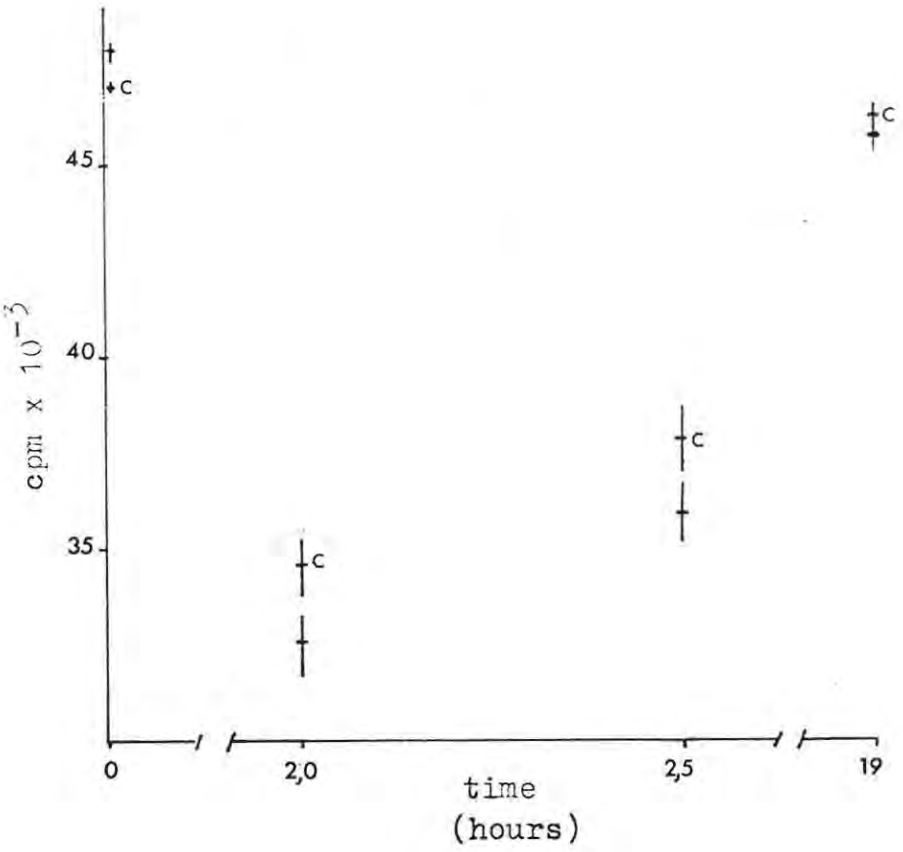


Fig. 3-3. Equilibrium dialysis at 21 °C of 6,4 μM PF4 (supplied by Pepper) with 18,8 μM [^{3}H]-serotonin. The cell contained a Spectra Por 4 membrane. The cell was incubated for 19 h, and weighed samples obtained during this period were counted for radioactivity. Samples were taken at 0,066, 2, 2,5 and 19 h. The range and mean of duplicate samples is given at each time. These values represent the sums of both sides of the membrane. C is control, i.e., without PF4 on either side of the membrane. Buffer: 50 μM $CaCl_2$, 2,5 mM ATP, 100 mM KCl, 600 mM NaCl, 5 mM $MgCl_2 \cdot 6\ H_2O$ and 20 mM Tris, pH 7,4.

soaked for several days and in which the experiment was conducted was greater than 0,7 (0,6 M NaCl; 0,1 M KCl in buffer). Thus, the charges on the PF4 molecule represented only a small proportion of the total charges present. A small diffusible charged molecule, such as serotonin, would be expected either to bind specifically to soluble protein and be concentrated on the protein side, or not to bind and distribute equally on either side of the membrane, rather than to bind to the membrane. Therefore, the results suggest that the enhanced binding of serotonin to the dialysis membrane *in vitro* in the presence of PF4 is related to a function of PF4 *in vivo*. Gel filtration results support this explanation (see this chapter, Section V) by indicating that at physiological concentrations PF4 apparently bound more serotonin than at nonphysiological concentrations.

The presence of 2% bovine serum albumin (BSA) in the cell affected the distribution of serotonin in dialysis experiments. In one experiment after 22 h incubation at ambient temperature, apparent $\bar{\nu}$ values of -2,2 and -1,9 were obtained in the absence of BSA, while an apparent $\bar{\nu}$ of 1,7 was obtained by difference in cells containing BSA and PF4, or BSA alone (Table 3-1). Serotonin-binding by platelet albumin has been reported previously (Tamir *et al.*, 1980a; Pignatti & Cavalli-Sforza, 1975). The results in Table 3-1 suggest that less serotonin is bound by the membrane when BSA is present than when BSA is absent.

In general, the results obtained from the equilibrium dialysis experiments were anomalous. The source of PF4 did not seem to make any difference: equilibrium did not appear to be achieved with PF4 received from Niewiarowski, Pepper or with PF4 prepared in this laboratory from fresh whole blood.

Moreover, evidence is accumulating which shows that 1) serotonin promotes the movement of proteins across biological membranes *in vivo* (Westergaard, 1978; Westergaard & Brightman, 1973; Costa *et al.*, 1974a) and that 2) PF4 can traverse biological membranes *in vivo* (Goldberg *et al.*, 1980; Dawes *et al.*, 1978). Furthermore, a heat-stable protein of M_r 30 000 that had antiheparin activity and was obtained from a human platelet acid extract has been shown to produce an increase in vascular permeability (Nachman *et al.*, 1972). Obviously, if PF4 does not remain entirely on one side of the membrane, or if PF4 increases permeability of the membrane to serotonin, no accurate binding parameters will be obtained from equilibrium dialysis experiments.

Table 3-1. Equilibrium dialysis of tritiated serotonin (1,1 μM) and PF4 (prepared in this laboratory) (0,032 μM). BSA*, when present, 2%. Buffer: 66 mM phosphate, 1 M NaCl, pH 7,4. Incubation for 22 h, 19-20 ^{o}C.

Protein	apparent $\bar{v}$
PF4 & BSA (bin)*	6,54
BSA only (bin)	4,80
PF4 (bin) (by difference)	1,74
PF4 alone (bin)	-1,85
PF4 alone (cs)*	-2,18

* Abbreviations as follows: BSA, bovine serum albumin; bin, radiolabelled serotonin binoxalate; cs, radiolabelled serotonin creatinine sulphate.

Even if PF4 does not actually permeate the membrane, if it binds to the membrane, which would not be surprising in view of PF4's known ability to bind to surfaces (Bolton et al., 1976a; Handin & Cohen, 1976; Nath et al., 1973), all binding data would be grossly distorted.

The results that indicate that serotonin was bound by the dialysis membrane confirm the findings of others (Tamir, 1982). Steinhardt and Reynolds (1969) have warned of adsorption of ligand onto the membrane when the ligand is combined with protein. Much larger amounts of ligand may be adsorbed than unassociated ligand.

In view of the above considerations, gel filtration was tried as a means for measuring serotonin binding by PF4.

V. Gel Filtration Results and Discussion

Application of ligand-protein mixtures to a column of Sephadex G-25, followed by elution with buffer, separates the mixture so that the eluant at the void volume contains the protein with bound ligand, while free ligand elutes much later. Chignell (1971) has warned that kinetic parameters obtained by this technique can be inaccurate, because the rate of elution is generally much lower than the rate of dissociation of the ligand-protein complex. However, this objection does not preclude the procedure as a comparative technique, as long as the same protein and ligand in various solutions are used. The method has the advantage that there are no membrane surfaces and thus no concomitant binding.

PF4's known ability to bind to Sephadex (Nath et al., 1973; Handin & Cohen, 1976) can be dealt with on theoretical grounds: in the absence of other macromolecules or complexes, serotonin that emerges at the void volume must be bound by PF4. Also, in the presence of another macromolecule, the values for the binding attributable to PF4 may be obtained by conducting a parallel experiment in which PF4 is omitted and the macromolecule is present in the same concentration as in the first gel filtration; the difference in radioactivity at the void volume would be due to PF4, if there is no interaction between PF4 and the macromolecule.

Comparative studies of radioactivity excluded by Sephadex G-25

were undertaken of solutions containing purified PF4 and [^{3}H]-serotonin binoxalate. No determination of kinetic parameters was attempted from these studies.

The results indicated that radioactive serotonin was bound by PF4, since the addition of PF4 in parallel experiments increased the percentage of total radioactivity in the fractions constituting the void volume peaks (Fig. 3-4 and Table 3-2).

Physiological concentrations of constituents were used in gel filtration experiment 9. The concentration of serotonin in normal human serum is 0,2 µg/ml (Davis, 1959), or 1,13 µM, that is, the same concentration as used in gel filtration experiment 9. The concentration of PF4 in platelet-poor plasma obtained from apparently healthy individuals is about 13 ng/ml, while that of serum is about 8000 ng/ml (Bolton et al.,(1976a); the concentration of PF4 employed in gel filtration experiment 9 was 480 ng/ml (0,0155 µM), which would obtain in partial platelet release. Holmsen et al. (1972) has shown that during platelet release metabolic ATP breaks down to hypoxanthine and the latter appears in the plasma. Holmsen incubated 2,7 µM radiolabelled adenine in platelet-rich plasma, and 14% of the labelled adenine was converted to hypoxanthine (0,38 µM). In the present study 0,35 µM hypoxanthine was used.

The percentage of total radioactivity eluted in the void volume peak seemed to be dependent on the ratio of the concentrations of serotonin and PF4. For instance, a comparison of the results of gel filtration experiments 5 and 6 and gel filtration experiments 9 and 10 (Table 3-2) shows that the amount of binding attributable to PF4 was very different in the two sets of experiments:

At a [serotonin]/[PF4] ratio of 0,070 (in gel filtration experiments 5 and 6), 5,51% of the total radioactivity was eluted in the void volume peak in the presence of 35,6 µM PF4 and 2% BSA, while 5,14% of the total radioactivity was eluted in the void volume peak when PF4 was absent. Therefore, 0,37% (5,51-5,14) of the total radioactivity is attributable to PF4, and 5,14% of the total radioactivity is attributable to BSA. However, BSA was present as a 2% solution (20 mg/ml), which is 294 µM (M_r 68 000), or 8,26 times the concentration of PF4 in the same solution. BSA was responsible for nearly 14 times, that is 5,14/0,37, as much void volume radioactivity as was PF4. Therefore, BSA effectively bound 1,7 times (14/8,26) as much serotonin per mole as did PF4.

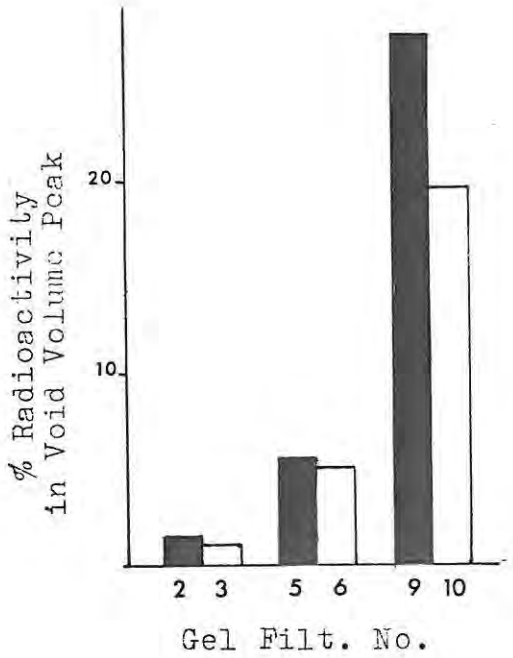


Figure 3-4. Comparative gel filtration experiments. Solid bars, with PF4. Open bars, without PF4, all other conditions the same within each pair. See Table 3-2 and text for further explanation.

Table 3-2. Gel filtration experiments on Sephadex G-25 at ambient temperature.
Buffer: 66 mM phosphate, 1 M NaCl, pH 7,3-7,4.

