AN INVESTIGATION INTO METHODS OF ASSESSING THE INTERACTION OF PLATELETS WITH IMMOBILISED COLLAGEN

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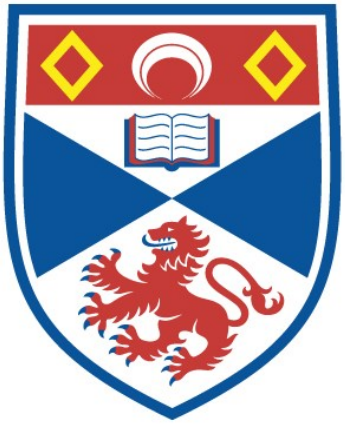
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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AN INVESTIGATION INTO METHODS OF ASSESSING THE INTERACTION OF PLATELETS WITH IMMOBILISED COLLAGEN

A THESIS PRESENTED BY

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to

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that it is my own composition and that no part of it has been previously presented for a higher degree.

The research was conducted in the Department of Biochemistry and Microbiology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr Graham Kemp.

CERTIFICATE

I hereby certify that Shana Thomson has spent nine terms engaged in research work under my direction and she has fulfilled the conditions of Ordinance General No. 12, and Resolution of the University Court, 1967, No. 1, and that she is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

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I matriculated as a research student in the Department of Biochemistry and Microbiology, University of St. Andrews in October, 1976.

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My husband also deserves my thanks for his patience during the writing-up period.

DEDICATION

This thesis is dedicated to my parents in gratitude for all they have done for me.

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SUMMARY

When platelets come into contact with collagen, they adhere to it then release the contents of their dense granules and α granules and aggregate together. This interaction is dependent on the conformation and degree of multimerisation of the collagen as well as the experimental conditions.

The fibrillar state of collagen varies with the pH and ionic strength of the solution so collagen was immobilised on to an insoluble support in order that its fibrillar state should remain unchanged during exposure to platelets and during modification. The first support used was polyamide sheet, but although the resulting collagen-strips induced measurable platelet adhesion and aggregation, there was insufficient collagen present to induce adequate aggregation or serotonin release to be of use in investigating these interactions. Sepharose 6B was then used as a support for collagen and this proved satisfactory, inducing approximately 20% serotonin release from platelets. The release reaction is nearer to the initial interaction between platelets and collagen than aggregation, which is the final step, and it therefore allows a better insight into the conditions necessary for the interaction to take place.

The ε-amino groups of collagen-Sepharose were modified in three ways, using succinic anhydride, trinitrobenzene sulphonic acid and dinitrofluorobenzene. Succinylation almost abolished serotonin release while di- and trinitrophenylation enhanced this reaction. ε-amino groups of collagen are therefore not in themselves important in the recognition of collagen by platelets and the subsequent reactions, since they were blocked by the modifications. The suggestion is made that succinylation disrupted the fibrils because of the change in charge whereas di- and trinitrophenylation merely neutralised the charge and permitted realignment of any previously misaligned areas of the molecule.

These results reinforce theories that the correct quaternary

structure of collagen must be present in order that it will be recognised by platelets.

1. INTRODUCTION

When a blood vessel is damaged, bleeding eventually stops due to the formation of a platelet plug and eventual coagulation of the blood. The initial steps in this healing of the wound involve the interaction of platelets with the collagen which is present in the blood vessel walls. The features of collagen which are recognised by platelets are (and have been) under investigation by a number of researchers, but as yet these features have not been identified. This thesis presents the findings of such an investigation.

1.1. Collagen structure

Collagen is the major structural protein of mammals, being the dominant protein in blood vessels, tendons, dentin, cartilage and bone (Jaffe, 1976). It can also be found in many other tissues.

Collagen contains the unusual amino acids hydroxyproline and hydroxylysine (Jaffe, 1976). It consists of a repeating tripeptide (glycine-X-Y) and at least one third of the primary structure has X = proline (Hannig & Nordwig, 1967): neutral regions consist mainly of this tripeptide (Hannig & Nordwig, 1967). Y is often hydroxyproline or alanine (Hannig & Nordwig, 1967).

The primary structure of collagen contains sections of mainly neutral amino acids (apolar) and sections of mainly polar residues, and this gives rise to striations when viewed with the electron microscope (Hannig & Nordwig, 1967). A small amount of carbohydrate (less than one percent) is present and is attached to collagen via hydroxylysine residues, forming galactosylhydroxylysine (gal-hyl) and glucosylgalactosylhydroxylysine (glc-gal-hyl) (Chesney et al., 1972; Jaffe, 1976).

The collagen molecule consists of three parallel polypeptide subunit chains (Piez, 1967) of approximately equal length (von

Hippel, 1967), each coiled in left hand helices (von Hippel, 1967). The three chains are coiled together to form a triple helix (polyproline type II) with a slight right hand twist (von Hippel, 1967) and the amino acid side chains point outwards (Jaffe, 1976). The molecule is rigid and measures about 300nm by 1.5nm and it has a molecular weight of about 3×10^5 (von Hippel, 1967).

At either end of the molecule there is a non-helical "telopeptide" portion which differs in amino acid content from the helical region (Jaffe, 1976). These telopeptides are important for intramolecular crosslinking and intermolecular interactions to form fibrils (Chesney et al., 1979) and are accessible to selective enzymatic and chemical cleavage (Piez, 1967).

The collagen molecule is held together both intra- and intermolecularly by non-covalent interactions (von Hippel, 1967), and the presence of covalent interchain interactions is important in fibril and fibre formation and subsequent insolubilisation (von Hippel, 1967). The following intermolecular crosslinks are found in collagen (Jaffe, 1976):-

(i) lysinonorleucine (adduct of lysine and allysine)
(ii) hydroxylysinonorleucine (adduct of hydroxylysine and allysine)
(iii) dihydroxylysinonorleucine (adduct of hydroxylysine and hydroxyallysine)
(iv) histidinylaldol (Michael addition of histidine and aldol)
(v) histidinylhydroxymerodesmosine (adduct of aldol, hydroxylysine and histidine)

Aldol intramolecular crosslinks (aldol condensation of two allysines) are also found in collagen (Jaffe, 1976). Allysine and hydroxyallysine are formed by the action of lysyl oxidase on lysine and hydroxylysine (Rowe et al., 1974). The lysine and hydroxylysine residues are therefore involved in most crosslinks of collagen.

Collagen molecules (tropocollagen) can self-polymerise to multimers and larger polymers (fibrils) of different types (Jaffe,

1976). Native-type fibrils are composed of collagen molecules arranged parallel to each other with a longitudinal displacement of approximately one quarter of the molecular length (Hodge, 1967). A periodic pattern of striations can be seen using the electron microscope, due to the different staining characteristics of the polar and apolar regions (Hodge, 1967), as mentioned above. The length of the collagen molecule is approximately 4.4 times that of the native collagen period (D) and it would appear that molecules may be arranged in hollow cylindrical filaments in a spiral manner, with 0.6D gaps between molecules and a cross section of five molecules (figure 1) (Smith, 1968). In this arrangement there would be an overlap zone of 0.4D and a hole zone of 0.6D in each period (Hodge, 1967).

Collagen can form fibrils of different types according to the experimental conditions (figure 2) (Muggli, 1978).

There are four subtypes of collagen (Jaffe, 1976). Type I collagen may be found in bone, mature skin, tendon and dentin and it consists of two types of subunit chain, $\alpha 1$ and $\alpha 2$: $[\alpha 1(I)]_2 \alpha 2$ (Jaffe, 1976). Type II collagen is present in articular cartilage and has three identical subunit chains, $[\alpha 1(II)]_3$, as does type III collagen, $[\alpha 1(III)]_3$, which is found in the media of muscular arteries and foetal skin (Jaffe, 1976). Basement membrane (type IV collagen) also has three identical subunit chains, $[\alpha 1(IV)]_3$.

The different types of collagen vary in the extent of hydroxylation of proline and lysine and in the amount of carbohydrate present (it is attached to these hydroxylated residues). Type IV is able to have a relatively large amount of carbohydrate due to the greater extent of hydroxylation (Jaffe, 1976).

Type I collagen is found in three different forms, depending on the maturity and extent of crosslinking: neutral salt soluble collagen, which is extracted from tissue using sodium chloride; acid soluble collagen, prepared by solubilising the tissue in citric acid or acetic acid; and insoluble collagen (Jaffe, 1976).

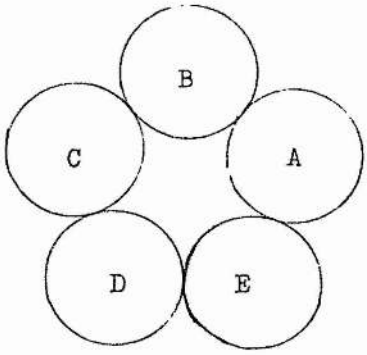


Figure 1a. Diagram of cross-section through a five molecule collagen filament (Smith, 1968).

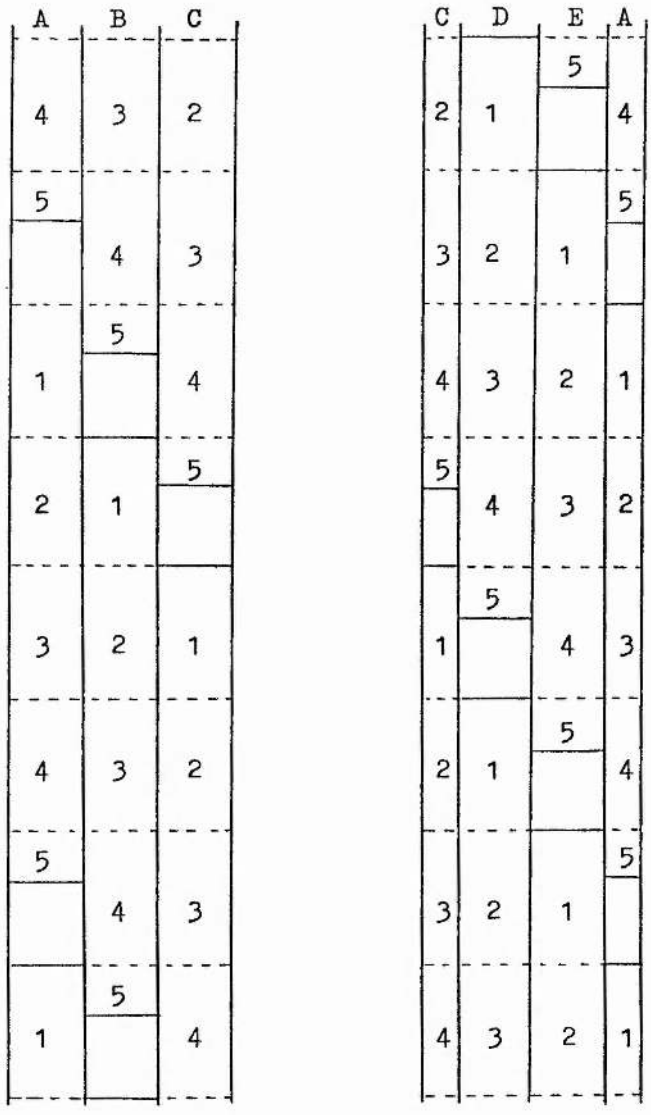


Figure 1b. The collagen filament in figure 1a seen from above (left) and from below (right) (Smith, 1968).

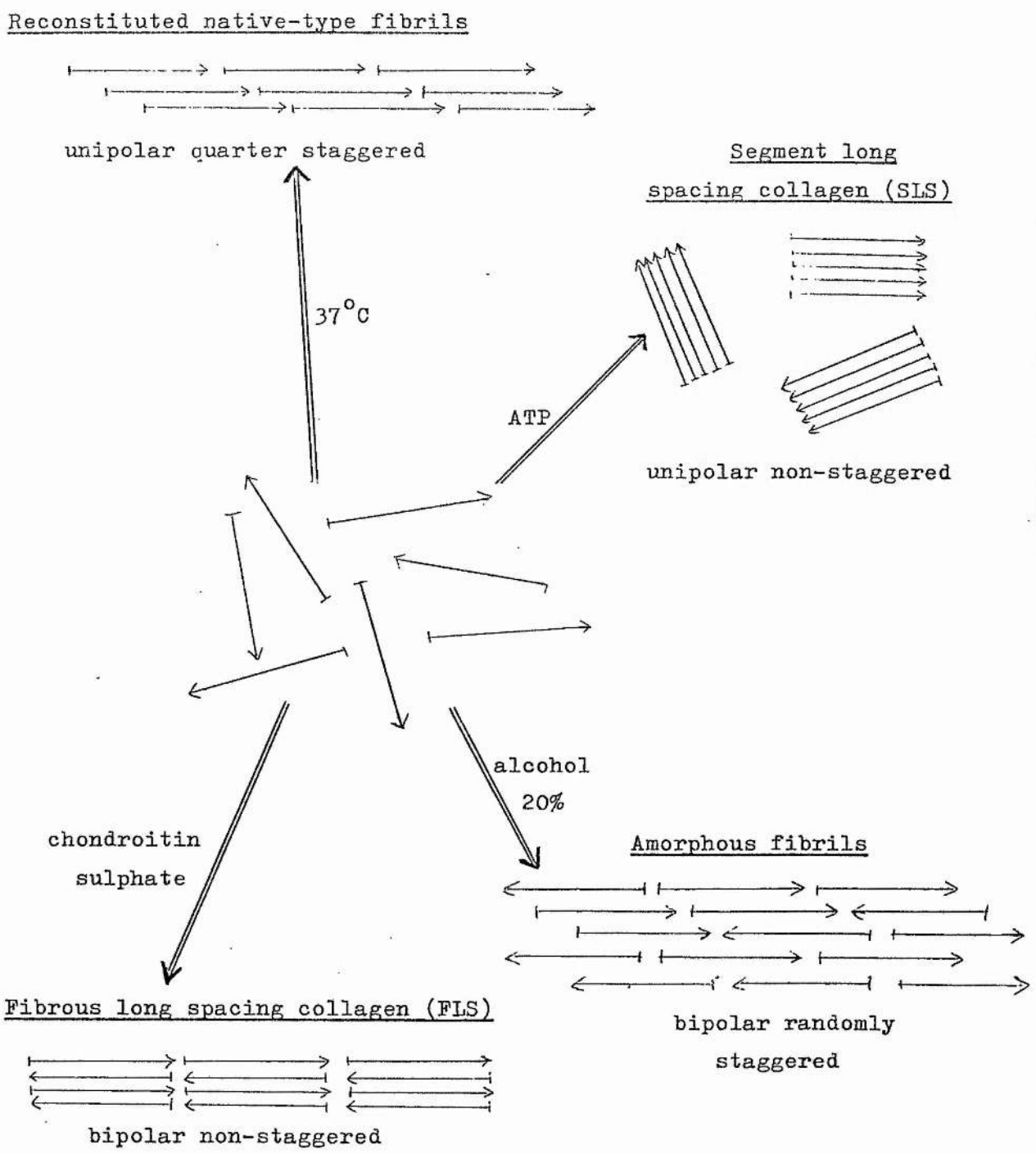


Figure 2. Formation of different types of collagen fibril (Muggli, 1978).