Gel Filt. No.	PF4 µM	5-HT µM	[5-HT]/[PF4]	Additional Constituents*	Incub. time, temp.	% Total Radioactivity in Void Volume Peak
1	198	10	0,05	1,3 mg Ch SO_4/0,1 ml	1h,15^{o}	3,7
2	154	2,5	0,02	0,6 mg Ch SO_4/0,1 ml, $4{,}86 \times 10^{-4}$ M DTT	1h,12-17^{o}	1,6
3	0	2,5	-	as in Gel Filt. 2	1h,13^{o}	1,1
4	64,8	5,0	0,08	2,76 mM ADP, 0,2 mg Ch SO_4/0,1 ml	1h,34-37^{o}	1,5
5	35,6	2,5	0,07	2% BSA	1h,25-37^{o}	5,51
6	0	2,5	-	2% BSA	1h,35-38^{o}	5,14
7	55,1	2,5	0,05	9,53 mM hypoxanthine	1h,37^{o}	0,89
8	29,1	880	30	6,6 mM hypoxanthine, 2% BSA	1h,37^{o}	8,46
9	0,0155	1,13	729	0,35 µM hypoxanthine, 2% BSA	0,33h,37^{o}	27,6
10	0	1,13	-	as in Gel Filt. 9	0,33h,37^{o}	19,75

* Abbreviations: 5-HT, serotonin; Ch SO_4, chondroitin sulphate; BSA, bovine serum albumin

At a [serotonin]/[PF4] ratio of 729 (in gel filtration experiments 9 and 10), 27,6% of the total radioactivity was eluted in the void volume peak in the presence of 0,0155 μM PF4 and 2% BSA. 19,75% of the total radioactivity was eluted in the void volume peak when PF4 was absent. Therefore (assuming, for the moment, that hypoxanthine's presence is irrelevant), 7,85% (27,6-19,75) of the total radioactivity is attributable to PF4, and 19,75% of the total radioactivity is attributable to BSA. However, BSA was again present in a concentration of 294 μM, that is, nearly 19 000 times the concentration of PF4 (294/0,0155). BSA was responsible for 2,5 times (19,75/7,85) as much void-volume radioactivity as PF4 was responsible for. Therefore, BSA effectively bound only 1,3 x 10^{-4} (2,5/19 000) as much serotonin per mole as did PF4. Moreover, at this higher [serotonin]/[PF4] ratio, PF4 seemed to bind much more serotonin than it did at the lower [serotonin]/[PF4] ratio (see Fig. 3-4).

The much greater ability to bind serotonin demonstrated by PF4 at the higher [serotonin]/[PF4] ratio cannot be due only to the presence of hypoxanthine. This is indicated by the lack of correlation between the concentration of hypoxanthine and the percentage of total radioactivity in the void volume peaks in gel filtration experiments 6-10.

It is not surprising that dithiothreitol does not enhance serotonin binding by PF4. The disulfide bonds in PF4 are believed to be intramolecular stabilizing structures (Hermodson et al., 1977).

The results of gel filtration 4 (see Table 3-2) suggest that ADP may inhibit serotonin-binding by PF4. Further evidence that ADP may inhibit serotonin binding by PF4 is presented in Chapter 5, Section IV.C.

In conclusion, the results presented in this chapter provide evidence for the following points:

1) (From gel filtration experiments) PF4 binds more serotonin per mole than does BSA at physiological concentrations of PF4 and serotonin.

2) (From gel filtration experiments) 2% BSA appears to enhance the ability of PF4 to bind serotonin. 10^{-4} M dithiothreitol appears to have no effect on the ability of PF4 to bind serotonin. 2,76 mM ADP appears to inhibit the binding of serotonin by PF4.

3) (From equilibrium dialysis experiments) PF4 appears to enhance the binding of serotonin to dialysis membrane.

From these equilibrium dialysis and gel filtration experiments, no

value was obtained for the association constant or for the number of binding sites for serotonin on PF4. These values will be obtained, undoubtedly, by use of another technique.

A potentially useful technique for determining the parameters of interest was ultrafiltration. However, PF4 has been shown to adsorb irreversibly onto ultrafiltration membranes (Handin & Cohen, 1976). Binding of βTG to ultrafiltration membranes has not been reported. The homology between βTG and PF4 prompted further investigation of serotonin binding by ultrafiltration of βTG. The results of this investigation are reported in the next chapter.

CHAPTER 4

Investigations into serotonin binding by purified human β-thromboglobulin (βTG) *in vitro*

I. Introduction

β-thromboglobulin (βTG) was originally isolated by Moore and Pepper (1976). Like PF4, βTG is found in the supernatant of thrombin-treated washed platelets and can be purified on heparin-agarose columns. Also, like PF4, it is a small protein (M_r about 35 000) and is composed of four protomers. Begg *et al.* (1978) showed the striking homology between these two proteins. When the amino acid sequence of βTG is aligned with that of PF4, 42 of the 81 residues are identical to residues of PF4. Notwithstanding these findings, the two proteins are immunologically distinct. It became necessary to determine whether βTG, like PF4, might bind serotonin. Two techniques were used for determining binding parameters, ultrafiltration on Millipore PSAC filters and the method of Hummel and Dreyer (1962). To provide additional evidence for the results from ultrafiltration and Hummel-Dreyer gel filtration, samples from both techniques were chromatographed on thin-layer plates and the radioactivity at various distances of migration was determined.

It had been shown by Tamir's group (see references in Chapter 1, section I) that Fe^{2+} enhanced binding of serotonin to a serotonin-binding protein they had isolated from rat brain. Therefore, many of the experiments described in this chapter were conducted in the presence of Fe^{2+}.

II. Materials

βTG: lyophilized purified βTG was a gift of D.S. Pepper (Edinburgh, Scotland)

Materials for ultrafiltration: PSAC membranes, 25 mm (17 ml), cell and other components were obtained from Millipore. PSAC membranes have a nominal molecular weight limit of 1000, which means that even if βTG monomerized upon binding serotonin (a possibility which was not tested in these studies), more than 95% of the monomers would probably be

retained by the membrane (Millipore, 1974, p. 55, Fig. 86). The membrane was placed in a 25 mm cell. Compressed nitrogen gas was used to provide the pressure (about 202 kPa) necessary to force the filtrate through the membrane.

Serotonin: $[^{14}C]$ -serotonin creatinine sulphate (specific activity 57 mCi/mmol) was obtained from the Radiochemical Centre, Amersham. The radioactivity of this compound is 1265,4 x 10^8 dpm/mmol serotonin.

Thin-layer chromatographic materials: Glass plates were coated with a 0,25 mm layer of GF 254 silica gel, and were developed in the upper phase of 4:1:5 (butanol:acetic acid:water) solvent (Amin et al., 1954).

Other chemicals: heparin (porcine, intestinal, 159 units/mg) was supplied by Sigma; Sephadex G-25 was obtained from Pharmacia.

Buffers: 66 mM phosphate buffer, pH 7,4-7,6, and 20 mM cacodylate buffer, pH 7,6, were used for the experiments described in this chapter.

III. Methods

The direct-binding method described in the handbook by Millipore (1974, pp. 46-51) was used. In ultrafiltration there is the danger that ligand may be adsorbed to the membrane. Ligand binding to ultrafiltration membrane has been demonstrated previously with methyl orange and bromphenol blue (Blatt et al., 1968). Millipore recommends that each system be inspected for such losses (Millipore, 1974, pp. 50, 51 and 63). They recommend discarding an initial aliquot which is depleted of the ligand due to membrane-binding of the ligand, as demonstrated by filtering a solution of the ligand with no protein present.

Losses due to adsorption by binding of serotonin to the membrane were determined by the procedure recommended by Millipore: a solution of radiolabelled serotonin (with no βTG present) was filtered and small aliquots were collected. The serotonin concentration was determined by radioactivity measurements and plotted against the volume collected, producing the curve shown in Fig. 4-1.

Initially 3,0 ml solutions ranging in concentrations from 0,04-2,6 μM βTG and containing 0,02 M cacodylate buffer, pH 7,6 and ferrous ammonium sulphate in concentrations ranging from 0,066-1,66 μM and heparin in concentrations ranging from 0,05-1,66 mg/ml were incubated

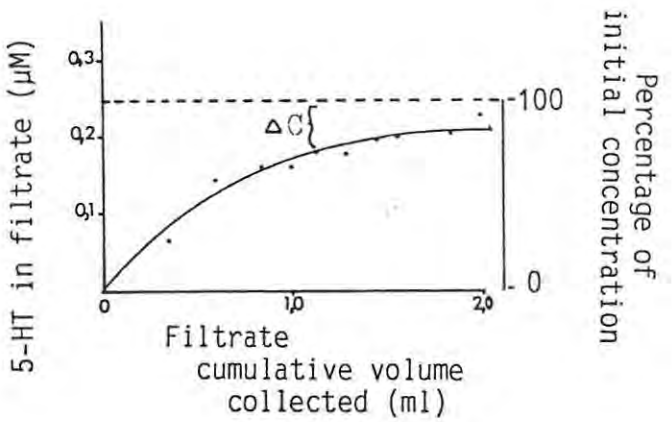


Fig. 4-1. Plot used to correct for nonspecific adsorption of serotonin on PSAC membrane. Solution contained 3,0 ml 0,02 M cacodylic acid, pH 7,6, 2 μl $[^{14}C]$-serotonin creatinine sulphate, 2 μl ferrous ammonium sulphate (0,066 μM) and 0,15 mg heparin. No βTG was included in the solution. The broken line represents the initial concentration of serotonin in the cell. ΔC refers to the percentage of initial concentration of serotonin in the filtrate.

for 10 min. After this period [^{14}C]-serotonin was added and the solutions were incubated for an additional 25 min. Ultrafiltration at 8-12 ^{o}C of the incubated solutions required 1-2 h.

In accordance with the Millipore recommendations for correcting for nonspecific binding of ligand to the membrane, the first 1,1 ml of the filtrate was discarded. The ratio of the apparent serotonin concentration and Δ C, the difference in the percentages of initial and final concentrations of serotonin, gave the true serotonin concentration in the filtrate assay mixture (see Discussion, this chapter).

Binding was determined from the following calculations (5-HT is serotonin):

$$dpm_{bound} = dpm_{retained} - dpm_{filtered\ corrected}$$

$$mmol\ 5\text{-}HT\ bound = (dpm_{bound})/(1265{,}4 \times 10^{8}\ dpm/mmol\ 5\text{-}HT)$$

$$\bar{\nu} = mmol\ serotonin\ bound/mmol\ \beta TG$$

$$[S] = 5\text{-}HT_{free\ at\ equilibrium} = \frac{dpm_{corrected\ filtered}}{1265{,}4 \times 10^{8}\ dpm/mmol\ 5\text{-}HT}$$

IV. Results

A. Ultrafiltration

The results in Table 4-1 show that in the presence of heparin and ferrous ion, 7-8 moles of serotonin were bound per mole of βTG tetramer. This was the value for $\bar{\nu}$ over a protein concentration range of 0,04-2,60 μM and a serotonin concentration range from 0,583 to 17,7 μM. However, it should be noted that in an experiment in which no ferrous ion was added, $\bar{\nu}$ had a value of 4, whereas when no heparin was present, the value for $\bar{\nu}$ was zero. (All data have been corrected for nonspecific binding by heparin and ferrous ion in the absence of βTG). In incubation mixtures containing much less serotonin than 8 times the βTG concentration, $\bar{\nu}$ values approximated the ratio of $[5\text{-HT}]/[\beta TG]$.

The amount of serotonin that was bound varied from 42 to 79% and increased with increasing $\bar{\nu}$ values for incubation mixtures containing

6,8 times (or greater) concentrations of serotonin over βTG, up to a maximum $\bar{\nu}$ value of about 8. In an experiment in which $CaCl_2$ had been added to the incubation mixture up to a final Ca^{2+} of 0,9 mM, the amount of serotonin bound was only 68% of that obtained without $CaCl_2$. However, in this experiment $\bar{\nu}$ (7,11) was 96% of the value obtained for $\bar{\nu}$ (7,34) in the absence of $CaCl_2$. The reason for including Ca^{2+} in this experiment was to determine whether the presence of Ca^{2+} might increase the binding of serotonin to βTG.

A Scatchard plot was prepared from the binding data by least squares regression. A value for K_D, which is equal to the negative slope of the Scatchard plot, that was obtained was $4,2 \times 10^{-8}$ M. The number of binding sites for serotonin on βTG is obtained from the intercept on the abscissa. This value was 8,1.

Table 4-1. βTG - serotonin ultrafiltration data

[5-HT] μM	[βTG] μM	$\frac{[5\text{-HT}]}{[\beta TG]}$	Fe^{2+} μM	heparin mg/3 ml	$CaCl_2$ mM	$\bar{\nu}$	$\bar{\nu}/[S] \times 10^8$	% binding
17,7	2,60	6,8	1,66	5,0	0	7,97	0,28	79
,583	,079	7,4	,066	0,15	0	7,39	3,99	61
,583	,079	7,4	0	0,15	0	3,95	4,29	63
,583	,040	14,5	,066	0,15	0	7,34	3,24	61
,583	,040	14,5	,066	0,15	0,9	7,11	3,59	42
,583	,079	7,4	,066	0	0	0	0	0
,583	2,60	0,23	1,66	5,0	0	0,26	0,19	71
1,17	2,60	0,45	1,66	5,0	0	0,41	0,23	74
2,92	2,60	1,12	1,66	5,0	0	1,21	0,15	66
,389	,079	4,9	,066	0,15	0	1,64	1,98	44

B. Hummel-Dreyer experiments, by the method originally described by Hummel and Dreyer (1962)

This is a method for measuring ligand-protein interaction on a column of Sephadex gel that excludes the protein. The column is equilibrated with a solution containing the ligand, and the protein, dissolved in the same solution, is passed down the column. The concentration of free ligand is reduced by an amount equal to the protein-ligand complex formed. As the complex peak emerges from the column, the total amount of ligand in the eluate increases. Also, after the protein peak the concentration of ligand in the eluate decreases. Thus, the elution profile displays a peak followed by a plateau and trough.

A K9/15 Sephadex G-25 column was equilibrated with a solution of 2,63 μM [^{14}C]-serotonin, 66 mM phosphate buffer, pH 7,5. The reservoir and column were covered with foil to protect serotonin from photodegradation. Purified βTG (0,2 mg) was incubated in the dark at 20 ^{o}C for 1 h with 0,1 ml of the 2,63 μM serotonin-phosphate solution. (In the incubation mixture [serotonin]/[βTG]was 0,046.) Then the βTG solution was applied to the column. The flow rate was 0,18 ml/min and the absorbance at 280 nm was monitored continuously with a LUV Monitor II, Model 1522 (LDC, Riviera Beach, Fla., USA). The monitor trace showed a small rounded peak, which emerged at the void volume. (This trace is reproduced in Fig. 4-2.) As can be seen, the trace did not show the typical Hummel-Dreyer plateau and trough. Radioactivity measurements for the 0,1 ml samples of eluate showed neither plateau nor trough, but did show a small increase at the void volume.

The column was washed well and re-equilibrated with the same serotonin-phosphate buffer. Next, 0,1 mg of purified βTG was dissolved in 0,1 ml of the same buffer, and 50 μl of freshly prepared $Fe(NH_4)_2SO_4$ was added. The βTG solution was incubated in the dark at 20 ^{o}C for 30 min. (The ratio of [serotonin]/[βTG]in the incubation mixture was 0,092.) Then the βTG solution was applied to the column. Again the flow rate was 0,18 ml/min. In this experiment the trace (Fig. 4-3) showed two 280 nm-absorption peaks, one immediately after the void volume and one immediately following the first peak. The void volume peak was larger than the void volume peak of the previous experiment, although twice as much βTG had been applied in the first experiment. The presence of the second peak obscured any plateau and trough that might have been present. The radioactivity measurements for the 0,1 ml

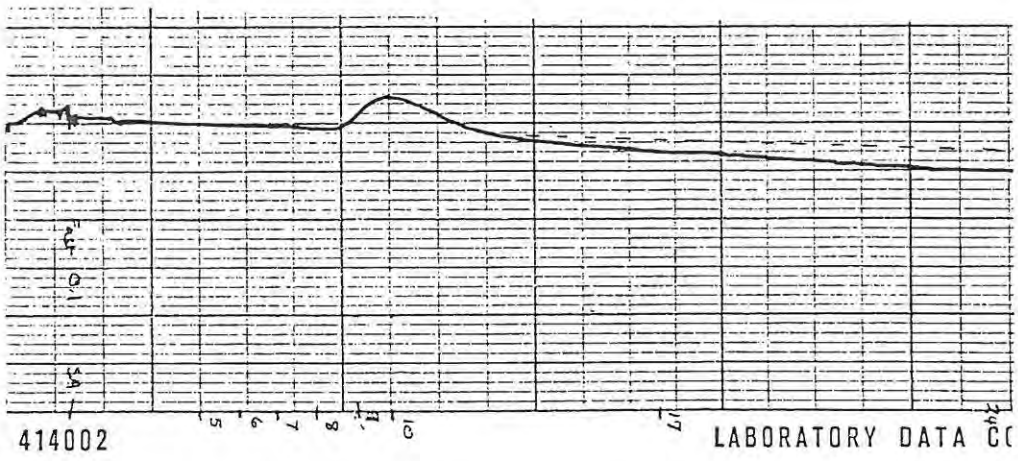


Fig. 4-2. Hummel-Dreyer experiment with 0,2 mg βTG chromatographed on Sephadex G-25 in the presence of 2,63 μM $[^{14}C]$-serotonin in phosphate buffer, pH 7,5. (No ferrous ion was added.) Void volume occurred at the end of fraction 9.

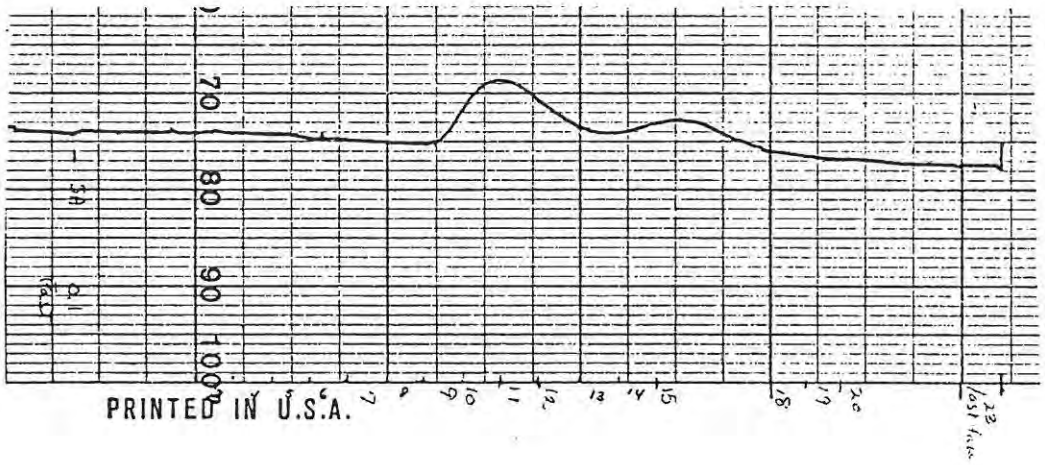


Fig. 4-3. Hummel-Dreyer experiment with 0,1 mg βTG chromatographed on the same column and in the same buffer as in Fig. 4-2, but with the addition of 33 μM ferrous ammonium sulphate. Void volume as in Fig. 4-2. Note that only half as much βTG was applied as in Fig. 4-2.

samples of eluate differed from those obtained in the previous experiment, but again there were no characteristic peak, plateau or trough of the typical Hummel-Dreyer ligand-binding experiments. Moreover, fractions 7-11 all contained less radioactive serotonin (values were 23 979 - 24 333 cpm) compared to values for pre-void volume fractions 1-6 (24 521 - 24 917) and post-void volume fractions.

C. Thin-layer chromatography

Samples which were chromatographed on thin-layer plates included 1) filtrates of the ultrafiltration experiments, 2) retentates of the ultrafiltration experiments and 3) fractions eluted near the void volume in the Hummel-Dreyer experiments. The samples were concentrated by evaporation on watchglasses in the refrigerator. Unbound serotonin standard migrated to R_f 0,37-0,47 and βTG would be expected at the origin. The cpm of each area was determined by the following procedure: first, each lane on the plate was lightly marked; then, successive square areas (0,5 cm on a side) were scraped, with the first square at the origin of each lane. When each area of gel had been loosened, it was shaken onto glassine paper and transferred to a scintillation vial containing Bray's scintillation fluid. The results are shown in Fig. 4-4.

It should be noted that in autoradiographs of thin-layer plates on which complexes of radiolabelled serotonin and serotonin-binding protein had been applied and developed, there was a blackening of the film at the point corresponding to each origin. Extending from the origin, streaking was apparent, probably due to dissociation of the complex in the presence of the solvent. In addition, a small spot at about R_f 0,12 appeared in lanes in which incubated mixtures of Fe^{2+} and serotonin had been applied. At this position a brown spot was sometimes visible in white light on the unsprayed developed plates, and when the serotonin was radiolabelled, the autoradiograph showed a blackening at R_f 0,12, as well as streaking. Thus, it appears that serotonin and Fe^{2+} form a complex which migrates to R_f 0,12, and dissociates in the thin-layer chromatographic solvent.

When the cpm obtained from near the origin was compared with the cpm of the area to which unbound serotonin migrated, the filtrates of

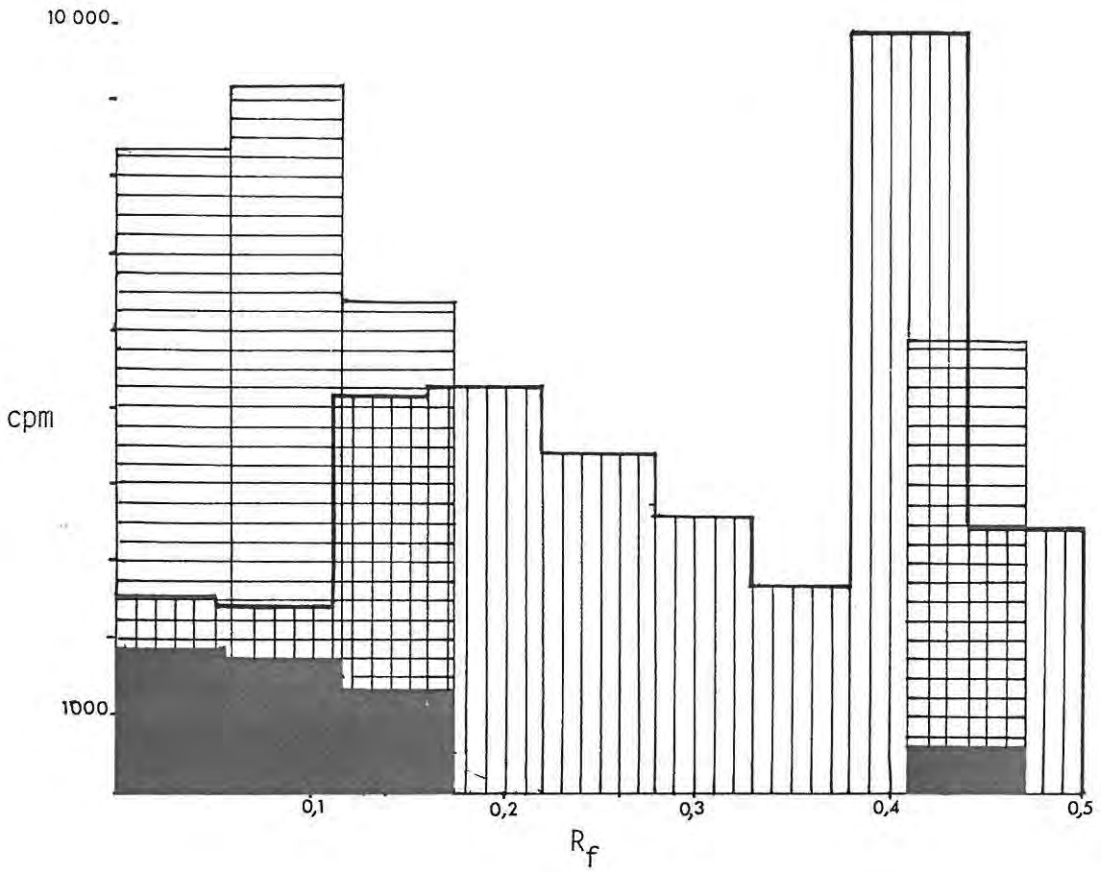


Fig. 4-4. Radioactivity of areas of various R_f when solutions from ultrafiltration and Hummel-Dreyer chromatographic experiments containing $[^{14}C]$-serotonin were chromatographed on thin-layer plates.

Solid bars, void volume fractions from Hummel-Dreyer experiments

Vertically striped bars, ultrafiltration filtrates

Horizontally striped bars, ultrafiltration retentates

the ultrafiltration experiments were obviously different from the retentate of the ultrafiltration experiments and also from the void volume fractions of the Hummel-Dreyer experiments. This is shown in Table 4-2. These results indicate that [^{14}C]-serotonin is bound by βTG and the protein-ligand complex is retained by the PSAC membrane and at least partly eluted from Sephadex G-25.

Table 4-2. Comparison of chromatographic results in Fig. 4-4.

Sample	cpm origin/serotonin R_f	Ratio*
UF filtrate	0,26	1
UF retentate	1,42	5,5
H-D void volume	3,34	12,8

* relative to UF filtrate.