1.2. Haemostasis

Collagen plays an important part in haemostasis. It can have a direct effect on platelets, causing the release of various constituents and eventual clotting (Huzoor-Akbar & Ardlie, 1976) and an indirect effect involving the activation of coagulation factors such as factor XI by collagen-activated platelets (Ardlie & Han, 1974) which is mediated by thrombin (Huzoor-Akbar & Ardlie, 1976). Collagen also activates factor XII, the Hageman factor (Wilner et al. 1968a), which is another factor of the intrinsic pathway of blood coagulation.

The direct effect of collagen on platelets has been extensively investigated during the past decade (Michaeli & Orloff, 1976) and it is generally agreed that there are several steps involved. The first step (Ardlie & Han, 1974; Crawford & Taylor, 1977) is the adhesion of circulating platelets to collagen which has been exposed by an injury to the blood vessel. These stimulated platelets activate factor XI and the intrinsic pathway of the blood coagulation system is initiated (Ardlie & Han, 1974), factor XII being activated directly by collagen (Wilner et al., 1968a).

The contents of the platelet dense granules (including ADP, serotonin (5-hydroxytryptamine) and ionised calcium) and α-granules (including fibrinogen and platelet factor 4) are released (Gordon & Milner, 1976) and thromboxane A_2 and the prostaglandin endoperoxides are formed (Mustard & Packham, 1977), all of which stimulates platelet aggregation (Gordon & Milner, 1976). The released ADP causes other platelets to change shape rapidly from discoid to spherical with pseudopodia and the microtubules disappear (Crawford & Taylor, 1977; Mustard & Packham, 1977). These altered platelets adhere to each other and to platelets which are already adhering to collagen (Mustard & Packham, 1977).

Aggregation is the next event to occur, accompanied by a further acceleration of blood coagulation (Mustard & Packham, 1977). Fibrin is formed as one of the last steps in the coagulation cascade and it forms a mesh around the aggregated platelets, trapping other

blood cells (Mustard & Packham, 1977) and the whole mass retracts into the wound to form a solid plug (Gordon & Milner, 1976). If the mesh contains no active thrombin it becomes relatively non-thrombogenic (Hovig et al., 1968).

To summarise, the main steps in platelet plug formation, none of which is a discrete process in itself (Crawford & Taylor, 1977), are adhesion, release and aggregation. Each step follows from and is affected by changes in the previous step(s).

1.3. Methods of investigation

There are many methods of investigating the interaction between platelets and collagen. This is because of the different stages of the interaction, each of which is greatly affected by the experimental conditions and by the preceding stages, and because of the different degrees of multimerisation of collagen and the effect of the experimental conditions on the rate of fibrillogenesis.

In recent years collagen has been used as insoluble fibres (Aznar et al., 1976), as hollow fibres (Miyata et al., 1976) and attached to a surface. The surface used has often been glass, in the form of cylinders (Cazenave et al., 1973), as coverslips (Meyer & Weisman, 1978) and as fibres (Meyer & Weisman, 1979). Plastic coverslips have also been used (Lyman et al., 1971). Collagen-coated gelatin tubes have been used to investigate platelet adhesion and aggregation (Muggli & Baumgartner, 1975). Collagen has also been attached to agarose in order to measure platelet adhesion and aggregation (Heene et al., 1975) and to measure adhesion and release (Brass & Bensusan, 1975).

There are many problems in the use of platelets since it is difficult to completely separate one step in their interaction with collagen from another. The choice of anticoagulant and resuspension medium is important since both may have a great effect on the interaction under investigation. For example EDTA, which reduces the concentration of ionised calcium and hence prevents aggregation

(Day et al., 1975), impairs the uptake of serotonin (Grant & Zucker, 1979; Hardeman & Heynens, 1974b). Sodium citrate enables more serotonin to be taken up (Born & Gillson, 1959) but it augments ADP-induced release (Heptinstall & Mulley, 1977), so care must be taken when measuring this parameter. Heparin inhibits collagen-induced release (Ardlie & Han, 1974; Heiden et al., 1977): it may affect the collagen since very low concentrations of heparin have been reported to retard fibril formation in solutions of acid-soluble collagen (Wood, 1960).

Acid-citrate-dextrose (ACD) prevents the pH of blood rising as high as when sodium citrate is used (Day et al., 1975) and serotonin uptake is greater than with EDTA (Hardeman & Heynens, 1974b). ACD removes the ionised calcium and maintains ionised magnesium (Ardlie et al., 1970).

It is advisable when using washed suspensions of platelets to include glucose in the medium in order to provide an energy source (Kinlough-Rathbone et al., 1969). The presence of a protein such as albumin is advantageous because it protects the platelets from lysis (Kinlough-Rathbone et al., 1977) but fibrinogen enhances the extent of aggregation and release induced by ADP and collagen (Kinlough-Rathbone et al., 1977).

Apyrase is often used to prevent platelet aggregation when adhesion or release is under investigation since it degrades ADP released from platelets (Cazenave et al., 1975) thus allowing adhesion but no aggregation while in a physiological concentration of divalent cations (Cazenave et al., 1973). EDTA can also be used to prevent aggregation while permitting collagen-induced adhesion (Salzman, 1971) but the concentration of divalent cations is reduced. Divalent cations are required for the collagen-induced release reaction (Kinlough-Rathbone et al., 1977).

There are many different methods in use for investigating the interaction of platelets and collagen, depending on which aspect of the interaction is to be examined. Aggregation is usually investigated with the aggregometer (Born & Cross, 1963) or, more

recently, the "lumiaggregometer" which measures ATP secretion as well as aggregation. Variations of this have been used by Charo et al. (1977), Feinman et al. (1977), Kronick and Jimenez (1976) and Larsson et al. (1977). Platelet adhesion to collagen has been measured by stopping the reaction by fixing the platelets with glutaraldehyde and counting the non-adherent platelets (MacKenzie et al., 1974). Sivertson (1976) used a similar method, but used whole blood and fixed the platelets with formaldehyde. Fauvel et al. (1976) separated non-adherent platelets from collagen by using Sepharose or Ficoll. Radioactive serotonin release has been measured by Ardlie and Han (1974) and by Brass and Bensusan (1974): the latter have also developed a method using collagen-Sepharose which can be used to measure adhesion as well as release (Brass & Bensusan, 1975; Brass et al., 1976).

More physiological approaches include the rotational viscometer (Anderson et al., 1978) and the perfusion chamber (Baumgartner & Haudenschild, 1972).

Platelets are often prepared by differential centrifugation followed by washing and resuspending (Ardlie & Han, 1974), but they may also be prepared by gel filtration (Tangen et al., 1971).

1.4. Theories of platelet-collagen interaction

There are many theories as to the mechanism of the platelet-collagen interaction and the active site on the collagen molecule which is recognised by platelets.

1.4.1. Collagen structure

The general consensus of opinion is that collagen must have some degree of quaternary structure in order to interact with platelets. Jaffe and Deykin (1974, 1975) showed that tropocollagen did not initiate binding or aggregation of platelets whereas fibrillar collagen did. Multimeric collagen caused binding and

aggregation after a short lag during which time fibrils were formed, and denatured collagen (gelatin) caused no aggregation. These experiments showed that collagen quaternary structure was required to cause platelet aggregation.

This evidence was reinforced by the discovery that the lag time before aggregation occurred was proportional to the degree of multimerisation (Brass & Bensusan, 1974; Simons et al., 1975) and that if polymeric collagen was added to the monomeric collagen, the lag period was decreased (Brass & Bensusan, 1974). However, Jaffe and Deykin (1974) found that the lag time was independent of the concentration of monomeric collagen. But small amounts of collagen were used and there may have been a synergistic effect with the released ADP (Packham et al., 1973).

When tropocollagen was modified in such a way as to impair or prevent multimerisation, platelet aggregation was also impaired or prevented (Jaffe & Deykin, 1974; Muggli & Baumgartner, 1973; Simons et al., 1975; Wang et al., 1978b).

But although fibrils are required for platelet aggregation, their effect is greater if they are finely dispersed, due to the greater surface area (Muggli & Baumgartner, 1973).

It has also been shown (Brass & Bensusan, 1975) that collagen quaternary structure is only required for platelet release and aggregation but not for adhesion. But collagen tends to bind non-specifically to surfaces, so some binding of collagen monomers to platelets may also be non-specific.

Although it would appear that platelets require collagen fibrils in order to aggregate, there is some evidence that the fibrils need not be native-type (Balleisen et al., 1976; Muggli, 1978). Indeed, Muggli (1978) showed platelet aggregation with SLS, FLS and non-striated amorphous collagen and observed no renaturation into native-type fibrils.

In spite of the wealth of evidence that collagen fibrils are

required for platelet aggregation, denatured ∝1 chains of chick skin collagen (which have no tertiary or quaternary structure) have been shown to cause aggregation (Kang et al., 1974; Katzman et al., 1973) but this effect has not been demonstrated with ∝1 chains from any other species (Puett et al., 1973). Whether chick skin collagen differs in any way from other collagens as far as the platelet is concerned, or whether reassociation of the monomers had taken place is unclear.

Other workers (Chesney et al., 1972; Puett et al., 1973; Wilner et al., 1968b) have shown that an intact tertiary structure is required for platelet aggregation, usually by using denatured collagen or chains.

Elements of the primary structure are necessary for adhesion and aggregation of platelets; the non-helical C-terminal end of the ∝1 chain has been shown to be important (Fauvel et al., 1978a,b).

1.4.2. Type of collagen

Type I collagen has been shown to be the most active type (Kronick & Jimenez, 1979; Michaeli & Orloff, 1976), and type III has also been shown to be very active (Hugues et al., 1976; Kronick & Jimenez, 1977). But Santoro and Cunningham (1977) have demonstrated that types I, II and III are all similar in action in their fibrillar form. The differences in activity are probably due to different rates of fibrillogenesis; type III may form fibrils quicker than type I (Santoro & Cunningham, 1977).

1.4.3. Functional groups

A number of groups or regions of the collagen molecule have been implicated in the recognition of collagen by platelets. The central, helical, portion of the collagen molecule is believed to contain the platelet-active groups (Wilner et al., 1968b, 1971).

Much investigation has been carried out into the role of the ε-amino groups of lysine and there are opposing schools of thought on this. It has been reported (Wilner et al., 1968b) that the ε-amino groups are required for platelet aggregation and that while poly-L-lysine will induce aggregation, succinylation of the amino groups causes a loss of this aggregating ability (Mohammad et al., 1977). But the apparent requirement for the amino groups may simply be a requirement for rigidly spaced positive charges (Nossel et al., 1969), which would explain the loss of activity when collagen is denatured from a rigid rod-shaped molecule to a random coil. It has also been reported (Wilner et al., 1971) that the rigidly spaced charges may be either positive or negative for aggregation to occur, since positively charged amino groups may be replaced by negatively charged carboxyl groups. But Miyata et al. (1976) disagree with this theory, having demonstrated that methylated collagen (which has fewer negative groups) caused more aggregation than did unmodified collagen, whereas succinylated collagen (which has negative groups instead of positive amino groups) caused less aggregation.

But the ε-amino group has also been shown to be unimportant for platelet aggregation (Wang et al., 1978a; Whitin & Simons, 1977) as have the carboxyl and hydroxyl groups (Wang et al., 1978a). Guanidination of lysine to homoarginine, which leaves the arrangement of polar groups intact, decreases the lag period before release of platelet constituents, but it also accelerates multimerisation (Brass & Bensusan, 1974). The enhanced platelet reaction is therefore probably due to the accelerated rate of fibril formation and not because the ε-amino groups are unimportant for platelet aggregation. Permethylation of the ε-amino groups does not inhibit aggregation nor impair fibrillogenesis (Jaffe & Deykin, 1975).

Arginine residues, which are also positively charged, are likewise thought to be important in the recognition of collagen by platelets (Wang et al., 1977, 1978a). "Bridges" have been observed between platelets and the dark bands (presumed to be groups of polar residues) on native-type fibrillar collagen (Hovig et al., 1968; Zucker-Franklin & Rosenberg, 1977) and it has been proposed (Wang et al., 1978a) that arginine residues may be involved in the

formation of these "bridges". Wang et al. (1978b) have also calculated that the minimum length of collagen required to cause aggregation and release is approximately three molecular lengths (900nm) and since there are five sites of polar residues at equidistant positions along the collagen molecule (Smith, 1968), there must be 15 such sites joined together to cause aggregation and release.

The importance of the telopeptides has been investigated and it is thought that they are required for fibril formation rather than having a direct effect on platelet aggregation (Chesney et al., 1979).

A recent theory is that there are proline-binding sites on platelets (Meyer & Weisman, 1978). The evidence for this is that platelet binding to collagen may be inhibited by preincubation with soluble collagen in helical or non-helical form, with or without telopeptides or sugars. This effect is also produced by cyanogen bromide peptides of collagen and by any polypeptide of adequate size which is rich in proline or hydroxyproline (Meyer & Weisman, 1978). Proline and hydroxyproline residues are found on the outer positions of the triple helix so they are available to any proline-binding sites which may exist on the platelet membrane.

Meyer and Weisman (1979) have shown that the morphology of collagen is also important in that collagen-coated glass fibres have a greater effect on platelets than do collagen-coated glass coverslips. This supports the observation of Muggli and Baumgartner (1973) that finely dispersed fibrils are more active than aggregates of fibrils.