V. Discussion

The data plotted in Figure 4-1 suggest that serotonin is bound to the PSAC membrane. This is a frequent defect of the ultrafiltration method. Non-specific binding by serotonin to filters, dialysis membrane and other materials has been noted previously (Tamir, 1982). As advised by Millipore (see this chapter, III), initial aliquots of filtrate were discarded. It can be assumed (Steinhardt & Reynolds, 1969) that equilibrium is reached very quickly by starting with a single solution containing both protein and ligand. If the binding sites for the ligand on the membrane are quickly saturated, there is no problem with the maintenence of equilibrium. However, direct ultrafiltration causes concentration of the protein. Therefore, the greater the volume of the aliquot that is discarded, the greater the danger of upsetting the equilibrium between complexed and free ligand and protein. Therefore, a compromise must be sought between the volume of solution that must be retained and the volume of filtrate that must be discarded. If a satisfactory compromise cannot be reached, alternative methods for measuring binding of that ligand must be sought.

Despite the arbitrary choice of the volume that was discarded, the results of the experiment conclusively show that binding does occur. In the present case, even if the value of ΔC were to be greatly in error, the results still indicate serotonin-binding by βTG. For example, an error of 10% in ΔC would not alter $\bar{\nu}$ by as much as 10%, provided the dpm of the retentate is much greater than the dpm of the filtrate. In the experiments with serotonin, the dpm of retentate was 3-6 times greater, e.g., 26 000 vs. 6 900. It can be shown by allowing a 10% error in ΔC (i.e. if ΔC is calculated at values of 0,70 or 0,77), the first line in Table 4-1 would yield $\bar{\nu}$ values of 7,90 or 8,11, respectively. Therefore, the results from the ultrafiltration experiments indicate that serotonin does bind to βTG.

When platelets undergo the release reaction, βTG and serotonin are released from their platelet storage sites at a similar rate into the blood (Kaplan et al., 1979; Harada & Zucker, 1971; Dawes et al., 1978; Gordon & Milner, 1976). Moreover, endogenous heparin is present at the time of the release reaction (Horner, 1974). In these experiments an attempt has been made to simulate in vivo conditions. At the time of release, the [serotonin]/[βTG] ratio is greater than 1.

The ratio [serotonin]/[βTG]in serum varies from 1,6 -3,6 from values reported by Davis (1959) for serotonin and by Ludlam et al. (1975) for βTG.

The alpha granules are the primary storage site of βTG (where the βTG is stored as LA-PF4). The serotonin content of the alpha granules is small. A recent report (Van der Meulen et al, 1982) localizes only about 4% of the platelet serotonin to the alpha granules. Thus, in the alpha granules the ratio [serotonin]/[βTG] would be expected to be much less than 1. In the light of this evidence, the results of the Hummel-Dreyer experiments, and results in Table 4-1 where the ratio is less than 1 are significant. In addition, the thin-layer chromatography results with the Hummel-Dreyer void volume fractions are pertinent. However, until further information is available regarding the binding of serotonin by LA-PF4 and the presence of glycosaminoglycan and ferrous ion in the alpha granules, no conclusions regarding the possibility of serotonin-binding by LA-PF4 in alpha granules should be drawn.

The dissociation constant for serotonin binding by βTG, determined from ultrafiltration data was found to be $4,2 \times 10^{-8}$ M. A serotonin-binding glycoprotein was isolated initially by ammonium sulphate fractionation, dialysis and Sephadex chromatography from rat platelet-rich plasma by Tamir et al. (1980a). The glycoprotein was soluble and had a molecular mass of 204 000. Although Tamir et al. reported two K_D values for their protein (K_D $4,2 \times 10^{-8}$ and $8,1 \times 10^{-7}$ M), it is of interest to note that one of these values is identical to that found in the present study. Their glycoprotein showed heat stability and a five-fold enhancement of serotonin binding by ferrous ion. This protein also had five isoelectric points (which were attributed to microheterogeneity of the carbohydrate moiety of the protein). The points of similarity between βTG and the protein isolated by Tamir et al. are striking. Further research in this area is warranted.

The results in the present investigation indicate that heparin and ferrous ion must be available to assure maximal binding of serotonin by βTG. It is possible that heparan sulphate, the glycosaminoglycan most commonly associated with endothelium, may fulfill the requirement for heparin. Heparan sulphate has been shown to have 75% of heparin's ability to complex with PF4 (Barber et al., 1972). The Fe^{2+} content of platelets has not yet been determined. Under normal physiological conditions, there is insufficient Fe^{2+} in serum to maximize serotonin-

binding to βTG. However, a preliminary experiment indicates that even in the absence of Fe^{2+}, serotonin can be bound at half the potential binding sites. This provides a field for further research.

The results obtained in the Hummel-Dreyer experiments were anomalous. These anomalous results may have been due to binding of βTG by Sephadex. PF4 binds to Sephadex (Nath et al., 1973; Handin & Cohen, 1976). It is possible that βTG, since it is homologous to PF4, might also bind to Sephadex, but has not been reported to do so. It is relevant that Tamir (1982) mentioned that new Sephadex columns should be used first with some "inert" protein to prevent loss of the serotonin-binding protein by non-specific adsorption onto the gel. However, the results in Chapter 2, Fig. 2-19, indicated that most of the radioactivity was eluted at the void volume when $[^{125}I]$ - βTG was chromatographed on Sephadex.

A necessary condition for Hummel-Dreyer experiments is that the protein should be excluded by the gel. βTG is known to monomerize at low concentrations (Bock et al., 1982). Since the nominal molecular mass exclusion of Sephadex G-25 for protein is about 5000, Sephadex G-25 would be expected to exclude both monomeric (M_r 8851) as well as tetrameric (M_r 35 404) forms of the protein. Therefore, the protein would be expected to be excluded from the gel. Also, as cited above, the results in Fig. 2-19 indicate that $[^{125}I]$- βTG was excluded from the gel.

In the absence of ferrous ion, βTG did emerge at the void volume (Fig. 4-2). However, in the presence of ferrous ion, the trace indicated that βTG emerged later than the void volume (Fig. 4-3). Thus, the βTG in the second Hummel-Dreyer experiment was not completely excluded from the gel, or else βTG became bound to the gel.

An apparent molecular mass of 5800 has been reported for βTG when the protein was electrophoresed on 7% SDS polyacrylamide gels subsequent to reduction of disulphide bonds (Moore & Pepper, 1976; Moore et al., 1975a). The authors attribute the low molecular mass to a conformational change, which follows the rupture of the intrachain disulphide bonds. Anomalous results for βTG on SDS polyacrylamide gel has also been noted by Varma et al. (1982). They attributed these results to the charges on the βTG molecule. Whether a protein of M_r 5800 were excluded by Sephadex G-25 would depend on the shape or conformation of the protein. It should be noted that no disulphide reducing agent, other than ferrous ion, was present in the buffer of

the Hummel-Dreyer experiments.

The anomalous trough that occurred in the radioactivity profile at the void volume was confirmed by multiple readings in the scintillation counter of more than one sample from each fraction. However, without confirmation obtained from further experiments, the results must be regarded as tentative.

The greater absorbance of the peak in Fig. 4-3 as compared to that in Fig. 4-2 might be accounted for by ferrous ion bound to βTG. Free Fe^{2+} would not elute so close to the void volume. It may be that the second peak in Fig. 4-3 represents the serotonin-iron complex. In fact, this may be the cause of the brown spot seen on thin-layer chromatograms at R_f 0,12. Further investigation into the existence of such a complex would be especially valuable if indeed Fe^{2+} does occur in the alpha granule.

Since the ratio [serotonin]/[βTG] affected binding of serotonin by βTG in molecular filtration experiments, it is possible that different results in the Hummel-Dreyer experiments might have been obtained at a [serotonin] / [βTG] ratio greater than one. Furthermore, a molecular filtration experiment indicated that no binding of serotonin occurred in the absence of heparin. Thus, the inclusion of heparin in Hummel-Dreyer experiments might be essential for demonstrating binding of serotonin by βTG.

The question arises, if βTG binds serotonin, why didn't the serotonin-affinity gel experiments described in Chapter 2 result in the purification of βTG as well as PF4? Three possible reasons can be offered: 1) βTG may require the alpha amino group of serotonin for binding, 2) a necessary cofactor (heparin or Fe^{2+}, for instance) may have been absent or present at sub-optimal concentration, or 3) the sample applied to the affinity gel would have contained LA-PF4, rather than βTG (see chapter 1), and LA-PF4 may not bind serotonin.

CHAPTER 5

Investigations into calcium binding by purified human PF4 *in vitro*

I. Introduction

In Chapter 2, affinity chromatography of platelet extracts resulting in the isolation of PF4 has been described. Of many eluting buffers tried, only two were effective in desorbing native, unlabelled PF4 and $[^{125}I]$-PF4 from serotonin-affinity columns. Acetic acid (1 M) eluted PF4 presumably because monomerization of PF4 occurs at low pH (Moore *et al.*, 1975b). The other eluant, 5% EDTA, probably removed PF4 from the serotonin gel by a mechanism involving chelation of divalent cations.

Calcium is known to play a most important role in platelet contraction and secretion (Feinstein 1978a & b; Bennett *et al.*, 1979 ; Robblee & Shepro, 1976 and references contained therein; D'Amore, 1978). During the "release reaction" (Detwiler & Feinman, 1973 a & b; Feinstein, 1980; Owen and LeBreton, 1981), in which serotonin and PF4 are released, calcium is also released from platelets (see Chapter 1 for references). Therefore, as part of this study, calcium binding by PF4 has been investigated and the results are presented in this chapter. Two methods were used to study calcium binding, equilibrium dialysis and a method of Statland *et al.* (1969).

The theory of equilibrium dialysis has been described in Chapter 3. By means of equilibrium dialysis an association constant, k, and n, the total number of identical independent binding sites, can be obtained from a Scatchard plot of the data if the Scatchard plot is linear (Scheinberg, 1982, and references therein).

Calcium uptake can also be demonstrated by the Statland technique. This method uses a dialysis cell which has a reaction mixture (Grette, 1963) containing 2,5 mM ATP and 50 µM calcium labelled with ^{45}Ca on both sides of a semi-permeable membrane. At zero time, a small sample of platelet membrane protein (suspended in reaction solution without $CaCl_2$) is injected into one compartment and samples are removed from the other side of the membrane at intervals for determination of radioactivity. Statland reported observable calcium uptake in less than 2 min and a steady state which was attained in less than 16 min. Although the amount of binding did not change with time after 16 min, it is unlikely that true equilibrium, that is, all the components

having the same chemical potential, was attained within 16 min (Steinhardt & Reynolds, 1969). Nevertheless, the Statland method does give an indication of the number of moles of ligand bound per mole of a purified protein. Inhibition by various agents can also be demonstrated.

The calcium concentrations used in the equilibrium dialysis experiments ranged from about 1-100 μM, and Statland experiments were performed in the presence of 50 μM calcium, since these are concentrations which are physiologically relevant: for example, 5-10 μM calcium stimulates adenosine triphosphatase activity in isolated calf platelet membranes (Robblee et al., 1973a); 1-100 μM calcium stimulates the same activity in isolated human platelet membranes (Käser-Glanzmann et al., 1977). Furthermore, in the presence of 50 μM calcium, uptake by "platelet relaxing factor" occurs (Statland et al., 1969). Release of serotonin by intact platelets is stimulated by 10-50 μM calcium (Robblee & Shepro, 1976; Knight & Scrutton, 1980). Increase in Ca^{2+} uptake by epinephrine stimulation is approximately 15 μM (Owen et al., 1980). Lysis of the platelet alpha granules (the site of PF4 storage), a response specific to calcium, occurs at calcium concentrations as low as 10 μM (Van der Meulen & Grinstein, 1982).

II. Materials

Membranes and Equilibrium dialysis cells: as described in Chapter 3
Calcium: $^{45}CaCl_2$ was obtained from New England Nuclear Chemicals, West Germany. The specific activity was 45,42 mCi/mg. Radioactivity was measured in a Beckman LS2800 scintillation counter. In early experiments, radioactivity was measured in Bray's scintillation fluid. In later work, Beckman Ready-Solv MP scintillation fluid was used. Window settings were 0 and 715 nm (as determined by a Spectrum Search, allowing 1% spillover, of a $^{45}CaCl_2$ sample). The quench was similar in all samples, the difference in H number for pairs of cells usually being less than 3.
Buffers: The buffer used for equilibrium dialysis (pH 7,4) contained 100 mM KCl, 600 mM NaCl, 5 mM $MgCl_2 \cdot H_2O$, 20 mM Tris HCl, and 2 mM ATP (disodium salt). This is the same buffer as was used in studies of calcium uptake by platelets, except for the addition of NaCl which was necessitated by PF4's low solubility at low ionic strength, and the

omission of oxalate (Robblee & Shepro, 1976; Robblee et al., 1973a and Käser-Glanzmann et al., 1977). The Statland experiments were carried out using the same buffer as for equilibrium dialysis except for the addition of 1 mM potassium oxalate and were 2,5 mM ATP instead of 2,0 mM and contained 50 μM calcium chloride.

PF4: purified PF4 was a gift from D.S. Pepper, Edinburgh, Scotland. Additional purified PF4 was prepared by the method of Wu et al. (1977).

Other chemicals: Amytal (amobarbital, from Lilly) and the disodium salt of ADP (Sigma) were used in inhibition studies.

III. Methods

Solutions for equilibrium dialysis cells were prepared with purified PF4 and calcium chloride to which $^{45}CaCl_2$ had been added in trace amounts. The concentration of PF4 ranged from 0,0067-1,2 μM. The calcium concentration was mainly 1-100 μM, except for a few experiments in which the calcium concentrations were less than 1, or above 100 μM.

After equilibrium was established (after about 10 h at ambient temperature), equal volumes of samples were taken from all cells and counted in duplicate in the scintillation counter. Binding was expressed by the formula:

$$\beta = \frac{P - N}{P + N}$$

where β is the fraction of total calcium bound, P is cpm of a sample from the cell containing PF4, and N is cpm of a sample of equal volume from the cell on the opposite side of the membrane.

The number of moles of ligand bound per mole protein is given by

$$\bar{v} = (\beta \cdot Ca_{total}) / PF4_{total}$$

$[Ca]_{total} = [Ca]_{bound} + [Ca]_{free}$ = the concentration of "cold" plus radioactive calcium in the system

For the Statland-type experiments, the method was modified slightly. At exactly 10 min after the introduction of purified PF4 into the dialysis cells, samples were withdrawn from both sides of the membrane and $\bar{v}$ was calculated as for the equilibrium dialysis experiments. The cells were shaken gently on CRC shakers throughout the Statland experiments. Due to technical difficulties, the cells could not be shaken for longer than 2 of the 10 or more hours as required in the equilibrium dialysis experiments. The Statland $\bar{v}$ values were used only for comparative purposes and not for obtaining values for n or k, since it was presumed that equilibrium had not been attained and these experiments were intended to demonstrate rapid uptake across the membrane.

IV. Results and Discussion

A. Determination of n

The plot of $\bar{v}$ against Ca^{2+}, the binding isotherm, is shown in Fig. 5-1. The curve is hyperbolic in shape, tending towards a plateau value around 4.

Binding may be expressed by a simple mass-action law (Scatchard, 1949):

$$k = \frac{\bar{v}}{(n-\bar{v})[S]}$$

$$\text{where } [S] = [Ca^{2+}]_{free}$$

and k is the equilibrium constant, an association constant, and n is the total number of identical independent binding sites on the protein. For the Scatchard plot, this equation is rearranged to:

$$\frac{\bar{v}}{[S]} = k(n-\bar{v})$$

and $\bar{v}/[S]$ plotted against $\bar{v}$. In the simplest case, in which no cooperativity exists among the binding sites, the plot is a straight

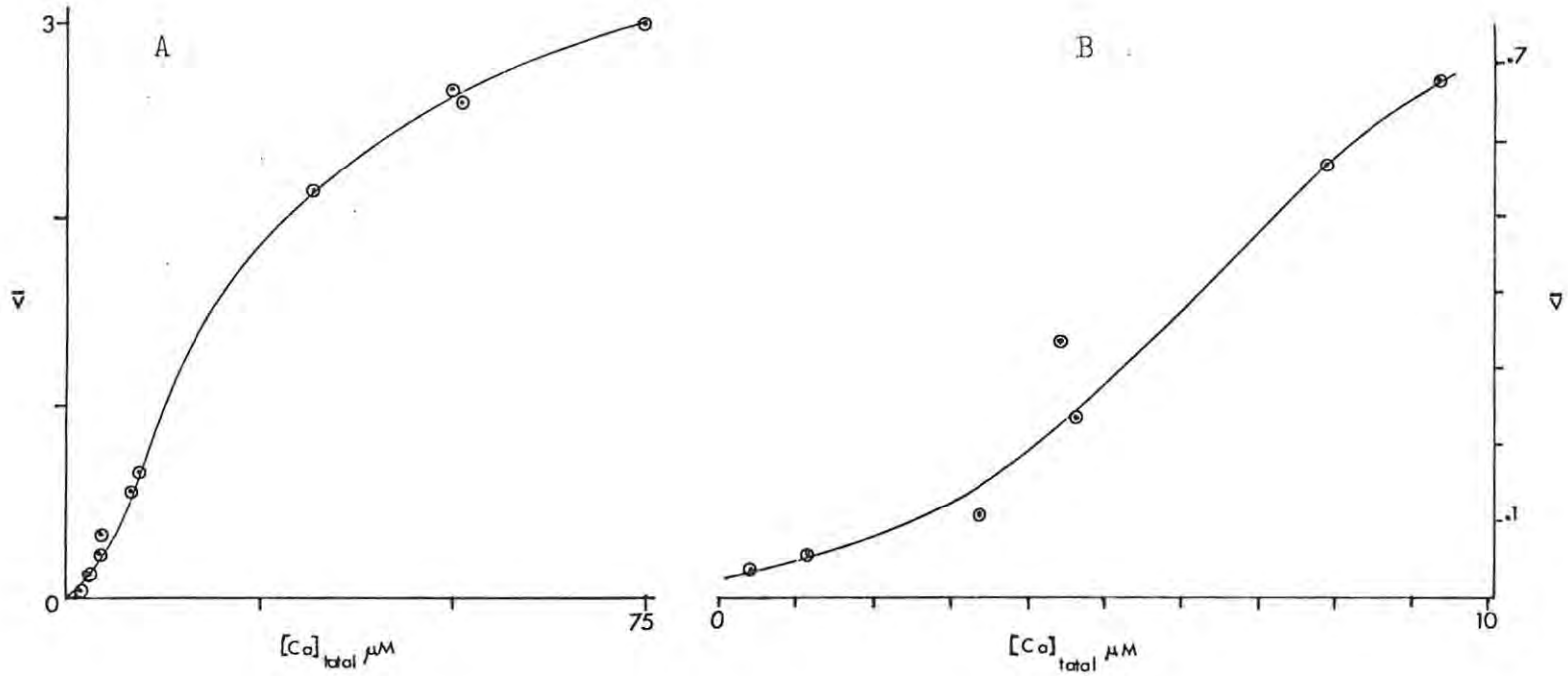


Fig. 5-1A and B. Plot of data from Table 5-1. PF4 = 0,15-0,25 μM.
A, 190-fold range of calcium concentrations
B, 23-fold range of calcium concentrations, to show sigmoidal curve at the low concentrations.

Table 5-1. Equilibrium dialysis experiments of calcium binding by purified PF4 at ambient temperature in pH 7,4 buffer containing 100 mM KCl, 600 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$, 20 mM Tris HCl and 2 mM ATP.

PF4 μM	β	Ca^{2+} (μM) total	Ca^{2+} (μM) bound	$\bar{\nu}$
0,15	0,007	56,1	0,390	2,60
0,15	0,014	0,40	0,006	0,037
0,15	0,011	7,84	0,086	0,573
0,25	0,013	1,12	0,014	0,056
0,25	0,008	3,36	0,027	0,108
0,25	0,018	4,48	0,082	0,330
0,20	0,008	50,0	0,400	2,67
0,15	0,010	32,0	0,320	2,14
0,15	0,020	75,0	0,450	3,00
0,15	0,0075	4,67	0,035	0,230
0,15	0,011	9,33	0,103	0,684
1,2	0,010	56,1	0,530	0,443
0,0067	0,003	56,1	0,168	25,1
0,0332	0,009	56,1	0,505	15,2
0,0665	0,012	56,1	0,673	10,1
0,15	0	100	0	0
0,15	0	500	0	0
0,15	0	700	0	0

line which has a negative slope and intercepts the abscissa. The intercept of the abscissa is equal to n. In the present study, the Scatchard plot of values obtained at PF4 concentrations of 0,15-0,25 μM does not produce a straight line (Fig. 5-2A, solid line and Table 5-1). However, if only those values obtained at the higher calcium concentrations are considered (32-75 uM), a line can be plotted that intercepts the abscissa at about 4 (Fig. 5-2A, broken line), i.e., 4 binding sites on the protein molecule. Additional support for the argument that n = 4 is a possible trend of the "hyperbolic" curve of Fig. 5-1 to a plateau value of 4.

Although the Scatchard plot is unusual in being curved, anomalous Scatchard plots have been reported (Judis, 1980a & b and references therein; Shen & Gibaldi, 1974; Eldefrawi & Eldefrawi, 1973). Such plots have been obtained for the binding of amphetamine, atropine, epinephrine, histamine, codeine, morphine and methadone to human serum albumin, and acetylcholine to its receptor. Scatchard plots with positive slope have been interpreted as indicative of cooperative binding in which an associated oligomeric protein shows an increasingly greater tendency to bind ligand at greater ligand concentrations. Bowmer and Lindup (1978) have reviewed reports of Scatchard plots having positive slopes, and have described the effect of change in albumin concentration on such plots as well as the effects of possible contamination of the protein. If the positive slope of the Scatchard plot is due to cooperativity, it should manifest itself in studies in which the protein concentration is changed, by a linear plot with positive slope. Figure 5-2B, which is the plot of $\bar{v}/[S]$ versus $\bar{v}$ for different protein concentrations, is linear with positive slope. By this criterion binding of Ca^{2+} to PF4 is cooperative.

The value of n can also be deduced from information about the amino acid composition of the protein (Gurd, 1970, p. 372). PF4 is an oligomeric protein composed of four protomers whose amino acid sequence at the NH_2-terminus reveals a clustering of negatively charged residues (Hermodson, 1977) similar to the proposed calcium-binding sites on calmodulin (Cheung, 1982) and on other proteins (Tufty & Kretzinger, 1975). Since there are four such sites per PF4 tetramer, this is support for n = 4, assuming these to be calcium-binding sites (see Table 5-2). (It should be noted that βTG contains no comparable site.)

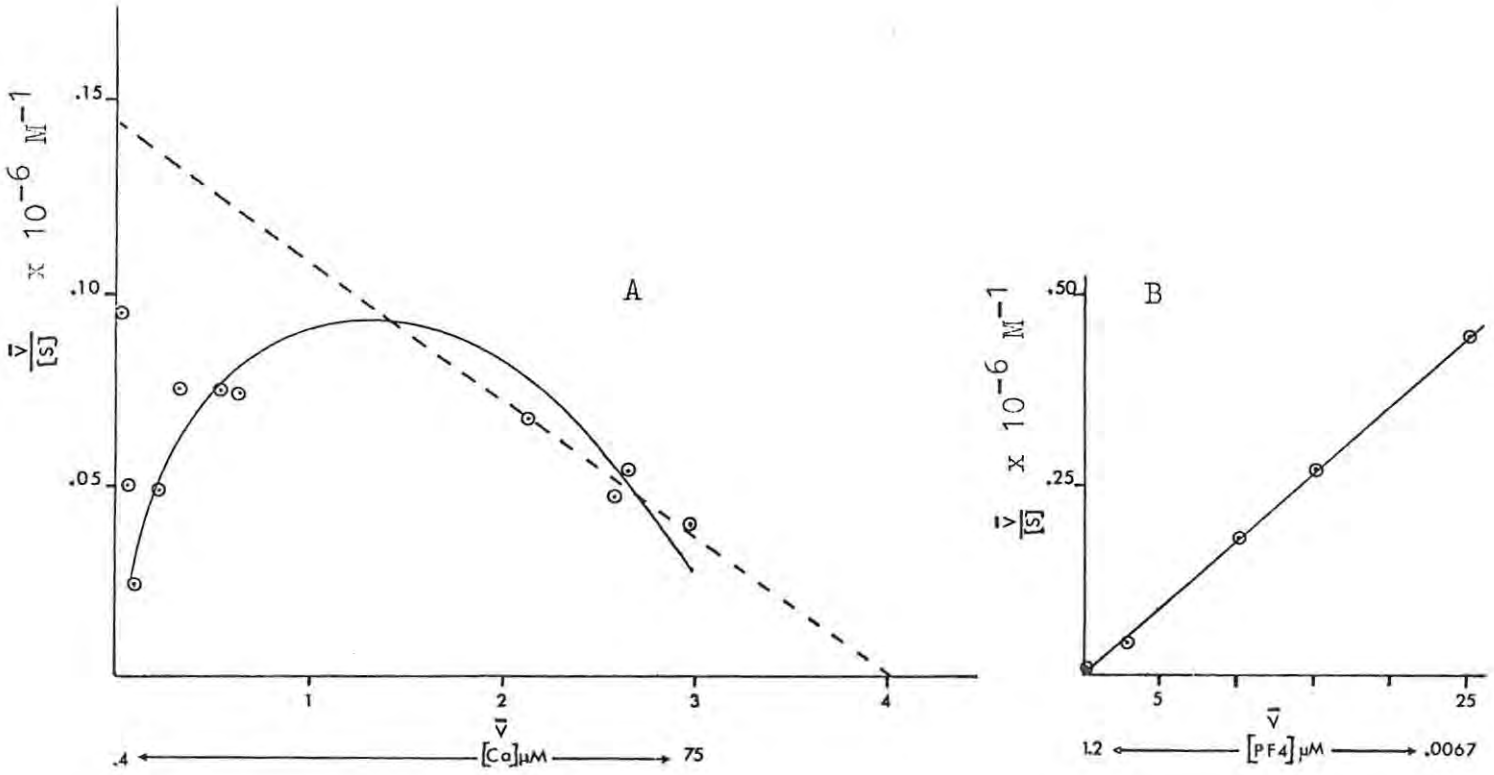


Fig. 5-2A and B. Scatchard plots of data from Table 5-1.
In A, PF4 was 0,15-0,25 μM. In B, Ca was 56,1 μM.