1.4.4. Carbohydrates and the glucosyl transferase theory

Hydroxylysine-linked carbohydrate groups have been shown to be necessary for platelet recognition of chick skin collagen (Katzman et al., 1973) and work using the α1-CB5 peptide of chick skin collagen identified the glycopeptide glycosylgalactosylhydroxylysine (glc-gal-hyl) as being important (Kang et al., 1974). Evidence for

this included observations that glc-gal-hyl inhibited collagen $\alpha 1$ chain-induced platelet aggregation (although at a large molar excess) and that when the carbohydrates of the $\alpha 1$-CB5 peptide were modified by periodate oxidation, no aggregation occurred.

But the importance of carbohydrate groups appears to be confined to chick skin collagen since when rat and human skin collagen were used (Puett et al., 1973), neither $\alpha 1$-CB5 nor glc-gal-hyl induced or inhibited native collagen-mediated platelet aggregation. Periodate oxidation of the collagen, which modified 90% of the carbohydrate, had no effect on its aggregating activity (Puett et al., 1973) so it is possible that the carbohydrate moieties do not act as platelet activators but that they might act as binding sites of collagen to the platelet membrane. These sites are probably non-specific since high concentrations of the glycopeptides are required for binding. Other workers (Muggli & Baumgartner, 1973; Santoro & Cunningham, 1977) have also oxidised the carbohydrate moieties of collagen and found that aggregation of platelets induced by the fibrillar forms of this collagen was unaffected.

Galactose oxidase has also been used to oxidise the galactose residues of collagen (Chesney et al., 1972; Harper et al., 1975) and no platelet aggregation was observed with collagen modified in this way. This evidence supported the theory that galactose receptors are important in platelet-collagen interaction. But galactose oxidase was then shown to impair fibril formation (Muggli & Baumgartner, 1973) and once fibrils had formed, the modified collagen caused normal aggregation. Fibrils formed both from periodate-oxidised collagen and from periodate-oxidised/borohydride-reduced collagen induced aggregation (Santoro & Cunningham, 1977). Other workers (Brass & Bensusan, 1976; Gordon & Simons, 1977a,b) also found that carbohydrate residues play a part in fibril formation.

An important theory explaining the platelet-collagen interaction is the glucosyl transferase theory, which also maintains that the carbohydrate residues are important. Collagen glucosyl transferase

was found in the outer part of the platelet membrane (Barber & Jamieson, 1971a,b; Bosmann, 1971) and since there are no endogenous acceptors it was thought that the enzyme might function for adhesion rather than for glycoprotein synthesis. The theory was developed by Jamieson et al. (1971), who suggested that collagen-platelet interaction occurs by formation of a complex between glucosyl transferase on the outer surface of the platelet membrane and galactosyl residues on collagen, which serve as acceptor molecules.

Further evidence to support this theory included the observation that glucosamine inhibited both glucosyl transferase and platelet-collagen adhesion (Barber & Jamieson, 1971b). But high concentrations of glucosamine were required, which casts doubts on its specificity as a glucosyl transferase inhibitor. It was later found (Brass & Bensusan, 1974, 1975; Hayashi & Nagai, 1972) that glucosamine inhibits the polymerisation of monomeric collagen, thus inhibiting the platelet-collagen interaction, but that it had no inhibitory effect on the action of fibrillar collagen on platelets.

Further doubts as to the veracity of the glucosyl transferase theory were raised when Menashi et al. (1976) reported that while only native collagen is the substrate in the collagen-platelet interaction, only denatured collagen acts as a substrate for platelet glucosyl transferase.

1.4.5. Other theories

There has been a recent report (Bensusan et al., 1978) that fibronectin might be at least one of the collagen receptors on the platelet membrane, but a conflicting report (Santoro & Cunningham, 1979) maintains that it has only a limited role in platelet adhesion to collagen.

There has been a suggestion (Lahav, 1979) that there may be more than one platelet component, or a complex consisting of several components for adhesion, and Puett et al. (1973) proposed that there may be two sites involved, which could be very close together. The

first would be a protein binding site of high specificity that activates aggregation and the second would be a carbohydrate site (possibly platelet glucosyl transferase) of lower affinity. This second site would be incapable of activating platelet aggregation.

1.4.6. Summary of theories

There is no generally accepted theory as to the method of recognition of collagen by platelets, but most investigators would agree that the tertiary and quaternary structures of collagen are of importance and that fibrils must be present in order that platelet aggregation may take place (Brass & Bensusan, 1974; Jaffe & Deykin, 1974; Simons et al., 1975). Chick skin collagen appears to be an exception to this since the α1-CB5 peptide has been shown to induce platelet aggregation (Kang et al., 1974; Katzman et al., 1973).

There is considerable evidence that the ε-amino groups of collagen are necessary for platelet aggregation (Mohammad et al., 1977; Wilner et al., 1968b) but it could simply be the positive charges on these groups which are recognised (Nossel et al., 1969). It has been suggested that a regular array of either positive or negative charges are required (Wilner et al., 1971), but it seems unlikely that the charges could be interchangeable. There is however some evidence against the importance of the ε-amino groups (Wang et al., 1978a; Whitin & Simons, 1977).

Many groups or portions of the collagen molecule are involved in fibrillogenesis, which is necessary for platelet recognition, and have no direct effect on platelets, e.g. telopeptides (Chesney et al., 1979), carbohydrates (Brass & Bensusan, 1976; Gordon & Simons, 1977a,b). Care must be taken when modifying the collagen molecule in any way or when using inhibitors since any effect on platelet aggregation may be due to inhibition of fibril formation rather than a direct effect on the platelets, e.g. glucosamine affects fibrillogenesis and therefore platelet aggregation (Brass & Bensusan, 1974, 1975; Hayashi & Nagai, 1972).

The carbohydrates present on the collagen molecule and the enzyme collagen glucosyl transferase on the platelet membrane have also been implicated in collagen-induced platelet aggregation (Bosmann, 1971; Jamieson et al., 1971; Kang et al., 1974; Katzman et al., 1973). It was suggested that a complex is formed between the glucosyl transferase on the outer surface of the platelet membrane and the galactosyl residues on collagen, which serve as acceptor molecules (Jamieson et al., 1971). But it has been shown that the carbohydrate residues on collagen are necessary for fibrillogenesis (Brass & Bensusan, 1976; Gordon & Simons, 1977a,b), which in turn is necessary for platelet aggregation, and that glucosamine, which inhibited both glucosyl transferase and platelet adhesion to collagen, also inhibited fibrillogenesis (Brass & Bensusan, 1974, 1975; Hayashi & Nagai, 1972). It was also discovered that platelet glucosyl transferase requires denatured collagen as a substrate whereas native collagen is required for the platelet-collagen interaction (Menashi et al., 1976).

Proline and hydroxyproline groups (Meyer & Weisman, 1978) and arginine groups (Wang et al., 1978a) have also been implicated in the platelet-collagen interaction, as has fibronectin (Bensusan et al., 1978), but as yet there is little evidence to support these theories.

1.5. Present investigation

Chemical modification of the collagen molecule is a potentially useful method of determining the structural features of collagen responsible for collagen-platelet interaction. But great care is necessary with this approach because such modifications (and indeed the buffer in which the platelets are suspended) can affect the kinetics and extent of collagen polymerisation, factors which are themselves important in collagen-platelet interaction. The approach of chemical modification would give more reliable information about this interaction if the collagen was immobilised on a suitable insoluble support in such a way that subsequent treatment had no effect on the extent of polymerisation.

The aim of the work described here was to investigate different methods of preparing immobilised collagen with particular emphasis on the insoluble support used and the method adopted for assaying collagen-platelet interaction.

2. METHODS

2.1. Analytical methods

2.1.1. Determination of collagen concentration

The microbiuret method of protein estimation (Itzhaki & Gill, 1964) was used to determine the concentration of collagen prepared from calf skin. Gelatin was used as a standard.

2.1.2. Determination of amount of collagen attached to polyamide strips

A sample of each batch of collagen-treated and activated polyamide strips was hydrolysed in 6M HCl (under nitrogen) in an oil bath at 110°C overnight (18 hours). Samples were then taken to dryness, washed with distilled water and redissolved in distilled water.

A hydroxyproline determination was carried out on each sample using the method of Stegemann (1958) and the amount of collagen present was estimated assuming hydroxyproline to be 10% by weight (Eastoe, 1967).

2.1.3. Effect of the presence of Sepharose on collagen determination

a) Collagen, activated Sepharose and collagen plus activated Sepharose were hydrolysed in 6M HCl as in section 2.1.2. Hydroxyproline determinations were carried out on these samples according to the method of Serafini-Cessi and Cessi (1964).

b) Equal volumes of hydrolysed sample (prepared as above) and ninhydrin reagent (as prepared for the Jeol amino acid analyser)

were placed in a boiling water bath for 30 minutes. Absorbance was measured at 570nm.

2.1.4. Use of amino acid analyser for collagen determination

Samples of collagen-Sepharose were hydrolysed in 6M HCl in sealed tubes at reduced pressure for 18 hours at 110°C. They were dried, washed with distilled water, then analysed using a Jeol JLC-5AH amino acid analyser. The collagen content of the collagen-Sepharose was estimated using glycine as 26% by weight (a measured weight of collagen had been found to contain this proportion of glycine).

2.2. Preparation of acid-soluble collagen

Acid-soluble collagen was prepared from calf skin by the method of Steven and Tristram (1962). This involved phosphate extraction followed by sodium chloride precipitation and acetic acid extraction.

2.3. Immobilisation of collagen

2.3.1. Polyamide support

a) Preparation of activated polyamide strips. A piece of polyamide sheet (BDH) was marked into strips 1cm x 4cm and incubated for 15 minutes with 75% (v/v) glutaraldehyde in 0.5M boric acid pH 9.0 at 90°C. The sheet was then washed thoroughly with distilled water for 15 minutes.

The use of a "spacer" was also tried in order to allow the attached collagen more freedom in subsequent interactions with platelets. The method used was to place the activated polyamide in 20% (w/v) polyethylene imine (the "spacer") for one hour, then wash it thoroughly with distilled water and reactivate it with glutaraldehyde and borate as before.

Activated polyamide (with or without spacer) was then washed with 0.1M phosphate buffer pH 7.5 and some was treated with collagen and some was left untreated as a control. Both activated and collagen-treated polyamide were soaked in 0.1M glycine for one hour in later experiments in order to block any remaining free aldehyde groups.

Brady's reagent (Gunstone et al., 1970) was used as a qualitative method for determining whether this method of activating polyamide was successful. An orange colour was produced on activated polyamide (but not on the non-activated control), indicating the presence of aldehyde groups and thus confirming that the polyamide had been activated.

b) Addition of collagen to activated strips. Two basic methods were used for attaching collagen to activated strips in order to determine the conditions under which collagen fibres (or fibrils) of the optimum conformation for inducing platelet aggregation would become attached to the activated strips. Either the strips were placed in visking tubing with collagen solution (2mg ml^{-1} in acetic acid) and dialysed against NaCl or NaCl/phosphate buffer with the intention that collagen fibres would "grow" on the activated strips, or they were mixed with collagen fibres formed by dialysing the collagen solution against NaCl or NaCl/phosphate buffer so that pre-formed fibres would attach to the activated strips.

Strips were dialysed with collagen against 0.4M, 0.5M or 0.6M NaCl and against NaCl (0.2M, 0.3M, 0.4M or 0.5M)/0.1M phosphate pH 7.0, 0.5M NaCl/0.1M phosphate pH 6.0 and 0.5M NaCl/0.01M phosphate pH 6.0, pH 6.5 or pH 7.0. Dialysis was normally carried out at 4°C, but occasionally at room temperature in order to compare the effect of different temperatures. Activated strips with spacer attached were also dialysed under different conditions.

Collagen fibres were used from dialysis against 0.4M, 0.5M or 0.6M NaCl and against 0.5M NaCl/0.1M phosphate at pH 6.0 or pH 7.0.

After preparation, the collagen-coated strips were washed with

distilled water then 0.1M phosphate pH 7.5 and stored in the latter at 4°C until required.

2.3.2. Sepharose 6B support

a) Activation of Sepharose 6B. Sepharose 6B (Pharmacia Fine Chemicals Ltd.) was activated using the method by which Brass et al. (1976) activated Sepharose 2B (i.e. using cyanogen bromide), except that 5M NaOH was used to maintain a pH of 11 and an ice bath was used to keep the temperature below 25°C.

b) Addition of insoluble collagen to activated Sepharose. A suspension of insoluble collagen (type I bovine achilles tendon, Sigma) was used at 1mg ml^{-1} in 0.01M acetic acid. One volume of collagen suspension was mixed carefully in an ice-cooled glass homogeniser with 1.5 volumes of a cold solution of 0.2M $NaHCO_3$/1M NaCl, pH 8.0, and the mixture was immediately added to activated Sepharose at a ratio of up to 1mg collagen per gram of Sepharose. This was mixed overnight at 4°C on a roller-mixer, then washed with a cold solution of 0.1M $NaHCO_3$/0.5M NaCl, pH 8.0, and stored in this solution at 4°C until required.

c) Addition of soluble collagen to activated Sepharose. Collagen was again used at a concentration of 1mg ml^{-1} in 0.01M acetic acid and added to activated Sepharose at a ratio of up to 1mg collagen per gram of Sepharose. Collagen was placed with the activated Sepharose in visking tubing and dialysed against a solution of 0.05M $NaHCO_3$/ 0.25M NaCl, pH 7.1, at room temperature overnight, then washed and stored at 4°C in fresh solution of the same type.

2.4. Modifications to collagen-Sepharose

The ε-amino groups of collagen were modified in various ways. The methods used by Wang et al. (1978) were adapted for use with collagen-Sepharose.

2.4.1. Succinylation

A 3ml aliquot of 2% (w/v) succinic anhydride in acetone was added dropwise to 15g collagen-Sepharose in 50ml 0.05M $NaHCO_3$/ 0.25M NaCl pH 7.1, using 5M NaOH to bring (and maintain) the suspension to pH 9.0. The succinylated collagen-Sepharose was then washed with water followed by 0.5M $NaHCO_3$/0.25M NaCl pH 7.1 and stored in the latter at 4°C.