Table 5-2. Correspondence of amino acid sequence in PF4 protomers with putative calcium-binding sites in other proteins.

										Reference
TEST	D*	O	D*	O	D*	G	O	I	D*	Tufty & Kretzinger, 1975
PF4	$_{1}$E	A	E	E	D	G	D	L	Q_{9}	Hermodson, 1977
CAL-I	$_{20}$D	K	D	G	N	G	T	I	T_{28}	Cheung, 1982
CAL-II	$_{56}$D	A	D	G	N	G	T	I	D_{64}	ibid.
CAL-III	$_{93}$D	K	D	G	N	G	Y	I	S_{101}	ibid.
CAL-IV	$_{129}$N	I	D	G	D	G	E	V	N_{137}	ibid.

Each calcium ion is thought to be bonded to six amino acids that occur in the test sequence. The relevant sequences of PF4 and the four calmodulin (CAL) binding sites are aligned by their correspondence to the test sequence in the first row. In the test sequence, D* has an oxygen atom as a ligand to the calcium and represents D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; S, serine; or T, threonine. G is glycine. I is a hydrophobic residue and represents I, isoleucine; L, leucine; or V, valine. O represents an amino acid of no importance in calcium binding according to the Tufty-Kretzinger test. The subscripts refer to the residue position in the protein's primary structure.

Levitzki (1978) has plotted binding data for noncooperative, positively cooperative and negatively cooperative ligand binding models. According to these models, a Scatchard plot associated with positively cooperative binding is a concave downward curve, and that of negatively cooperative binding, concave upward. Positive cooperative binding yields a concave upward curve on the double reciprocal plot of $1/\bar{v}$ against $1/[S]$, whereas negatively cooperative binding is concave downward. The Scatchard and reciprocal plots of calcium binding by PF4 shown in Fig. 5-2A and 5-3 are therefore consistent with positively cooperative binding. A Scatchard plot line with positive slope is interpreted as representing the lower concentrations of a positively cooperative system (Judis, 1980 a & b). It is considered that if a larger range of concentrations were used, a concave downward Scatchard plot would result. Obviously, when seeking values for n and k, it is

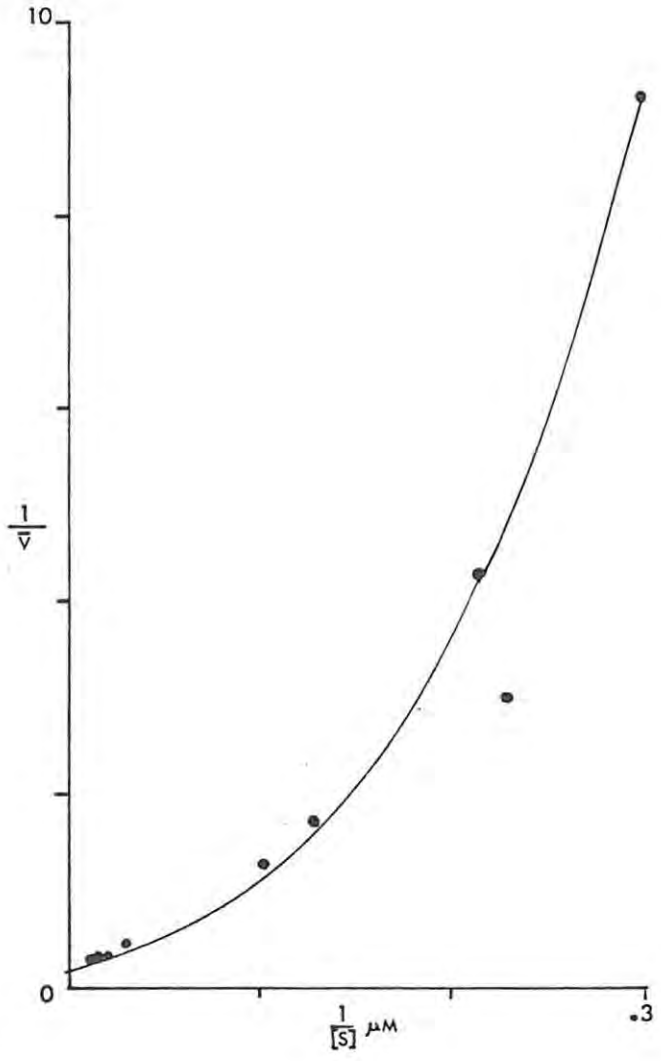


Figure 5-3. Reciprocal plot of data from Table 5-1.

an advantage to include a large range of concentrations of both ligand and protein. However, at very low concentrations, total ligand and free ligand are both small numbers and Klotz (1953) has warned that the difference between the two is subject to a relatively high degree of uncertainty. On the other hand, at very high ligand concentrations, total ligand and free ligand are both large, and again their difference is imprecise.

It is important to distinguish between positive cooperativity and systems in which the affinity for the ligand depends on the state of aggregation of the molecule, since both possibilities result in curvature of the Scatchard plot. Although, in general, binding is independent of the total protein concentration for positive cooperativity, where binding is affected by protein association, the binding will depend on the total protein concentration. The latter phenomenon has been investigated by Steiner (1980). Since this situation and positive cooperativity can both serve to explain similarly curved Scatchard plots, binding measurements should be made over a wide range of protein concentrations to distinguish conclusively between the two possibilities. Alternatively, direct molecular mass determinations should be used. In addition, heterogeneity of binding sites and negative cooperativity can also result in curvature of the Scatchard plot. In these instances, however, curvature is opposite or otherwise different from the curvature in cases of positive cooperativity (Levitzki, 1978; Klotz & Hunston, 1971; Nørby et al., 1980 and references therein).

Moreover, as evidenced by the variety of values obtained for PF4 by SDS electrophoresis (prior to the amino acid analysis of PF4), accurate values for direct molecular weight determinations of PF4 are difficult to obtain. Also, because of the anomalous chromatographic behaviour of PF4 on Sephadex (Handin & Cohen, 1976), chromatography is precluded as a means for accurately determining molecular mass. Thus, equilibrium dialysis over a 179-fold range of protein concentrations justifies acceptance of positive cooperative binding of calcium by PF4 rather than association of PF4 to explain the shape of the Scatchard plot.

Additional evidence that the curvature of the Scatchard plot is due to positive cooperative effects is the anisotropy of the fluorescence of dansyl (5-dimethylaminonaphthalene-1-sulphonyl) labelled PF4 (Bock et al., 1982) These studies have indicated that the

aggregation state of PF4 (but not of βTG) is unaffected by changes in ionic strength of the medium between 0,3 M to 2 M with various protein concentrations.

It should be noted that calcium ions can promote the association of some proteins (tyrosinase, neurophysin and hemocyanin) (Klotz, 1953). It is thus tempting to invoke calcium-induced association of PF4 instead of positive cooperativity to explain positive cooperativity, because this implies that EDTA removes PF4 from the serotonin-affinity column by the same mechanism by which acetic acid removes PF4 from the affinity column, namely through dissociation of the PF4 tetramer. However, the Scatchard plots with positive slope for various protein concentrations tend to corroborate positive cooperativity. Therefore, EDTA must act by a mechanism that is different from that used by acetic acid.

In the Bjerrum plot (Fig. 5-4), the shape of the calcium association curve of PF4, consistent with interaction among Ca^{2+} binding sites, is similar to the oxygen association curve of hemoglobin. Oxygen binding by hemoglobin, first described nearly 75 years ago, is the classic case of positive cooperative binding. Hemoglobin contains four equivalent binding sites for oxygen, and normal hemoglobin is composed of two α and two β subunits. Subunit interaction within the tetramer (which does not change its aggregation state in the presence of oxygen) has not yet been explained. Even as recently as December, 1982, new findings in oxygen binding by hemoglobin were reported (Weber, 1982). Since the binding of one calcium ion apparently influences the binding of another calcium ion at a comparable site on another PF4 monomer, this effect may be regarded as homotropic, like oxygen binding by hemoglobin (Monod et al., 1963).

The Hill coefficient (h), which can be obtained by plotting the experimental values according to the following equation (Levitzki, 1978, p. 12), is used as an index for cooperativity.

$$\log \frac{Y}{1-Y} = h \log[S] - \log K_D$$

where K_D is k^{-1} and Y is the degree of saturation.
In the present case, using an assumed value of 4 for n,

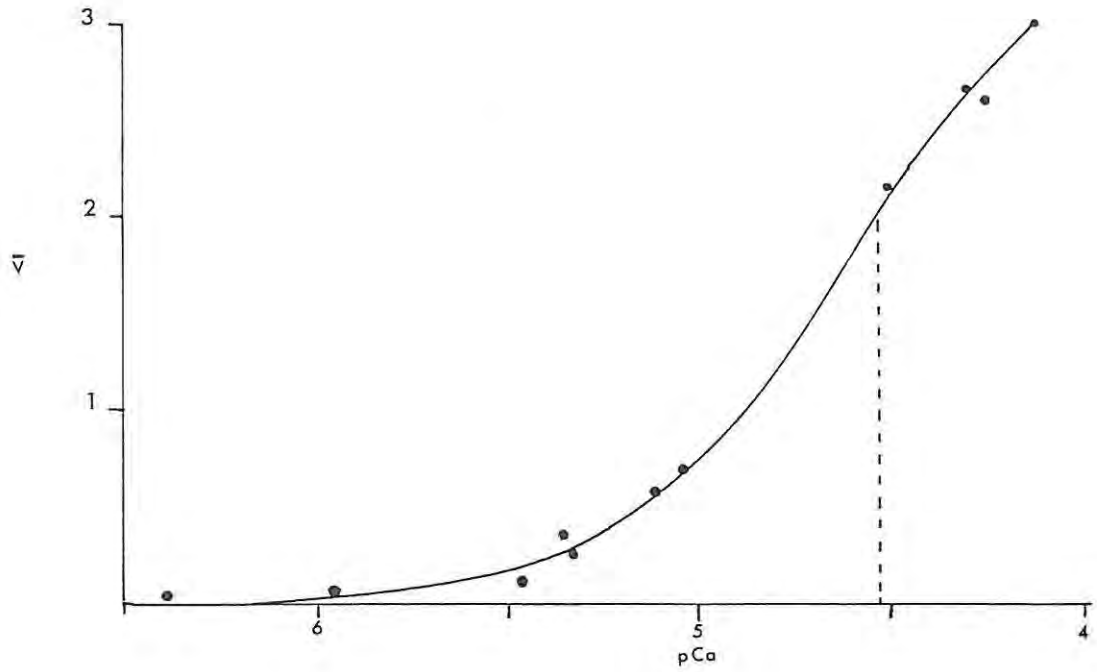
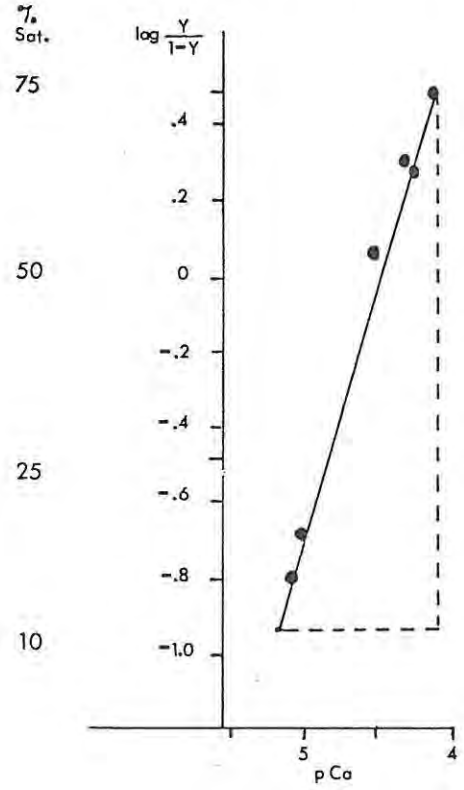


Figure 5-4 (above). Bjerrum plot of data from Table 5-1.

Figure 5-5 (below). Hill plot of data from Table 5-1. The slope of the line, h, is 3,4.



$$\frac{Y}{1-Y} = \frac{\bar{v}}{4-\bar{v}}$$

A high value for h signifies a high degree of cooperativity. At the upper limit, h is equal to the number of binding sites on a protein (Fersht, 1977). With positively cooperative effects, h is above 1,0; with noncooperativity, h is 1,0; and with negatively cooperative effects, h is below 1,0. It has been demonstrated that Hill plots are not always straight lines over the entire ligand concentration range. In the case of positive cooperativity they are generally sigmoidal with only the portion of the curve in the 10-90% saturation range producing a straight line (Wyman, 1964; Levitzki,1978). When the corresponding points are plotted for calcium binding by PF4 (Fig. 5-5), a straight line is produced for the points representing calcium concentrations between 7,8 and 75 μM. The slope of this line, which is equal to h, is 3,4, and implies a high degree of cooperativity.

B. Determination of k

Methods for the determination of k in positively cooperative systems have been discussed by Judis (1980a & b). In positive cooperativity, because the equilibrium constant for binding at one site is influenced by binding at other sites, it is difficult to estimate an accurate value for k. However, an approximate value can be obtained for k when $\bar{v} = 0,5\ n$. Thus, when the equation of the mass-action law is transformed, by taking logarithms, i.e.,

$$\log k + \log[S] = \log \frac{\bar{v}}{n-\bar{v}}$$

log k is equal to $-\log[S]$ when $\bar{v}$ is equal to n/2.
Then, from Fig. 5-4, at $\bar{v} = 2$, log k is approximately 4,5 and, therefore, K_D is approximately 3×10^{-5} M.

An approximate value for k can also be determined from the Scatchard plot (Fig. 5-2A) by extending the broken line to its intercept with the ordinate axis, kn. Corresponding to a value of $0,145 \times 10^{6}$ M for the intercept, K_D can be shown to have a value of 3×10^{-5} M.

C. Inhibition of Ca^{2+} binding by ADP and Amytal

Statland experiments were performed with and without inhibitors. The results are summarized in Tables 5-3, 5-4 and 5-5. Samples were taken 10 min after addition of purified PF4. The average $\bar{\nu}$ after 10 min incubation was 1,0 for the uninhibited system (Table 5-3), and 0,1 for both the ADP and the Amytal-containing systems (Tables 5-4 and 5-5). Inhibition of Ca^{2+} binding at these concentrations of ADP and with Amytal was reported by Statland et al. (1969) in experiments to study the platelet "relaxing factor". Statland et al. used a procedure which removed most of the alpha granules (membrane fractions of washed 14 000 - 100 000 g platelet pellets which had been subjected to sucrose gradient centrifugation were used). Therefore, a low PF4 concentration could be expected. From their studies, these investigators concluded that the relaxing factor is associated with membrane-enclosed vesicles. The significance of inhibition by ADP and Amytal on the binding of calcium ions by PF4 has not been determined. For reasons that are discussed in Chapter 6, it is considered unlikely that PF4 is the relaxing factor described by Statland et al.

Allosteric binding mechanisms are involved in the complex regulation of physiological systems. Since evidence has been presented that PF4 is located in or on the platelet surface immediately after release (George & Onofre, 1982; Gjesdal, 1977, p. 137), it is possible that PF4 regulates calcium ion transport through the platelet membrane, thereby controlling further platelet release. However, considerably more data will have to be obtained before a role for the observed positive cooperativity in the binding of calcium by PF4 can be accounted for.

Table 5-3. Statland experiments with a buffer containing 50 μM calcium, 2,5 mM ATP, 1 mM potassium oxalate, 100 mM KCl, 600 mM NaCl, 5 mM $MgCl_2 \cdot 6H_2O$ and 20 mM Tris-HCl. pH of buffer: 7,4.

temp.	PF4 μM	$\bar{v}$
14	0,645	1,6
14	0,32	0,78
14	1,45	0,72
16	0,32	0,78
16	0,80	0,50
17	0,32	2,19
17	1,12	0,54
17	2,24	0,94

Table 5-4. Statland experiments with ADP. Buffer as in Table 5-3, with the addition of 2,5 mM ADP. Temp. 17 °C.

PF4 μM	$\bar{v}$
0,96	0,208
1,45	0,055
3,2	0,030

Table 5-5. Statland experiments with Amytal. Buffer as in Table 5-3, with the addition of 5 mM Amytal. Temp. 16 °C.

PF4 μM	$\bar{v}$
0,64	0
0,96	0,052
1,28	0,04
4,00	0,088
0,96	0,26
1,60	0,125

CHAPTER 6
Discussion

This study began with the hypothesis that a releasable platelet-specific protein might bind serotonin. It was thought that serotonin might be important to the protein's function and that a knowledge of the protein's function might help to explain some of serotonin's functions. By means of affinity chromatography of native and purified PF4 (Chapter 2), ultrafiltration of purified βTG (Chapter 4) and gel filtration of purified PF4 (Chapter 3), evidence has been obtained to show that two platelet-specific releasable proteins, PF4 and βTG, bind serotonin.

Twenty affinity chromatography experiments showed that protein from disrupted platelets bound to serotonin-affinity gel. After many unsuccessful attempts to elute the protein from the affinity gel with a variety of buffers, two effective solutions were found. In these experiments, the protein was frequently lost because of unusual properties. The unusual properties of this protein suggested that it might be PF4, a platelet-specific releasable protein hitherto not known to bind serotonin. Besides PF4, a second platelet-specific protein, βTG, which has a homologous structure to PF4, was found to bind serotonin.

The identity of endogenous PF4 was verified by several means (Chapter 2) which included: a) radioimmunoassays which demonstrated that platelet extract protein reacted 1:1 with PF4 antibody, but only weakly with βTG antibody; b) strong binding of unpurified native PF4 and purified $[^{125}I]$-PF4 only on serotonin-affinity columns, and not on control columns; c) similar elution of native PF4 and purified $[^{125}I]$-PF4 from the serotonin-affinity columns; d) migration on SDS polyacrylamide gels of the PF4 from platelet extracts to the same position as attained by $[^{125}I]$-PF4; and e) similar fluorescence pattern of purified PF4 and the exhaustively dialysed serotonin-binding protein from the affinity gel.

Experiments which reconfirm many of the reported physicochemical characteristics of PF4 are described in Chapter 2. These include: low molecular mass, association with a carbohydrate (probably the PF4 proteoglycan carrier), low aromatic amino acid content (as evidenced by a lack of an A_{280} peak), permeation of dialysis membrane, anomalous

behaviour on Sephadex and heat stability.

It is difficult to account for the observations from the experiments with elution of PF4 (both native PF4 and [^{125}I]-PF4) from the serotonin-affinity columns. Elution problems are usually associated causally with binding systems in which K_D is 10^{-8} M or lower (Pharmacia, 1979, p. 7). Because of other problems (discussed below and in Chapter 3,), a value for K_D could not be determined for the serotonin-PF4 complex. Therefore, it could not be assessed whether the K_D for the complex is as low as 10^{-8} M. However, it is pertinent that 1 M acetic acid, suggested by Pharmacia as a desorbing agent for immune complexes which usually have very high affinities, was one of the two successful eluants for PF4.

PF4 may have been eluted from the serotonin-affinity column by 1 M acetic acid as a result of acid-induced monomerization. Monomerization by acetic acid is a well-known characteristic of PF4. Very accurate measurement using [^{125}I]-PF4 showed the same amount of PF4 to be eluted by 5% EDTA as by 1 M acetic acid. This may suggest a common mechanism, as yet unknown, for these two reagents. Calmodulin is freed from calcium at low pH (Haiech et al., 1981). A similar mechanism could operate during the elution of PF4 from serotonin-affinity columns. In this study no evidence has been obtained for calcium-mediated association as described by Klotz (1953) or Steiner (1980), that would result in monomerization due to chelation of Ca^{2+} by 5% EDTA. On the contrary, calcium-binding by PF4 seems to be an example of positive cooperativity, that is, PF4 in its tetrameric (associated) state shows an increasingly greater tendency to bind calcium at higher calcium concentrations, an effect which is independent of the total PF4 concentration.

The increased binding of serotonin-binding protein from platelet extracts to the serotonin-affinity columns in the presence of Fe^{2+} (as discussed in Chapter 2) could be due to non-specific binding. Alternatively, Fe^{2+} may promote the binding of PF4 to serotonin. Fe^{2+} has been shown to enhance binding of serotonin to the serotonin-binding proteins isolated by Tamir's group (see Chapter 1). It was because of these findings that Fe^{2+} was originally incorporated in the starting buffer for some of the affinity chromatographic experiments in the present investigation. Fe^{2+} was excluded from later experiments because of subsequently acquired information concerning the paucity of Fe^{2+} in vivo.

After demonstrating that serotonin bound to an identified protein, it was of interest to investigate how binding by serotonin affected the function of PF4 and βTG or how PF4 or βTG affected the function of serotonin. Thus, as Scatchard advised (see Introduction of Chapter 1), "how many?" and "how tightly?" were the next questions to answer.

Although there are many methods to obtain values of n and k for other binding systems (Steinhardt & Reynolds, 1969) (e.g., equilibrium dialysis, ultrafiltration, gel filtration according to Hummel & Dreyer (1962) or according to Tamir (1982), fluorescence and ultracentrifugation), systems containing PF4 or βTG in which serotonin-binding is measured may cause more difficulties than most other systems. This is because of the inherent characteristics of these molecules. Not only is serotonin notorious for its ability to bind to surfaces such as dialysis membrane and filters (Tamir, 1982), but also PF4 disappears on Sephadex columns by binding to the gel, as well as permeating intact dialysis membrane or biological membranes (as does βTG) (Handin & Cohen, 1976; Nath et al., 1973; Goldberg et al., 1980). In the presence of its carrier, PF4 gives speciously low values in the Lowry protein determination.

In short, every method applied (equilibrium dialysis, ultrafiltration, Hummel-Dreyer's and Tamir's gel filtration techniques) required compromises or allowances for nonspecific binding by the ligand or the protein to a membrane, filter or gel. Although considerable qualitative data eventually indicated that serotonin was bound by PF4 and βTG, no value could be obtained for n and k for the serotonin-PF4 system. Values of n and k for the serotonin- βTG system were obtained by ultrafiltration through a filter whose binding sites for serotonin were not saturated.

The technique described by Shih and Rho (1977) as a means for establishing n and k values by fluorescence offers promise for determining binding parameters of interactions between various ligands and serotonin-binding protein. But, as can be seen from their published figures, similarities in the excitation and emission spectra of serotonin and the protein preclude the use of this technique to measure interaction between serotonin and the serotonin-binding protein. For the same reason other fluorescence techniques described by Steinhardt & Reynolds (1969) cannot be used.

Ultracentrifugation has not yet been used to examine serotonin-binding by PF4. This technique was not used because the limits of

sensitivity of the method would be exceeded if the stoichiometry of the interaction involved binding of only a few moles of serotonin (M_r 176) per mole of tetrameric PF4. However, ultracentrifugation is still a potentially useful technique for investigating the serotonin-PF4 system. In the equilibrium dialysis experiments described in Chapter 3, apparent $\bar{v}$ values of about 2 were often obtained. This value, equivalent to half a serotonin-binding site per monomer, implies that a dimeric form of PF4 may occur and is consistent with Klingenberg's hypothesis (1981) for proteins that traverse membranes. PF4 dimers have not been described previously, although PF4 does traverse membranes (see references in Chapter 1).

Monomerization of PF4 by changing the protein concentration or ionic strength of the solution does not occur, although the state of aggregation of βTG is affected by both these factors. This could complicate the analysis of results of binding studies by ultracentrifugation.