The extent of succinylation was determined by the analysis of available ε-amino groups, as described by Wang et al. (1978).

2.4.2. Trinitrophenylation

30ml of 0.1% (w/v) aqueous 2,4,6-trinitrobenzene sulphonic acid (TNBS) solution were mixed with 30ml collagen-Sepharose suspension (15g in 4% (w/v) aqueous $NaHCO_3$) and incubated at 30°C for 72 hours. The resulting trinitrophenyl (TNP)-collagen-Sepharose was washed thoroughly with water, then 0.5M $NaHCO_3$/0.25M NaCl pH7.1 and stored in the latter at 4°C.

Amino acid analysis was used to determine the amount of collagen attached to Sepharose and the extent of modification was calculated from the results of this analysis. The number of moles of (lysine + hydroxylysine) per mole of glycine present in TNP-collagen-Sepharose was compared with the ratio for collagen-Sepharose and the percentage modification was calculated from this.

2.4.3. Dinitrophenylation

20ml saturated aqueous $NaHCO_3$, 40ml ethanol and 2ml 2,4-dinitrofluorobenzene (DNFB) were mixed with 15g collagen-Sepharose and kept in a stoppered flask at room temperature for five days. Then the dinitrophenyl (DNP)-collagen-Sepharose was washed with water followed by ethanol. It was then subjected to Soxhlet extraction with ethanol for 16 hours, washed with ether then with 0.05M $NaHCO_3$/0.25M NaCl

pH 7.1 and stored in the latter at 4°C.

The extent of dinitrophenylation was determined by amino acid analysis.

2.5. Preparation of platelets

Bovine blood was collected from the jugular vein or carotid artery of a bullock immediately after slaughter at the St Andrews abbatoir. Care was taken that the bullock was fairly calm before slaughter and that the blood was fast-flowing (in order to minimise contact with the damaged blood vessel wall). The blood was collected into the appropriate anticoagulant and mixed carefully.

Platelet-rich plasma (PRP) was prepared by centrifuging the anticoagulated blood in an MSE bench top centrifuge for ten minutes at 50g. The PRP was pipetted off and the remaining blood was re-centrifuged for ten minutes at 1400g to give platelet-poor plasma (PPP).

Plastic measuring cylinders and centrifuge tubes were used throughout, but pipettes were glass. Silicone treatment of glass has been thought unnecessary (Packham et al., 1969) and untreated glass was therefore used in initial experiments. However, platelets sometimes aggregated unexpectedly so silicone-treated pipettes were used in later experiments (all those using ^{14}C-serotonin and all but the initial ^{3}H-serotonin experiments). Pipettes were soaked in 4% (v/v) silicone MS 200/350 c/s in ethyl acetate then drained well and dried in an oven at 110°C overnight (18 hours).

2.6. Experiments using collagen-strips

2.6.1. Aggregometer method

Nine volumes of bovine blood were collected into one volume of 3.8% (w/v) sodium citrate and mixed thoroughly. PRP and PPP were

prepared and kept at 37°C until used.

1.2ml PPP was placed in a plastic cuvette in a Unicam SP600 (series 2) spectrophotometer with a thermostatted cell carriage (37°C) and was used as a blank. 1.2ml PRP was placed in another plastic cuvette in the spectrometer and was stirred from above by means of a small battery-driven motor with a stirrer attached. The absorbance at 600nm was measured and recorded on a chart recorder (Pye Unicam AR45 Linear/Log 1.0 Decade Recorder).

When a steady baseline had been obtained, collagen (50μl, 2mg ml^{-1} in 0.1M acetic acid), ADP (50μl, 1mM in 139mM NaCl/15.4mM tris (hydroxymethyl) aminoethane hydrochloride pH 7.4 - "Tris-saline") or an activated or collagen-treated strip was added to the PRP in order to determine whether platelet aggregation would be initiated. If aggregation was not seen within 15 minutes, no aggregation was considered to have been induced by the stimulus. If, in the case of the strips, no aggregation had taken place withen 15 minutes, 50μl ADP (1mM) was added in order to check the viability of the platelets.

Strips with collagen added in various ways (section 2.3.1.b) were tested using this method.

2.6.2. Method using platelet counts in whole blood

Four parts of bovine blood were collected into one part of 3.8% (w/v) sodium citrate and this was kept at room temperature in order to minimise spontaneous aggregation during storage (Sivertson, 1976; Hardeman & Heynens, 1974b).

The method of Sivertson (1976) was used. It involved incubating 0.7ml aliquots of anticoagulated blood with ADP (50μl, 1mM in Tris-saline), collagen (50μl, 2mg ml^{-1} in 0.1M acetic acid) or polyamide strips (cut into small pieces) for 30 or 120 seconds. A glass bead was placed in the (plastic) reaction tube in order that any platelet aggregates formed might adhere to it, and the tube was shaken at about one shake per second in a water bath at 37°C. The reaction

(if any) was stopped by adding 13.3ml 0.2% (w/v) sodium citrate solution containing 1.5% (v/v) formaldehyde (this haemolysed erythrocytes and fixed platelets) and non-aggregated platelets were counted under a microscope, using an Improved Neubauer haemacytometer.

2.6.3. Method using ^{3}H-serotonin release from platelets

Eight volumes of bovine blood were collected into one volume of 2% (w/v) EDTA pH 7.4 and PRP and PPP were prepared as previously described (section 2.5.). The platelet count of PRP was adjusted to 4×10^{8} ml^{-1} with PPP.

^{3}H-serotonin (5-hydroxy [G-H3] tryptamine creatinine sulphate, 500mCi mmol^{-1} from Radiochemical Centre Ltd., Amersham) was dissolved in sterile 0.85% (w/v) NaCl to give 10μCi ml^{-1} (8.33μg ml^{-1}). This was added to the PRP to give 0.3μCi (10^{9} platelets)$^{-1}$, (0.36μg (10^{9} platelets)$^{-1}$) and incubated for 15 minutes in a water bath at 37°C. The platelets were collected by centrifugation (ten minutes at 1400g) and resuspended in a solution containing NaCl (140mM), Tris-HCl (25mM), EDTA (0.3mM) and glucose (5mM) at pH 7.4 ("resuspension solution").

Tests were carried out in a water bath at 37°C using 1ml aliquots of washed platelets. They were incubated for varying times with 50μl collagen (2mg ml^{-1} in 0.1M acetic acid) or activated or collagen-treated polyamide strips. ADP and $CaCl_2$ were also used. The reaction was stopped by cooling the tubes on ice, then they were centrifuged for ten minutes at 1400g. The supernatants were decanted and 0.2ml samples (in duplicate) were added to 10ml toluene/Triton X-100 scintillation mixture (two volumes of toluene/PPO (4g l^{-1})/POPOP (0.8g l^{-1}) to one volume of Triton X-100). In later experiments 1ml water was added to each vial in order to form a clear solution rather than a cloudy one.

^{3}H-serotonin was counted using an Intertechnique SL 30 scintillation spectrometer. Background counts were determined using washed platelets which had not been incubated with ^{3}H-serotonin. Percentage release of serotonin was calculated as follows:-

$$\% \text{ release} = \frac{A - B}{C - B} \times 100$$

where A = counts in supernatant of test
(i.e. extracellular counts)
B = counts in supernatant of control
(i.e. initial extracellular counts)
C = counts in total control
(C - B = initial intracellular counts)

Details of the counting efficiency of the liquid scintillation spectrometer are described in the Appendix.

Since the contents of plastic tubes do not cool quickly when the tubes are put into an ice bath and there is a time interval at the beginning of centrifugation during which any reaction may continue, another method of stopping the reaction was tried. 1ml of ice-cold resuspension solution was added to the reaction mixture which was then centrifuged as usual (0.4ml aliquots of supernatant were counted instead of 0.2ml). This method was used for experiments involving adsorption of serotonin to strips (section 3.1.4.d) but made little difference to results and therefore the original method was used in subsequent experiments.

2.7. Experiments using Sepharose

2.7.1. Anticoagulant

For the preliminary experiments using ^{3}H-serotonin (sections 3.3.1. and 3.3.2.), eight volumes of bovine blood were collected into one volume of 2% (w/v) EDTA. In initial experiments the pH of the EDTA solution was 7.4. The pH of the blood anticoagulated in this way rose, even in a capped bottle, so in later experiments an EDTA solution of pH 5.5 was used. The blood pH still rose, but remained below pH 7.4.

For the remainder of the experiments using ^{3}H-serotonin and all those using ^{14}C-serotonin, acid-citrate-dextrose (ACD) was used as

an anticoagulant. Ten volumes of bovine blood were collected into 1.5 volumes of ACD (22g l^{-1} sodium citrate, 8g l^{-1} citric acid, 22g l^{-1} glucose).

2.7.2. Incubation with serotonin

PRP was incubated at 24°C with ^{3}H-serotonin (0.3μCi (10^{9} platelets)$^{-1}$) for 40 minutes, centrifuged for ten minutes at 1400g and resuspended in "washing buffer" (140mM NaCl, 25mM Tris-HCl, 3.3mM EDTA, 5mM glucose and 5mg ml^{-1} bovine serum albumin (fraction V, Sigma) at pH 7.4). The platelet suspension was then centrifuged as before and resuspended in "platelet buffer A" (identical to the washing buffer except that the EDTA was 0.3mM). This was centrifuged to remove any erythrocytes present (five minutes at minimum speed, about 10-15g) and the supernatant platelet suspension was kept at 24°C until required.

^{14}C-serotonin (5-hydroxy-[side chain-2-C14] tryptamine creatinine sulphate, 58mCi $mmol^{-1}$ from Radiochemical Centre Ltd., Amersham) was used in later experiments because of the possibility of hydrogen exchange when using ^{3}H-serotonin. It was diluted with 0.85% NaCl to a concentration of 1nCi μl^{-1} (7.0ng μl^{-1}) and non-radioactive serotonin was added to give a final concentration of 20nCi and 2.5μg per millilitre of platelets (unless stated otherwise). Incubations with platelets were carried out in the same way as with ^{3}H-serotonin, usually using platelets at 4 x 10^{8} ml^{-1}.

2.7.3. Preparation and use of column

A jacketed plastic column (0.7 x 15cm) was used, with water at 37°C circulating through the jacket. The column was filled half full with platelet buffer A at 37°C and sufficient Sepharose or collagen-Sepharose was added to give a 2.5ml (approximate) bed volume (about 1g Sepharose). After the gel had settled, the column was equilibrated with platelet buffer A (37°C) by passing several bed volumes of the buffer through the gel. Bubbles forming in the

warmed gel were removed by gentle resuspension of the column, which was repacked at a low flow rate.

When the column was ready, 1ml (or more) of the labelled platelet suspension was gently pipetted on top of the gel and allowed to flow through it. Once it had entered the gel, an additional 10ml of platelet buffer A (at 37°C) were added to the column and allowed to drain through at a flow rate of between 0.5ml and 1.0ml per minute.

The effluent was collected in a single plastic tube at room temperature, then mixed gently and sampled. Two 1ml samples were used for determination of the total effluent radioactivity (after correction for effluent volume) and two 1.5ml samples were centrifuged (ten minutes at 1400g) for determination of released radioactivity (1ml of each sample was used in order to overcome any errors due to supernatant remaining on the sides of the tube or trapped in the pellet). The liquid scintillant used was the same as before (10ml of toluene/Triton X-100 scintillation mixture, section 2.6.3.). Per cent release of serotonin was calculated in the same way as for experiments using polyamide strips (section 2.6.3.).

Later experiments showed that there was some spontaneous release or leakage of serotonin from platelets during the course of the experiment (section 3.3.1.), so a separate control sample of radioactive platelets was taken at the same time as each column was run. (Serotonin uptake is energy-linked and there is a passive outward flux (Okuda & Nemerson, 1971), so when there is no serotonin present in the resuspension medium, platelets will lose some of their serotonin content.) Each experiment was completed within five to six hours of collecting the blood (usually within five hours) to minimise any loss of viability of the platelets.

When platelets at 24°C were added to the Sepharose column at 37°C, more bubbles tended to form, so in a few experiments an aliquot of platelet suspension was brought to 37°C just before applying to the column. Fewer bubbles formed, but the results were unaffected, so this method was not used further since serotonin

uptake has been found to be optimal at 24°C (Hardeman & Heynens, 1974b) and maintained better during storage at that temperature (Lemmer et al., 1977).

2.8. Statistical analysis of results

Student's t-test was applied to paired results in order to compare results of incubation of platelets with collagen-strips (section 3.1.3.) and for comparison of serotonin release induced by various types of collagen-Sepharose (section 3.5.).

The following method was used:-

$$\text{Average difference between pairs, } \bar{d} = \frac{\sum_{i=1}^{i=n} (x_i - y_i)}{n}$$

where n = number of paired comparisons, x and y.

$$\text{Standard deviation of differences, } S_d = \sqrt{\frac{\sum_{i=1}^{i=n} (\bar{d} - d_i)^2}{n - 1}}$$

$$t = \frac{\bar{d}\sqrt{n}}{S_d}$$

3. RESULTS

3.1. Use of polyamide strips

3.1.1. Method of attaching collagen to activated polyamide strips

Collagen was attached to activated polyamide strips of size 1cm x 4cm using two methods; either by incubating activated strips with collagen fibres formed by dialysing soluble collagen against a suitable solution or by dialysing activated strips with soluble collagen against a similar solution (section 2.3.1.b). Most collagen was present on strips which had been added to collagen fibres formed by dialysing collagen against 0.5M NaCl (780μg collagen) and on strips which had been dialysed with collagen against 0.5M NaCl/0.01M phosphate pH 6.0 and pH 7.0 (1025μg and 545μg collagen). Least collagen was present on strips which had been dialysed with collagen against 0.2M and 0.3M NaCl/0.1M phosphate pH 7.0 (140μg and 110μg collagen).

Collagen-treated polyamide strips used in subsequent experiments were prepared by dialysing activated strips with collagen (2mg ml^{-1} in 0.1M acetic acid) against 0.5M NaCl/0.01M phosphate pH 6.0 (for whole blood, counting technique) and pH 6.5 (for ^{3}H-serotonin release) since these conditions caused the greatest weight of collagen to become attached to the activated polyamide strips.