The study of calcium binding by PF4 was amenable to investigation by equilibrium dialysis, in contrast to serotonin-binding by PF4. As long as binding could be shown, it was assumed that PF4 was still on the side of the membrane where it had been introduced, if serotonin was excluded. Although it was hoped to obtain binding parameters for the binding of Ca^{2+} by PF4, analysis of accumulating data indicated that the Ca^{2+}-PF4 interaction is not simple and appears to involve positive cooperativity. A value of 3,4 obtained for the Hill coefficient tends to confirm the evidence of positive cooperativity shown by the Scatchard plots. The values for n and k could be determined only over a limited range of calcium concentrations.

It seems unusual that serotonin-binding by PF4 and βTG has not been described previously, since these proteins are among the most abundant in the platelet (Moore & Pepper, 1976). Although there are many studies of serotonin uptake in human platelets (see Chapter 1), only one report has been published on human platelet serotonin-binding proteins (Pignatti & Cavalli-Sforza, 1975). These authors found that platelet albumin and two soluble glycoproteins bind serotonin. However, albumin and one of the glycoproteins can be presumed to bind serotonin only weakly, since these were both bound and unbound on the serotonin-affinity column. The other soluble glycoprotein was eluted from their column by 10^{-3} M serotonin, which implies a K_D of 10^{-4} - 10^{-6} M (Pharmacia, 1979, p. 87). Pignatti and Cavalli-Sforza did not wash

their affinity columns thoroughly after the main unbound protein peak emerged. This can be seen in their figure (Pignatti & Cavalli-Sforza, 1975). A more thorough wash of the affinity columns in the present investigation may have caused a loss of the glycoprotein which these authors described.

On the other hand, a protein with the properties of PF4 was not reported among their serotonin-binding proteins. The absence of PF4 can be explained if their platelets had undergone release during preparation. This is a likely possibility since they used no anticoagulant in their platelet isolation procedure.

It has been reported by another group of investigators who have isolated serotonin-binding proteins from platelets (Tamir et al., 1980a; Kupsky et al., 1981; Tamir et al., 1983), that a glycoprotein and platelet albumin bound serotonin in the rat platelet. These investigators obtained K_D values of $4,2 \times 10^{-8}$ M and $8,1 \times 10^{-7}$ M for the serotonin-glycoprotein complex. This glycoprotein, named serotonectin (Kupsky et al., 1981), has an identical K_D value for its complex with serotonin to that found in the present study for βTG. Serotonectin and PF4 are also similar in that both are heat-stable, both show enhanced binding of serotonin in the presence of ferrous ion, both are present in the plasma when there is no evidence of platelet cytosol proteins in the plasma, and both are associated, at least briefly, with the platelet plasma membrane. Moreover, PF4 has been shown to be present in the megakaryocyte (McLaren & Pepper, 1982) and serotonectin is thought to be synthesized in megakaryocytes (Tamir et al., 1983).

However, the protein serotonectin described by Tamir's group is not βTG nor PF4. Serotonectin produces a single band which stains for both carbohydrate and protein on SDS polyacrylamide electrophoresis gels, and corresponds to M_r 204 000, and serotonectin is very labile whereas PF4 is stable.

Tamir's group did not discover that PF4 or βTG binds serotonin, probably because they used a different procedure from that used in the present study. One step in their procedure that may be responsible for loss of PF4 is the ammonium sulphate precipitation and subsequent dissolution of the precipitate. When in the present study platelet extract proteins were precipitated with ammonium sulphate at 52% saturation (see Chapter 2), some of the precipitate did not dissolve on

dialysis against water, dilute phosphate buffer or starting buffer with Fe^{2+}, even after more than 30 h dialysis. The precipitate was gel-like and sticky and clung tenaciously to any solid surface with which it made contact. This precipitate was salted back into solution and subsequently bound to serotonin-affinity columns. Tamir et al. do not mention such a precipitate nor the need to salt it back into solution, and this suggests that they may have lost PF4 at this stage in their procedure.

Two other steps in the procedure of Tamir et al. which may also have been responsible for loss of PF4 are dialysis and chromatography on Sephadex.

Two reasons can be offered for not obtaining in the present study the glycoprotein described by Tamir et al. These are differences in species, rat platelets used by Tamir et al. as opposed to human platelets in the present study, and differences in techniques. Tamir et al. followed a general purification procedure involving freeze-thawing of platelet-rich plasma, high-speed centrifugation, ammonium sulphate precipitation, dialysis and Sephadex G-200 chromatography. In this study serotonin-affinity chromatography was used together with a gel to which serotonin was covalently bound via its alpha amino group. Only molecules that bind serotonin and that do not require the amino group of serotonin to be free for binding will be obtained on columns containing this gel. It is possible that the glycoprotein isolated by Tamir et al. binds to the alpha amino group of serotonin.

The present study was intended to elucidate the functions of serotonin and of the platelet-specific releasable proteins which bound serotonin. As has been described in Chapter 1, soon after platelet release, PF4, serotonin and calcium are transiently associated with the platelet plasma membrane (Phillips et al., 1980; Jaffe et al., 1982; George & Onofre, 1982; Lingjaerde, 1981, p. 164; Massini et al., 1978; Skaer et al., 1974). PF4 exists in a form that is different from the characteristic form it possesses in the alpha granules (Hagen et al., 1982). The released form shows greater homogeneity of charge and does not bind to thrombin, although its precursor did bind to thrombin.

Since Ca^{2+} binding to PF4 is positively cooperative, a possible role for PF4 in Ca^{2+} transport through platelet plasma membranes should be examined. A Ca^{2+}-dependent ATPase (called "relaxing factor") has been postulated to be present in the platelet plasma membrane (Statland et al., 1969). It has been proposed that this enzyme may provide the

source of calcium involved in initiating the platelet release reaction (Rubin, 1974). From considerations of M_r and K_D (Dean & Sullivan, 1982) (see Chapter 1) and the presence of the Ca^{2+}-ATPase activity in the plasma membrane, open canalicular system and dense tubular system in platelets that have not undergone release (ibid.) (see Chapter 1), it does not seem very likely that this protein is PF4. Preliminary experiments in this laboratory indicated that purified PF4 has only very weak ATPase activity. For these reasons, a role for PF4 in calcium transport through the platelet plasma membrane is rejected.

A possible function of a serotonin-Ca^{2+}-PF4 complex on the platelet plasma membrane at the time of platelet release is induction of platelet aggregation (Lüscher & Käser-Glanzmann, 1974). The present study lends credence to this possibility. The results reported in Chapter 3 indicate that serotonin was bound to dialysis membrane in a time-dependent manner, and that this binding was enhanced by the presence of PF4. The stickiness of the ammonium sulphate-precipitated PF4 (described earlier in this chapter) is indicative of agglutinating properties. Release inducers cause platelets to become sticky (Holmsen et al., 1969) and a sticky platelet may act to aggregate other platelets (ibid.). In addition, the difficulties with elution of native and purified PF4 from the serotonin-affinity columns could be interpreted as evidence for agglutinating properties. PF4 has been shown to agglutinate formaldehyde-fixed, trypsin-treated erythrocytes, washed platelets and formaldehyde-fixed washed platelets (Phillips & Gartner, 1980, p. 424-5). Moreover, a drug, dipyridamole, that is known to cause significant inhibition of PF4 secretion also reduces platelet aggregation and adhesiveness (Akman, 1980; Gray et al., 1968).

The agglutinating activity of PF4 is not trypsin-sensitive (Phillips & Gartner, 1980, p. 425). As has been described in Chapter 1, trypsin treatment of PF4 has produced varying results (Moore et al., 1975b; Farbizewski et al., 1966; Nath et al., 1973 and 1975). There are only three sites on the PF4 molecule at which trypsin is likely to attack (Haschemeyer & Haschemeyer, 1973, p. 81), and these sites may not always be exposed, especially in the native PF4 molecule. It has been noted previously that thrombin does not bind to the form of PF4 that exists after platelet release, and that this form has greater electrophoretic homogeneity than has the unreleased form of PF4 (Hagen et al., 1982). It may be that binding of Ca^{2+} to PF4 is responsible for both effects, that is, protection of PF4 from proteolytic

degradation and production of greater homogeneity of charge. There are precedents for both effects by Ca^{2+}. Thrombin and trypsin are both inhibited by Ca^{2+} binding at certain, but not all, vulnerable sites on the Glycoprotein G molecule (Lawler et al., 1982; Lawler & Slayter, 1981), and on the calmodulin, troponin C and modular protein molecules (Walsh et al., 1977). The electrophoretic mobility of the proteins calcineurin B, calmodulin and troponin C is affected by Ca^{2+} binding, as well (Klee et al., 1979). It can be assumed that binding of cations or anions changes the net charge on a protein and the isoelectric point increases with binding of cations and decreases with binding of anions (Bull, 1964, p. 136). Calcium binding to PF4 would thus make the protein more cationic.

After leaving the platelet plasma membrane, PF4 exists only briefly in the plasma before becoming associated with the endothelium (Dawes et al., 1978 & 1982; Busch et al., 1980). The gel filtration results presented in Chapter 3 indicate that PF4 binding of serotonin was enhanced by 2% bovine serum albumin, and that, at physiological concentrations of serotonin and PF4, the PF4 binds much more serotonin per mole than at lower [serotonin]/[PF4] ratios. These results suggest that serotonin remains associated with PF4 as it moves from the platelet plasma membrane to the endothelium.

PF4 enters endothelial cells after injury (Goldberg et al., 1980). Serotonin accumulates on the walls of arteries, veins and capillaries within an area of injury (Costa et al.,(1974 a). Costa et al. wrote "It seemed, in some instances, as if 5-hydroxytryptamine was localized in the endothelial lining or muscular coat of the vessels." It is also thought that serotonin alters the permeability of endothelium to protein tracers, even across the blood-brain barrier (Westergaard & Brightman, 1973; Westergaard, 1978).

Nachman et al. (1972) described a factor (probably PF4, in view of Goldberg et al.'s results, paragraph above) from human platelets that enhanced vascular permeability as a stable anti-heparin cationic protein of M_r about 30 000. The vascular permeability response was blocked by prior treatment of the animal with antihistamine. The permeability response was biphasic. The acute response was characterized by edema and vasodilation, while the delayed response (at about 3 h) was characterized by leukocytic infiltration (Nachman & Weksler, 1972). These findings have been confirmed by others (Anderson & Scotti, 1976, p. 77). Anderson and Scotti cite serotonin as one of

the better known chemical mediators likely to be responsible for the increased vascular permeability. These results suggest a relationship between serotonin and PF4 in vascular permeability responses.

The results presented in Chapter 2, Table 2-13, indicate that some drugs shown to affect serotonin storage or uptake in platelets and brain have an effect on the PF4 antigen-antibody reaction. It was noted in Chapter 1 that serotonin storage in platelets is almost entirely within the dense granules and does not involve proteins. Serotonin uptake at the platelet plasma membrane has also been described and attributed to so-called "Site B," a tricyclic antidepressant-sensitive site. Although the Site B protein shares at least two characteristics with PF4, namely, binding to serotonin with high affinity and sensitivity to tricyclic antidepressants, no substantive evidence has been put forward to show that the Site B protein is identical to PF4. On the contrary, the Site B protein appears to be a permanent member of the platelet plasma membrane, while PF4 only resides on the membrane briefly at the time of platelet release. However, imipramine and other tricyclic antidepressants inhibit serotonin uptake not only into platelets, but also by endothelial cells (Shepro et al., 1975). Thus, the results presented in Chapter 2 showing inhibition by tricyclic antidepressants in the PF4 radioimmunoassay are consistent with the involvement of a serotonin-PF4 complex in the vascular permeation of endothelial tissue.

PF4's main function may be heparin-neutralization (Pepper, 1980). Binding of heparin probably does not preclude the binding of serotonin by PF4, since: 1) native PF4 did bind to the serotonin-affinity column and was removed with an associated carbohydrate (Chapter 2); 2) heparin binds to the lysine residues near the carboxyl terminus of the PF4 molecule (Handin & Cohen, 1976), leaving the remainder of the PF4 molecule free for other interactions; and 3) βTG (which also neutralizes heparin) does bind serotonin in the presence of heparin (Chapter 4).

The leukocytic infiltration of injured tissue, which has been described as the second phase of the biphasic permeability response, probably involves βTG (Senior et al., 1983), but binding of serotonin by βTG does not appear to be required for this cytotaxic function, or for any other putative function of βTG (see Chapter 1). Thus, a role for the serotonin-βTG complex remains to be ascribed.

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