3.1.2. Collagen-induced platelet aggregation caused by collagen-treated polyamide (using aggregometer)

No collagen-treated polyamide strip induced platelet aggregation, not even those with the most collagen present. Inhibition of collagen- and ADP-induced aggregation occurred once with a strip dialysed with collagen against 0.5M NaCl, but this result could not be reproduced.

This lack of effect of collagen-strips on platelets could be due to the conformation of the collagen fibrils or to the reactive sites being used for attachment to the polyamide rather than being free to be "recognised" by platelets. The most probable reason is that there was insufficient collagen present to induce aggregation.

3.1.3. Platelet adhesion and aggregation using the whole blood method of Sivertson (1976)

The amount of collagen exposed to platelets was calculated to be 0.96μg from the surface area of a polyamide strip, assuming that only the outer layer of collagen would be exposed. Burns (1976) found that minimal aggregation was induced by 20μg collagen in the aggregometer used here, so the amount of collagen on a strip which was exposed to platelets was insufficient to induce aggregation. Nylon beads and tubing were also calculated to have an inadequate surface area to volume ratio for collagen exposure to platelets.

Sivertson's method (1976) involving whole blood was used. Although developed for work involving platelet aggregation it was thought possible to measure adhesion in the same way when using treated polyamide strips on which there was too little collagen to induce aggregation.

Results of platelet adhesion and aggregation obtained using this method are shown at table 1. Blood samples which gave a platelet concentration in the control (where the blood was incubated with a glass bead only) outside the limits of 300 000 to 500 000 μl^{-1} $\pm$ 1% after incubation for 30 seconds were disregarded.

After incubating blood with activated strips for 30 seconds the platelet count decreased slightly, but not significantly ($p>0.1$), so any effect of collagen strips on platelets was not due to the activated strip itself. But when blood was incubated with collagen strips for 30 seconds, the resulting decrease in platelet count was highly significant ($p<0.01$) both when compared with the controls and with activated strips. New strips (i.e. not activated) gave

Table 1. Platelet adhesion and aggregation induced by collagen-treated polyamide and other agents

Animal No.	Platelet count μl^{-1} 30s after addition of control	new strip	activated strip	collagen strip	collagen*	ADP**	Wt. of collagen on strip (µg)
1	487 500	372 500	397 500	295 000	127 500	192 500	54
2	297 500	232 500	228 750	188 750	105 000	153 750	40
3	417 500	321 250	388 750	260 000	153 750	140 000	40
4	392 500	345 000	375 000	265 000	132 500	173 750	40
5	332 500	-	347 500	296 250	51 250	61 250	20

* Collagen: 100 µg in 0.1M acetic acid
** ADP: 50 nmoles in Tris-saline

Table 2. Stability of platelet aggregates

Animal No.	Platelet count μl^{-1} 120s after addition of						Wt. of collagen on strip (μg)
	control	new strip	activated strip	collagen strip	collagen*	ADP**	
1	395 000	367 500	437 500	330 000	172 500	70 000	54
2	253 750	187 500	227 500	160 000	137 500	78 750	40
3	325 000	343 750	378 750	312 500	135 000	42 500	40
4	303 750	343 750	335 000	300 000	115 000	51 250	40
5	271 250	-	292 500	255 000	52 500	20 000	20

* Collagen: 100 μg in 0.1M acetic acid
** ADP: 50 nmoles in Tris-saline

similar results to activated strips.

Samples were also incubated for 120 seconds in order to investigate whether aggregates formed were stable or reversible (table 2).

Both soluble collagen and ADP induced marked aggregation of platelets, but aggregates induced by collagen were unstable (i.e. primary, reversible, aggregation had occurred) whereas those induced by ADP were stable and in most cases continued to form between 30 and 120 seconds (i.e. secondary, irreversible, aggregation had occurred). Platelet adhesion to collagen strips (or slight aggregation induced by these strips) was reversible in three cases (animals 1, 3 and 4) but slightly increased in the other two (animals 2 and 5).

The decrease in platelet count between 30 seconds and 120 seconds in the control tubes was probably due to platelets adhering to the glass bead and possibly also to the plastic tube.

3.1.4. Release of ^{3}H-serotonin

Since the previous method (Sivertson, 1976) did not distinguish between adhesion and aggregation - indeed it measured both - the release reaction was now used as a means of determining the effect on platelets of collagen-treated polyamide. Preliminary experiments were carried out to determine the optimum incubation period for platelets with ^{3}H-serotonin and to ascertain the effects of ADP and collagen on the incubated platelets before trying treated polyamide strips.

a) Optimum incubation time of platelets with ^{3}H-serotonin at 37°C. Platelets were incubated with serotonin for varying periods of up to 60 minutes (at 37°C) in order to determine the optimum incubation time (table 3). Serotonin was present at a ratio of 25ng $(10^{8}$ platelets$)^{-1}$.

Table 3. Effect of different incubation times on serotonin incorporation by platelets

Incubation period (min)	Serotonin incorporated (ng $(10^8$ platelets$)^{-1}$)*
15	3.9 ± 0.8
30	2.0 ± 0.6
45	3.0 ± 1.4
60	2.1 ± 0.8

* mean ± standard deviation (4 experiments).

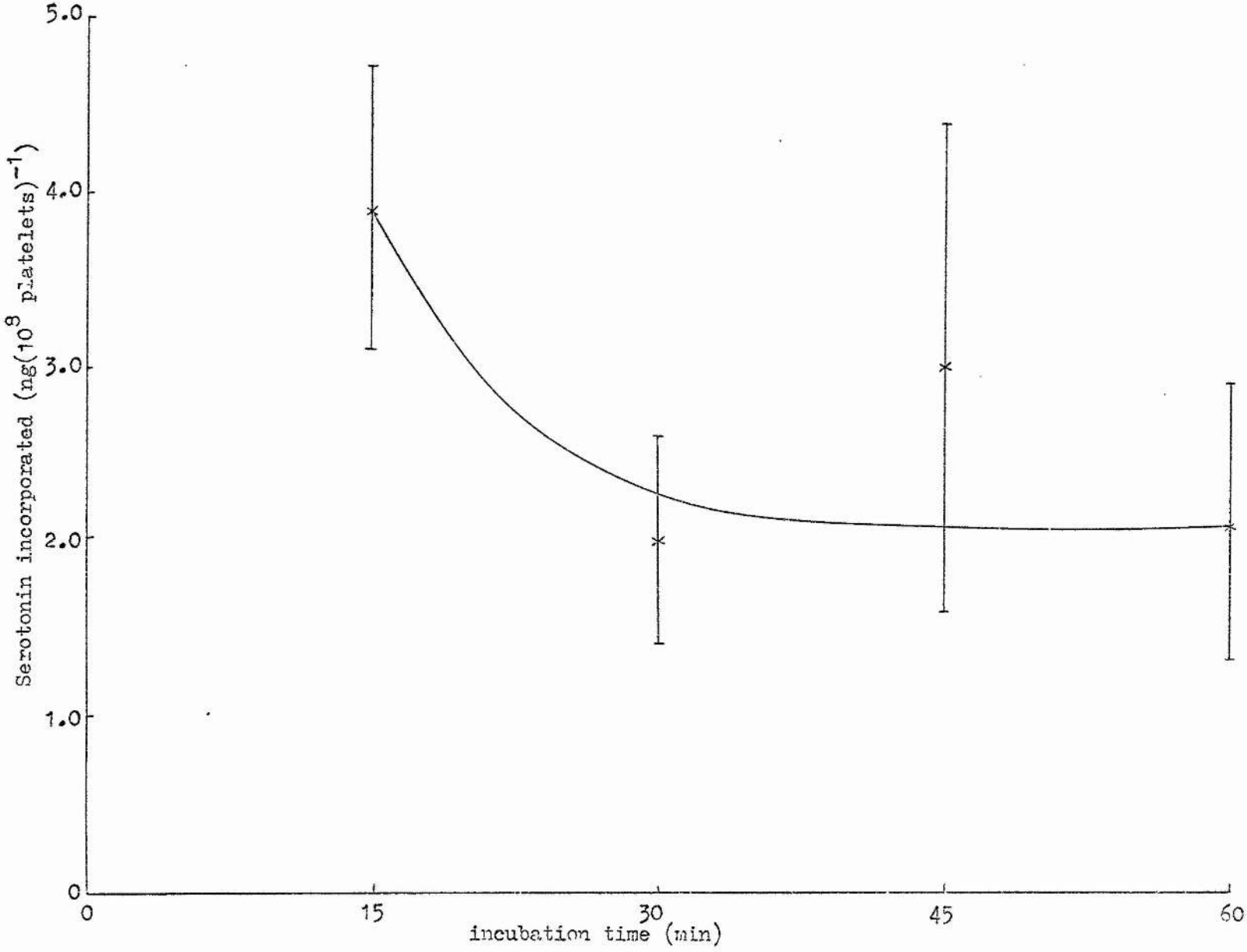
Incorporation of serotonin by platelets varied with the length of incubation but most serotonin was taken in during a 15 minute incubation at 37°C (see graph 1), so this length of incubation was used in subsequent experiments.

b) Effect of ADP and $CaCl_2$ on serotonin release from platelets. Platelets were incubated with ADP and/or $CaCl_2$ in order to check that ^{3}H-serotonin would be released as expected (table 4).

Table 4. Effect of ADP and $CaCl_2$ on serotonin release

5 min incubation with ADP	5 min incubation with $CaCl_2$	% serotonin release	N*
50 nmoles	-	1.3 ± 0.7	8
-	30 µmoles	3.8 ± 4.5	5
-	50 µmoles	4.4 ± 3.6	9
50 nmoles	30 µmoles	8.0 ± 3.9	5
50 nmoles	50 µmoles	7.2 ± 4.3	9

* N = number of experiments.



Graph 1. Optimum incubation time of platelets with ^{3}H-serotonin at 37°C.

ADP alone induced very little serotonin release from platelets (table 4) since it requires the presence of Ca^{2+}, which was chelated by the EDTA present in the resuspension buffer. $CaCl_2$ alone induced some release (but gave very variable results) but ADP plus $CaCl_2$ induced a greater degree of serotonin release.

c) Effect of collagen on serotonin release. Having found that platelets would release serotonin as expected on incubation with ADP, platelets were incubated with collagen to investigate collagen-induced serotonin release (table 5).

1ml aliquots of washed platelets were incubated with 50μl soluble collagen in 0.1M acetic acid for various periods of time (table 5). Serotonin release reached a maximum after five to ten minutes' incubation with collagen and then levelled off (graph 2), the platelets having released the maximum amount of serotonin for the given amount of collagen. As expected, more collagen induced greater serotonin release (graph 3), since the stimulus to release was greater.

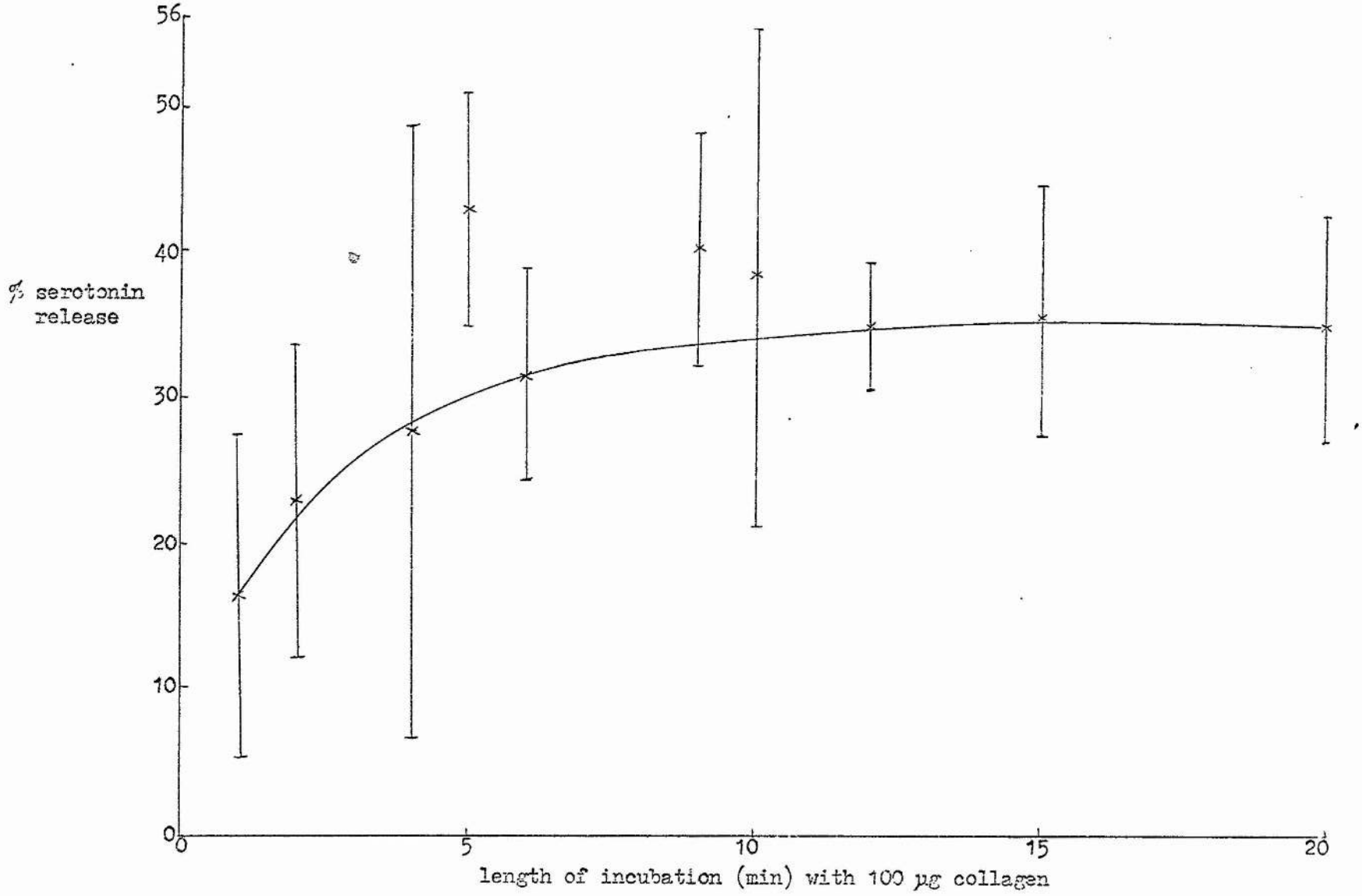
d) Adsorption of serotonin to strips. Serotonin released from platelets might adhere to treated polyamide strips and cause a decreased apparent release. This possibility was investigated by incubating serotonin with treated strips cut up in two different ways (it was necessary to cut the strips in order that the platelet suspension would cover them) since the way they were cut might affect any adsorption (table 6).

Cutting strips into 0.2cm x 1cm pieces caused slightly greater serotonin adsorption than cutting them into 1cm x 1cm pieces (table 6), probably because there were more cut surfaces to which the serotonin could adsorb. A larger amount of polyamide caused more serotonin adsorption, mostly due to the greater surface area available. There was little difference in adsorption to activated strips and collagen-strips cut in the same way.

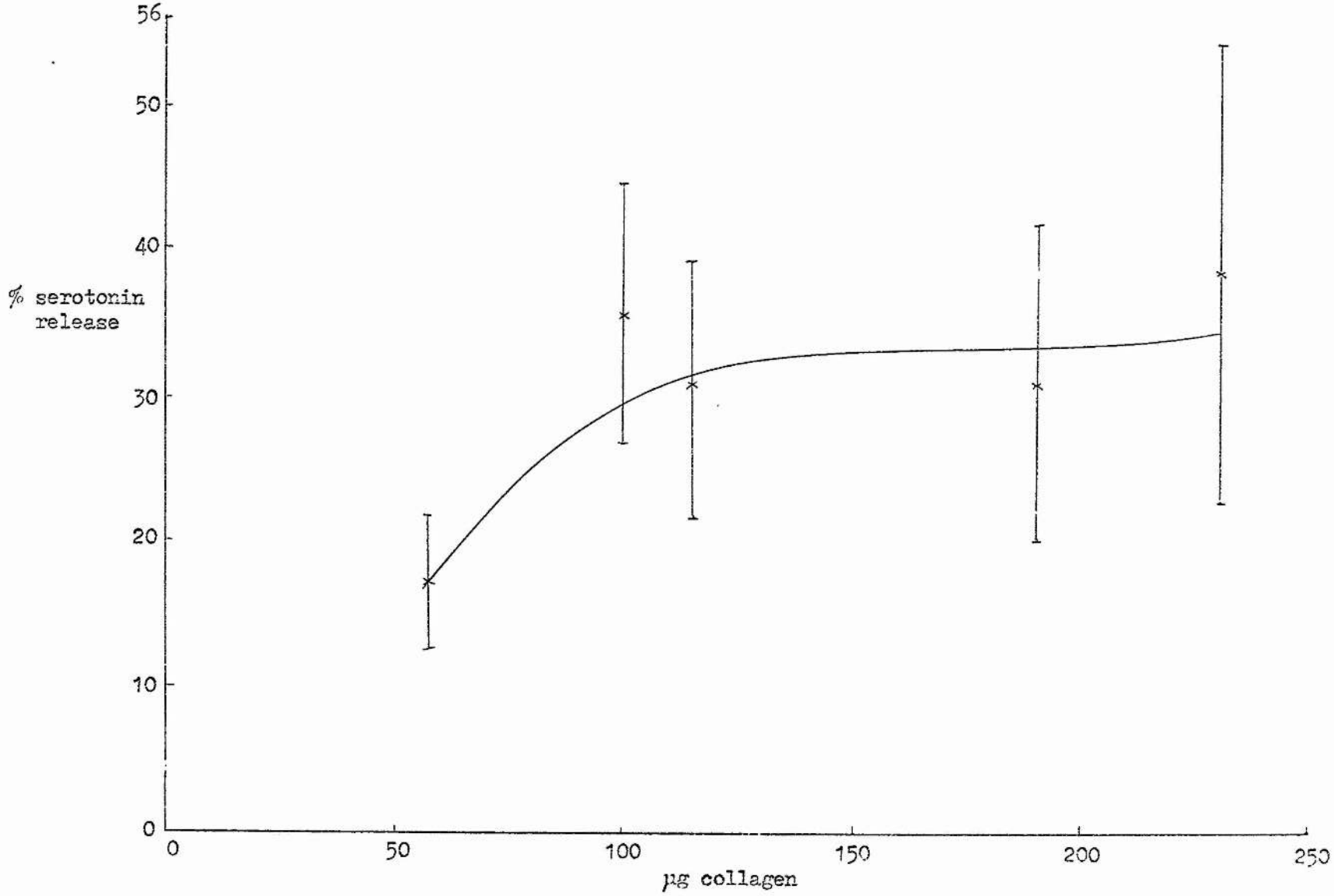
e) Effect of treated polyamide strips on ^{3}H-serotonin release from platelets. Knowing that there is little adsorption of released

Table 5. Incubation of platelets with collagen

Incubation time (min)	% serotonin release after incubation with collagen (N)				
	57.5 µg	100 µg	115 µg	190 µg	230 µg
1	7.0 ± 2.1(4)	16.5 ± 11.1(10)	20.1 ± 8.5(4)	25.1 ± 20.5(10)	38.4 ± 4.0(4)
2	12.4 ± 1.1(4)	23.0 ± 10.8(11)	30.7 ± 8.5(4)	26.6 ± 14.9(10)	42.4 ± 22.0(4)
4	-	27.7 ± 21.0(3)	-	-	-
5	15.4 ± 7.4(4)	43.1 ± 8.1(8)	22.8 ± 9.8(4)	33.0 ± 13.8(10)	48.0 ± 14.3(4)
6	-	31.7 ± 7.4(3)	-	-	-
9	-	40.5 ± 8.0(3)	-	-	-
10	17.0 ± 6.8(4)	38.4 ± 16.9(8)	23.9 ± 11.4(4)	38.2 ± 16.6(10)	35.9 ± 16.1(4)
12	-	34.9 ± 4.5(3)	-	-	-
15	17.1 ± 4.7(4)	35.6 ± 9.0(11)	30.5 ± 9.0(4)	30.8 ± 11.1(10)	38.6 ± 15.8(4)
20	15.3 ± 4.8(4)	35.0 ± 7.5(11)	19.5 ± 6.5(4)	32.8 ± 15.7(10)	34.1 ± 11.2(4)



Graph 2. Effect of incubation time with 100 µg collagen on serotonin release from platelets



Graph 3. Effect of amount of collagen on serotonin release after 15 minutes incubation

Table 6. Adsorption of serotonin to strips

Size (& cut) of strip	% total counts on strip	
	activated strip	collagen-strip*
$4cm^2$ (0.2cm x 1cm)	5.7	6.5
$4cm^2$ (1cm x 1cm)	4.1	3.5
$2cm^2$ (0.2cm x 1cm)	4.7	3.1
$2cm^2$ (1cm x 1cm)	2.6	2.9

* 22μg collagen cm^{-2} of strip.

serotonin to strips, platelets were incubated with treated strips and the percentage serotonin release was measured (table 7).

More serotonin was released when platelets were incubated with strips cut into small pieces (0.2cm x 1cm) than with $1cm^2$ pieces (table 7), possibly because of the presence of more cut surfaces in the former. More polyamide induced more release due to the larger surface area. Collagen-strips induced marginally more serotonin release than did activated strips.

Table 7. Serotonin release from platelets in contact with treated strips.

Size (& cut) of strip	% serotonin release after 20 min incubation with		N
	activated strip	collagen-strip*	
$2cm^2$ (1cm x 1cm)	2.0 $\pm$ 1.3	4.1 $\pm$ 1.1	2
$2cm^2$ (0.2cm x 1cm)	4.7 $\pm$ 0.8	5.0 $\pm$ 1.7	4
$4cm^2$ (0.2cm x 1cm)	3.8 $\pm$ 0.8	7.8 $\pm$ 3.4	4

* 35μg collagen (pH 6.5 buffer) cm^{-2} of strip.

f) Effect of platelet-strip incubation time on ^{3}H-serotonin release. When platelets are incubated with a stimulus which induces serotonin release, the amount of serotonin released increases with increasing incubation time until a maximum is reached (graph 2). So platelets were incubated with treated strips for different periods in order to determine the optimum incubation time (table 8).

The length of incubation had little effect on release (table 8); presumably release reached a maximum during the first five minutes' incubation with the polyamide. The buffer used when preparing collagen-strips (0.5M NaCl/0.01M phosphate) could be pH 6.5 or pH 7.0 without making much difference to serotonin release (table 8).

g) Summary of results of ^{3}H-serotonin release experiments. Amounts of serotonin released by platelets in contact with collagen-treated polyamide were small and differed little from amounts released by platelets in contact with activated polyamide. This could be due to insufficient collagen being present on the polyamide, but this is unlikely since appreciable release was measured when incubating platelets with a similar weight of soluble collagen; 17% release was obtained after 15 minutes' incubation with 57.5μg soluble collagen (table 5) whereas only 5% release was obtained after a similar incubation with 70μg collagen attached to 2cm^2 polyamide (table 7).

It is possible however that insufficient collagen was available to the platelets to induce aggregation (as calculated in section 3.1.2.), but that there was sufficient available collagen to induce adhesion (section 3.1.3.). The release reaction follows adhesion and once sufficient release has occurred, aggregation will follow. So the available collagen present on a polyamide strip may not in fact have been enough to induce sufficient serotonin release to measure using this method.

There are other possible reasons for the small amount of serotonin release. The collagen attached to the polyamide may not have formed the required conformation for platelets to recognise, or the groups involved in adhesion may have been inaccessible to

Table 8. Effect of varying incubation time of platelets with strips

Length of incubation (min)	% serotonin release after incubation with * activated strip (N)	collagen-strip (pH6.5) (N)	collagen-strip (pH7.0) (N)
5	5.8 ± 2.3 (8)	10.0 ± 6.9 (8)	8.8 ± 1.3 (5)
10	6.1 ± 3.3 (8)	7.9 ± 5.0 (8)	8.0 ± 2.8 (5)
15	6.2 ± 2.6 (7)	6.1 ± 2.2 (7)	6.3 ± 1.6 (5)
µg collagen strip^{-1}	-	109 ± 52	124 ± 50

* strips: 4cm^2, in 0.2cm x 1cm pieces

platelets or involved in the bonds with the polyamide.

Because of the presumed insufficiency of available collagen present on polyamide strips, Sepharose 6B was used as the insoluble support for collagen for further investigation of the collagen-induced release reaction of platelets.

3.2. Determination of collagen on Sepharose

In order to compare the amount of serotonin released from platelets passed through collagen-Sepharose prepared on different occasions, it was necessary to know how much collagen was attached to the Sepharose in case different batches of collagen-Sepharose varied greatly in collagen content. Several different methods of collagen determination were used and the effect of activated Sepharose on the amount of collagen measured was noted.

3.2.1. Hydroxyproline determination (Stegemann, 1958)

Collagen contains the unusual amino acid hydroxyproline (hyp). Because this amino acid is so uncommon, collagen concentration can be determined from an assay of hydroxyproline. In these assays hydroxyproline is taken to be 10% (by weight) of collagen.

Comparing weights of collagen measured by microbiuret and hydroxyproline methods (table 9), one can see that not only did the hydroxyproline method of Stegemann give lower results but that the difference was not constant. Also, the presence of activated Sepharose interfered with the detection of hydroxyproline in collagen by reacting with the p-dimethylaminobenzaldehyde (table 9, line c). When the effect of the activated Sepharose is taken into account (table 9, line e), the two methods gave similar ratios for 108μg collagen (line e) and 920μg collagen (line b). But for 240μg collagen the ratio obtained is much lower (line c).

Table 9. Effect of activated Sepharose on collagen determination using the hydroxyproline method of Stegemann

line	Sample activated Sepharose (g)	µg collagen expected*	µg collagen from hyp assay	assay/ expected (%)
a	-	240	109	45
b	-	920	628	68
c	0.1	-	14	-
d	0.1	108	89	82
e=(d-c)	0.1	108	75	69

*by microbiuret method (Itzhaki & Gill, 1964)

3.2.2. Hydroxyproline determination (Serafini-Cessi & Cessi, 1964)

Because of the reaction of activated Sepharose when determining hydroxyproline concentration and the variability of results, an alternative method of hydroxyproline determination, that of Serafini-Cessi and Cessi (1964) was tried. In this the hydrolysed collagen or collagen plus activated Sepharose was reacted then distilled before the colour reaction (with p-dimethylaminobenzaldehyde) took place, and it was hoped that only the reacted hydroxyproline would be distilled, leaving behind the reacted activated Sepharose.

Table 10 shows no direct relationship between the expected weight of collagen and that obtained from the hydroxyproline determination, percentages for collagen alone varying between 60% and 87% and those for collagen plus activated Sepharose varying between 50% and 78%. The presence of activated Sepharose had varying effects on the amount of hydroxyproline measured using this method, the ratios of percentage collagen detected in collagen and in collagen plus activated Sepharose varying between 0.79 and 1.54.

In case more consistent results could be obtained by using different time intervals between reacting (incubating) and distilling the sample or between distilling it and measuring the absorbance at 550nm, results obtained using a standard solution of hydroxyproline and varying these time intervals were compared (table 11).

The length of time between reacting (incubating) and distilling the samples affected the absorbance (table 11), in spite of the fact that Serafini-Cessi and Cessi maintained that waiting for up to an hour before distillation had no effect. Results were slightly more reproducible if distillation was carried out between 20 and 40 minutes after incubation (graph 5). The colour produced was most stable between five and 15 minutes after distillation; about five minutes were required to obtain full colour development and this colour tended to decrease again after 15 minutes (graph 4).

Table 10. Effect of activated Sepharose on collagen determination using the hydroxyproline method of Serafini-Cessi and Cessi

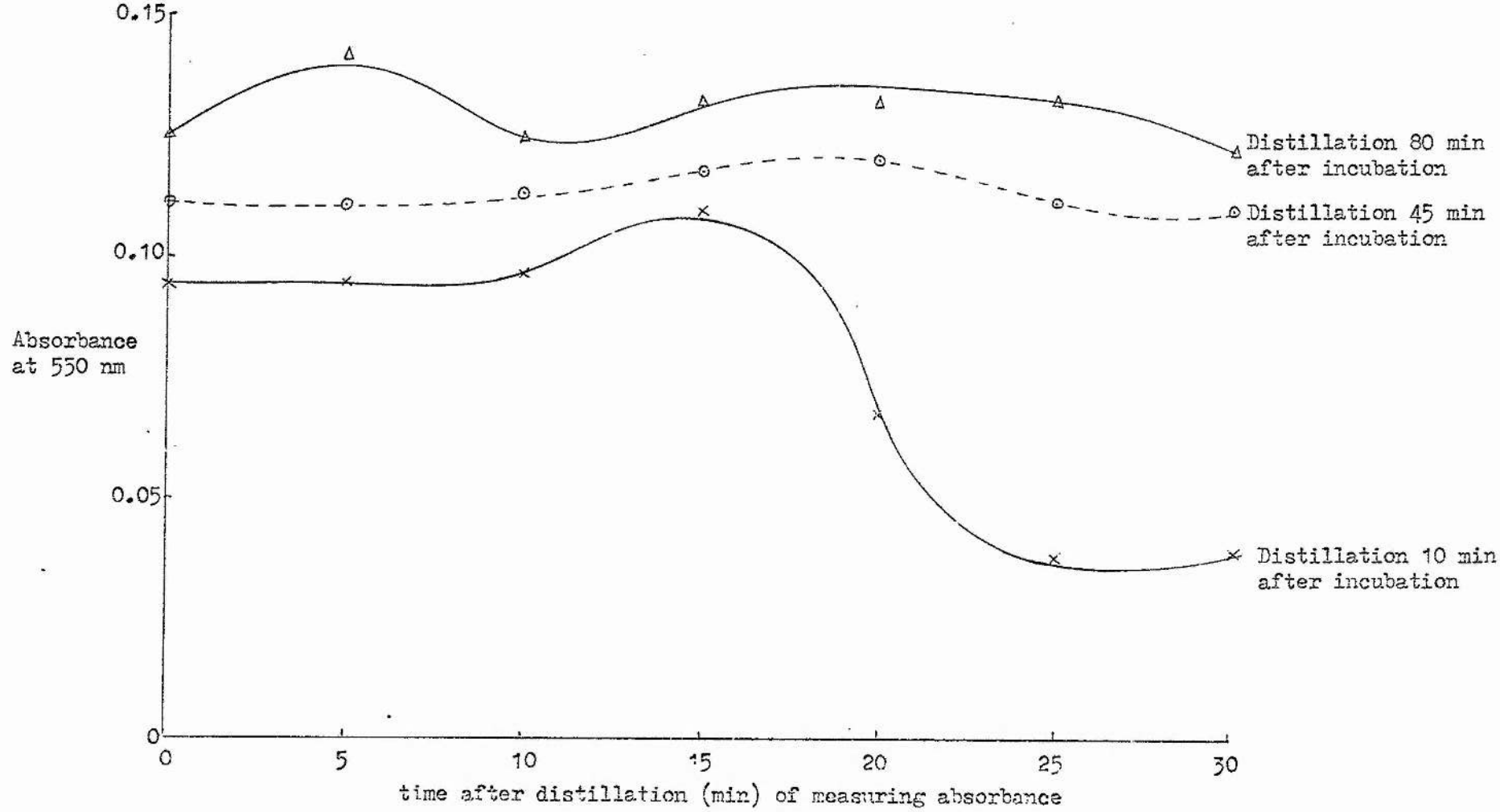
Sample: activated Sepharose (g)	collagen expected*(µg)	µg collagen from hyp assay	assay(av.)/ expected (%)	collagen/ collagen with Sepharose (from assay)
0.1 (not activated)	-	25		
-	240	148	62	
-	230	200	87	
0.1	-	0		
0.2	-	0		
0.1	240	196, 165**	75	1.01
-	240	144, 220	76	
0.1	230	185, 163	76	0.79
-	230	110, 166	60	
0.2	480	402, 342	78	1.09
-	480	450, 368	85	
0.2	460	220, 243	50	1.54
-	460	382, 325	77	

* by microbiuret method (Itzhaki & Gill, 1964)

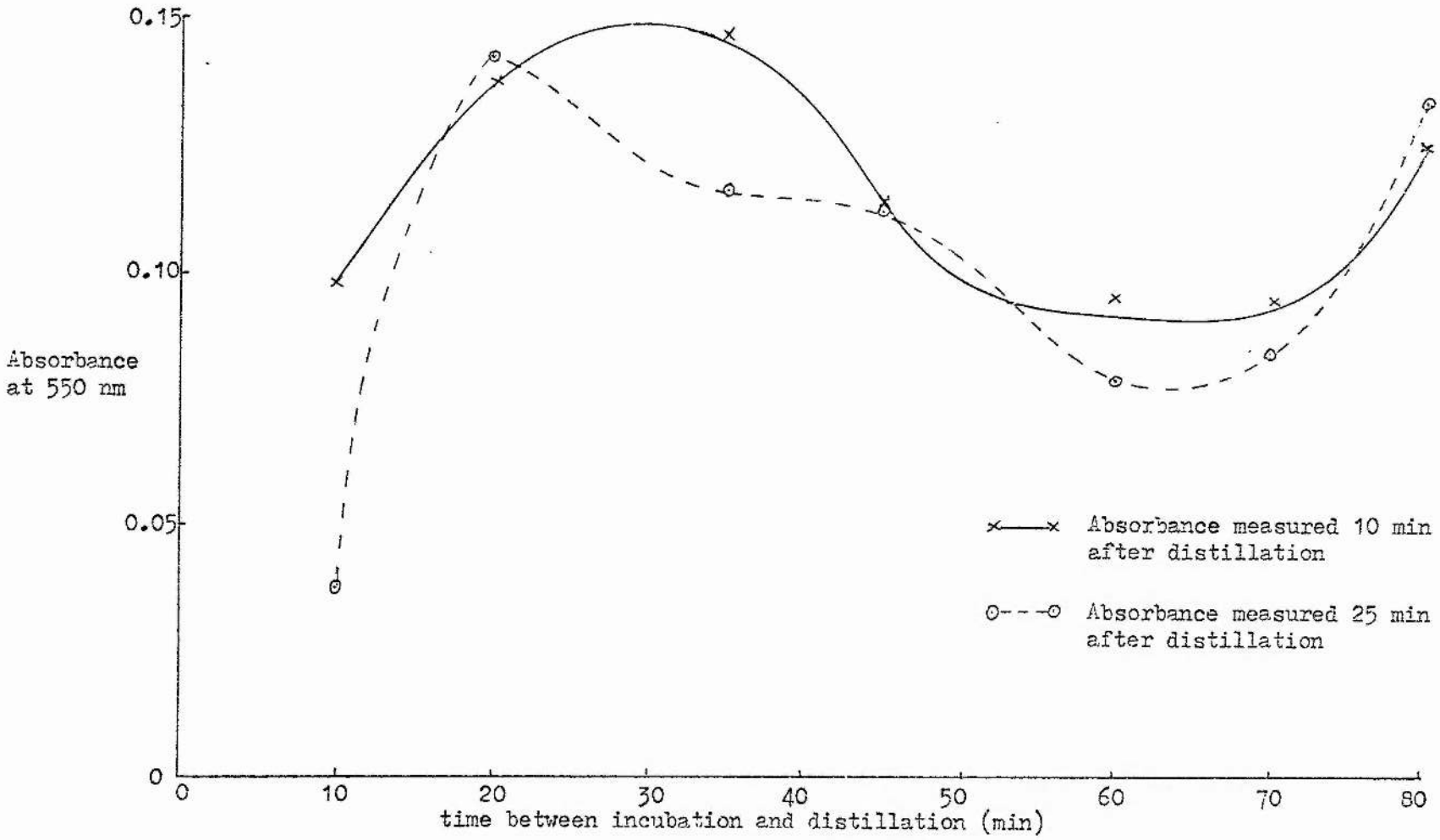
** Repeated values of assay results were from duplicate assays using the same collagen solution

Table 11. Effect of changing incubation and distillation times on hydroxyproline determination

Conc. of hyp ($\mu g\ ml^{-1}$)	Time (min) after incubation	Absorbance (550nm) at time (min) after distillation						
		0	5	10	15	20	25	30
0	5	-0.04	-0.04	-0.04	-0.04	-0.04	-0.031	-0.031
5	10	0.095	0.095	0.097	0.110	0.069	0.038	0.040
5	20	0.162	0.140	0.138	0.156	0.160	0.143	0.136
5	35	0.110	0.120	0.147	0.127	0.131	0.116	0.118
5	45	0.111	0.111	0.114	0.119	0.121	0.112	0.111
5	60	0.078	0.079	0.096	0.096	0.081	0.079	0.078
5	70	0.080	0.079	0.095	0.096	0.096	0.084	0.083
5	80	0.126	0.142	0.125	0.133	0.133	0.134	0.123



Graph 4. Effect of varying distillation times on absorbance for different periods after incubation



Graph 5. Effect on absorbance of varying times between incubation and distillation

3.2.3. Ninhydrin reaction

Ninhydrin may be used to determine amino acid concentration. Because of the effect of activated Sepharose on collagen determination using hydroxyproline methods, reaction with ninhydrin was tried. Samples of collagen, activated Sepharose and mixtures of the two were hydrolysed and then reacted with ninhydrin.

Table 12. Effect of activated Sepharose on ninhydrin

Sample		Absorbance
Activated Sepharose (mg)	Collagen (μg)	at 570nm
20	—	6.5
20	48	6.4
—	48	3.6
20	51	5.5
—	51	4.2

Hydrolysed activated Sepharose reacted so strongly with ninhydrin (table 12) that it masked the reaction of the amino acids obtained by hydrolysing collagen.

3.2.4. Amino acid analyser

The amino acid composition of collagen may be determined by using an amino acid analyser. Once the composition is known, the concentration of subsequent collagen solutions may be calculated from the amount of a single amino acid present. It is wise to ensure that the amino acid composition is similar each time a sample is analysed.

A typical amino acid analysis of acid-soluble calf skin collagen used for adding to Sepharose is shown at table 13. The

Table 13. Amino acid composition of acid-soluble calf skin collagen

Amino acid	No. residues/1000 amino acid residues	
	Test	Published*
hydroxyproline	86	93
aspartic acid	45	43
threonine	16	16
serine	30	32
glutamic acid	75	70
proline	132	141
glycine	327	330
alanine	111	112
valine	23	19
methionine	trace	2
isoleucine	11	11
leucine	24	22
tyrosine	5	3
phenylalanine	15	15
hydroxylysine	8	10
lysine	29	29
histidine	5	7
arginine	59	47

* Eastoe, 1967

composition of the same type of collagen, as quoted by Eastoe (1967), is also given for comparison. The two preparations are very similar.

The weight of glycine detected by the amino acid analyser was 26% of the weight of collagen analysed.

Although the amino acid analyser uses ninhydrin to detect the amino acids, and hydrolysed activated Sepharose has been shown (section 3.2.3.) to react strongly with ninhydrin, the amino acid analyser may be used to determine the collagen content of collagen-Sepharose because the reaction products of ninhydrin and activated Sepharose are separated from the various ninhydrin-amino acid products by the cation exchange resin. Results of amino acid analysis of collagen and collagen plus activated Sepharose are shown at table 14.

The proportions of collagen to activated Sepharose analysed in table 14 were chosen to correspond with the approximate proportions expected in collagen-Sepharose. The maximum amount of collagen possible was 1mg per gram of Sepharose (since that was the amount added to the activated Sepharose) so approximately that proportion (1.15mg collagen per gram of Sepharose) was analysed. It was unlikely that all of the collagen would become attached to the Sepharose so half this proportion (575μg collagen per gram of Sepharose) was also analysed. In fact when samples of different preparations of collagen-Sepharose were analysed later, amounts of collagen per gram of Sepharose varied from 580μg to 970μg (usually between 700 - 800μg).

The results using the amino acid analyser (table 14) were more consistent than those from other methods. There was still a difference between collagen and activated Sepharose plus collagen, but the ratios of (% glycine measured in collagen) to (% glycine measured in collagen plus activated Sepharose) were similar (0.70 - 0.75).

The amino acid analyser could also be used to determine the amount of collagen present on modified collagen-Sepharose and to determine the extent of modification of ϵ-amino groups by comparison of amounts of lysine and hydroxylysine present in modified and unmodified collagen.

Table 14. Effect of activated Sepharose on collagen determination using amino acid analyser

Sample hydrolysed		glycine		assay/ expected (%)	collagen/ (from glycine) (collagen plus Sepharose)
activated Sepharose ($mgml^{-1}$)	collagen (μgml^{-1})	expected* ($nmoles\ ml^{-1}$)	measured ($nmoles\ ml^{-1}$)		
87	100	346.3	287.6	83	0.75
-	100	346.3	216.8	63	
87	50	173.2	215.3	124	0.72
-	50	173.2	155.9	90	
87	50	173.2	216.8	125	0.70
-	50	173.2	151.3	87	

* calculated as 26% by weight of collagen

3.3. ^{3}H-serotonin release from platelets

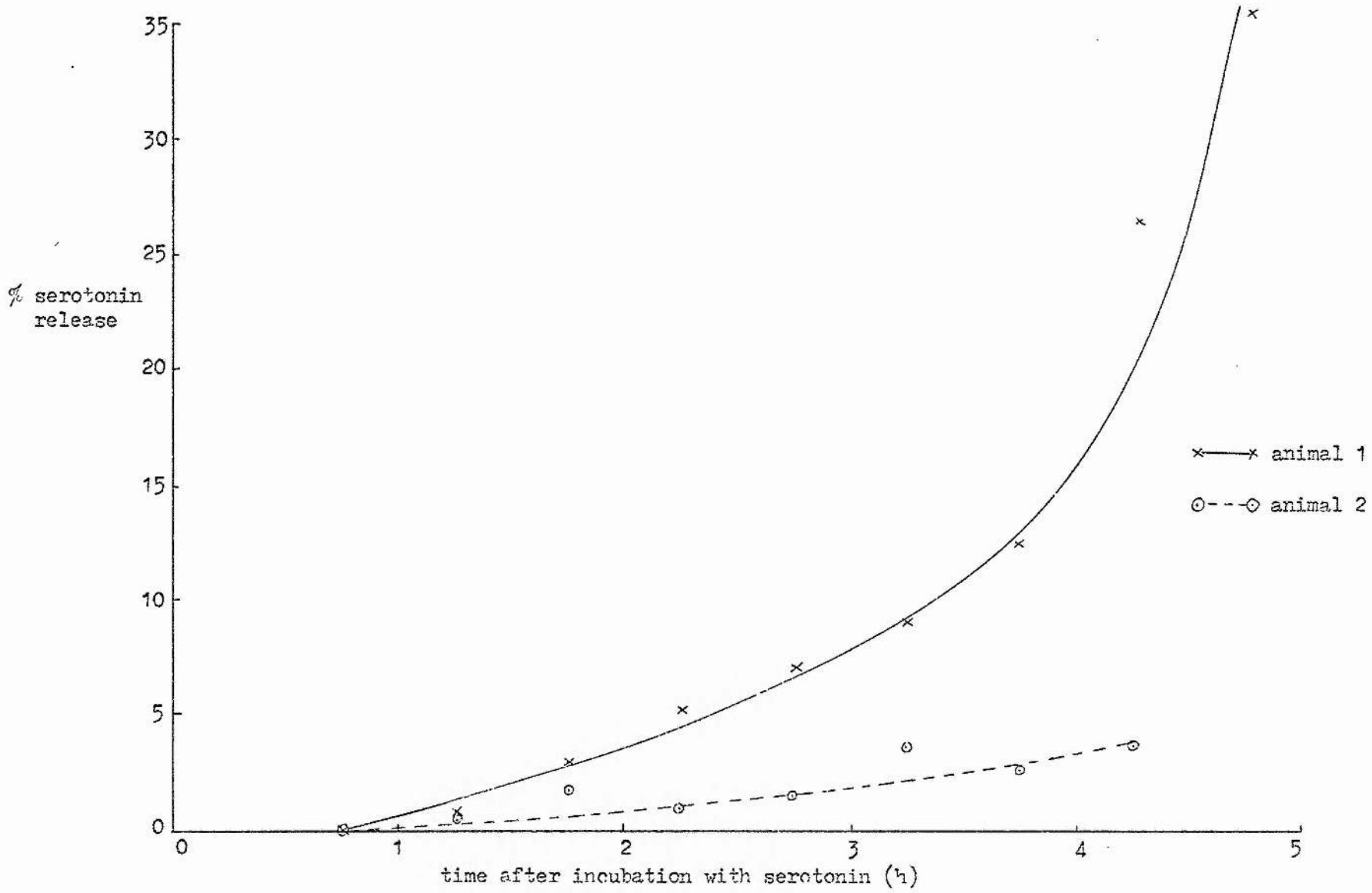
3.3.1. Spontaneous serotonin release from platelets

After incubating platelets with ^{3}H-serotonin, they were kept at 24°C until required. This storage period varied since the platelets were used one aliquot at a time. During the storage period, which could be as long as four hours, it is possible that ^{3}H-serotonin might leak or be released from the platelets. Platelets were therefore stored for various lengths of time after incubation with ^{3}H-serotonin and any ^{3}H-serotonin released was measured.

Table 15. Spontaneous serotonin release (or leakage) from platelets

Time after incubation (h)	% serotonin release	
	animal 1	animal 2
0.75	0	0
1.25	0.9	0.5
1.75	3.1	1.8
2.25	5.3	0.9
2.75	7.2	1.5
3.25	9.3	3.9
3.75	12.6	2.8
4.25	26.7	3.7
4.75	35.7	-
Time between collection & incubation	3h 10min	2h 15min

Serotonin was released from platelets during storage (table 15). This spontaneous release (or leakage) increased with increasing



Graph 6. Spontaneous release of serotonin from platelets

storage time (graph 6) and was also greater if platelets were kept longer before incubating them with ^{3}H-serotonin. Consequently when an aliquot of ^{3}H-serotonin-containing platelets was taken for application to a Sepharose column, a duplicate aliquot was also taken to use as a control.

3.3.2. Serotonin release induced by various types of Sepharose column

Platelets were applied to three different types of Sepharose column in order to determine whether serotonin release would be induced. Sepharose columns were used as a control, insoluble collagen-Sepharose was used in order to follow the method of Brass et al. (1976) as closely as possible and soluble collagen-Sepharose was the type under investigation (table 16).

Table 16. Effect of different Sepharose columns on serotonin release

Type of column	% serotonin release	μg collagen (g Sepharose)$^{-1}$	N
Sepharose	4.2 $\pm$ 3.3	-	22
insoluble collagen-Sepharose	8.4 $\pm$ 5.4	251 $\pm$ 57	8
soluble collagen-Sepharose	9.0 $\pm$ 6.6	728 $\pm$ 123	19

Serotonin release was much lower than that obtained by Brass et al. (1976) and in these experiments it was very variable, probably partly due to differences in individual animals (table 16). Various parameters were therefore investigated in order to obtain greater serotonin release before continuing with experiments using soluble collagen-Sepharose and its modifications.

3.3.3. Adsorption of serotonin by (collagen-) Sepharose

Figures for serotonin release obtained so far were very low and it is possible that released serotonin was adhering to Sepharose and soluble collagen-Sepharose, causing a decrease in apparent serotonin release. So serotonin in a similar concentration to that present in an incubated and washed platelet suspension (10nCi ml^{-1}, 8.33ng ml^{-1} in platelet buffer A) was passed through both types of column and collected either in one tube as usual, or in seven separate 1.5ml fractions (to give accurate 1.0ml volumes for scintillation counting). The total counts in the fractions and in the single tubes were calculated in order to compare the two methods of collection (table 17).

Table 17. Adsorption of serotonin by Sepharose

Type of column	Method of collection	% initial counts
Sepharose	fractions	98.0
"	mixed	102.3
collagen-Sepharose	fractions	97.4
" "	mixed	99.9

There was very little difference between the two methods of collecting the column effluent (table 17) and serotonin did not appear to adsorb on to (collagen-) Sepharose. Figures calculated from fractions were less accurate than those from mixed effluent, due to greater counting errors in the former.

3.3.4. Effect on measured serotonin release of different methods of collecting column effluent

Released serotonin might have been taken up by non-adherent platelets while in the collection tube, so the column effluents were collected either in seven 1.5ml fractions (to attempt to separate

released serotonin from non-adherent platelets) or in a single tube as before. Platelets were also incubated with soluble collagen (62.5μg (10^8 platelets)$^{-1}$) for ten minutes to ensure that released serotonin was present, then passed through Sepharose. The column effluents were collected in fractions or in a single tube as in section 3.3.3. (table 18).

Table 18. Possible uptake of released serotonin by non-adherent platelets

Incubation with soluble collagen before Sepharose	Method of collection after Sepharose columns	% serotonin release
no	fractions	2.2
no	mixed	4.8
yes	fractions	4.5
yes	mixed	10.8

On comparing percentage serotonin release (table 18) with that obtained in table 16, it would seem that non-adherent platelets do not take up released serotonin to any great extent. However, serotonin and platelets were only slightly separated by the columns, so collection of the effluent in separate fractions may in fact have been very similar to collection in one tube.

3.3.5. Effect of Sepharose on collagen-induced serotonin release

Sepharose appeared to have an effect on released serotonin. This was investigated while determining the amount of serotonin release induced by various quantities of collagen (soluble or insoluble).

a) Effects of Sepharose and different types of collagen on release. Platelets were incubated for ten minutes with either insoluble or soluble collagen in order to determine whether greater

serotonin release could be induced than in previous experiments. A sample of platelets which had been incubated with each type of collagen was passed through a Sepharose column in order to discover whether Sepharose had any effect on released serotonin (table 19).

Table 19. Effects on release of Sepharose and incubation with different types of collagen

Collagen (μg (10^8 platelets)$^{-1}$)*	Type of collagen	Sepharose column	% serotonin release
0	-	yes	10.3
0 (acetic acid)**	-	no	0.5
62.5	insoluble	no	45.2
62.5	insoluble	yes	7.3
62.5	soluble	no	64.6
62.5	soluble	yes	69.2
250	insoluble	no	56.1
250	soluble	no	87.9

* collagen: 1mg ml^{-1} in 1mM acetic acid.
platelets: 4 x 10^8 ml^{-1}.
** acetic acid: 62.5nmole (10^8 platelets)$^{-1}$.

Acetic acid alone did not induce serotonin release from platelets. 250μg collagen induced greater release of serotonin than did 62.5μg (table 19). After incubation of platelets with soluble collagen, passage through a Sepharose column had no effect on serotonin released, but released serotonin appeared to be "lost" in the Sepharose column after incubation with insoluble collagen. Results from soluble collagen incubations were probably rather inaccurate since it formed a gel and hence sampling was difficult (insoluble collagen formed large masses of fibres). Platelets passed through a Sepharose column after incubation with soluble collagen were probably in contact with the collagen for longer than

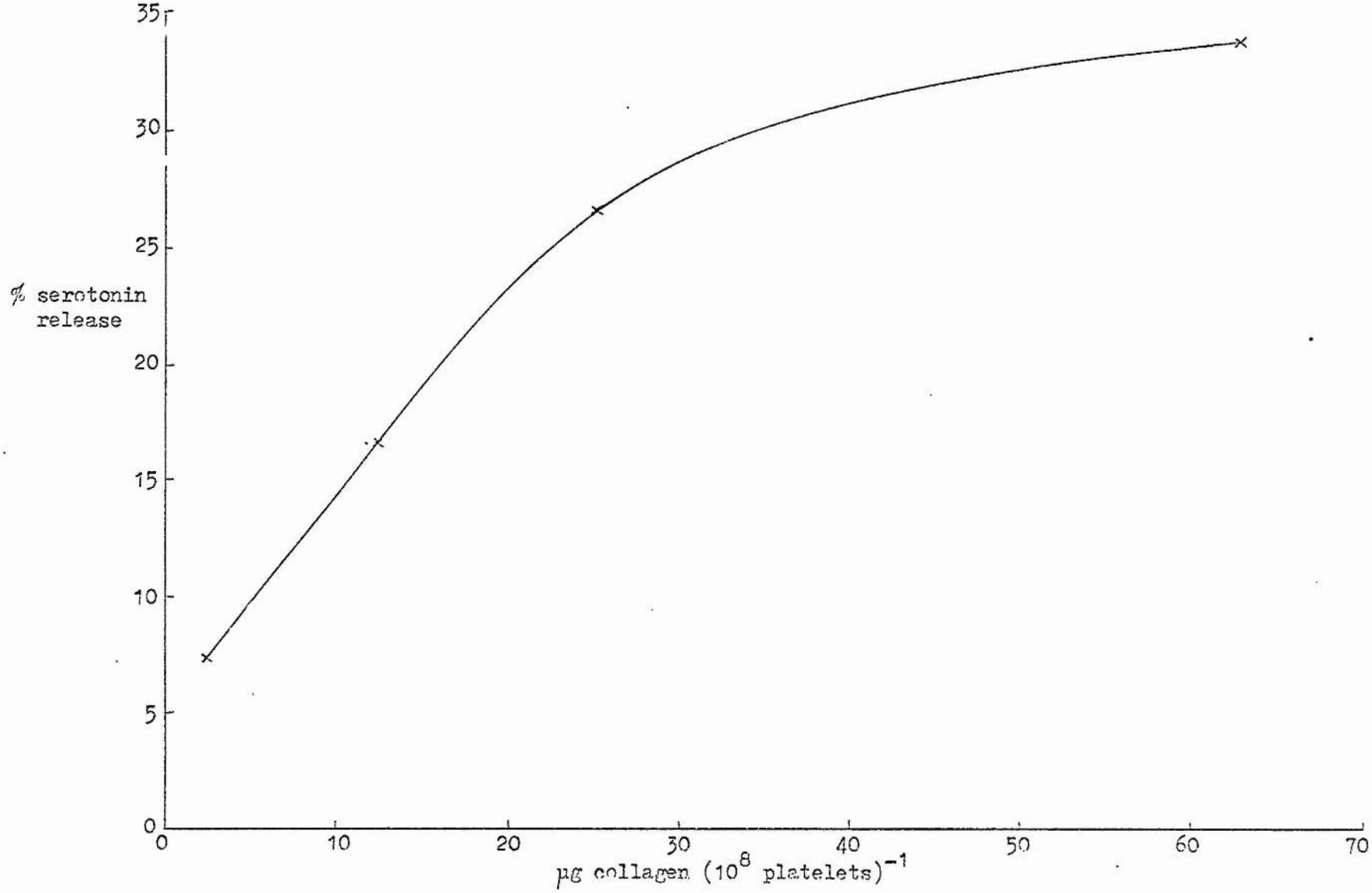
those incubated with insoluble collagen. This is because the insoluble collagen formed large masses of fibres during incubation with platelets and these would be unable to pass through the Sepharose with the platelets, whereas the soluble collagen formed a gel which apparently consisted of smaller fibres and some of the smallest ones could probably pass through part of the Sepharose column with the platelets.

b) Effect of amount of collagen and length of incubation on release. Collagen-induced serotonin release was investigated further by incubating platelets with various amount of insoluble collagen for different periods in order to determine the effect of these parameters on serotonin release. Samples of the platelet-collagen mixture were also passed through a Sepharose column (table 20).

Table 20. Effect of Sepharose and different incubation conditions on release

μg collagen (10^8 platelets)$^{-1}$*	Incubation (min)	Sepharose column	% serotonin release
0	0	yes	2.3
2.5	10	no	7.3
12.5	10	no	16.7
25	10	yes	4.7
25	10	no	26.7
25	20	no	26.8
25	30	no	32.9
62.5	10	yes	2.3
62.5	10	no	33.9
62.5	20	no	41.8
62.5	30	no	30.2

* concentrations of insoluble collagen and platelets as in table 19.



Graph 7. Serotonin release from platelets after 10 minutes incubation with collagen

More collagen induced greater serotonin release (table 20, graph 7), and maximum release was attained within ten minutes' incubation. Released serotonin appeared to be "lost" on Sepharose columns.

c) Effect of different treatments with Sepharose on serotonin release. The previous section showed that released serotonin appeared to be "lost" on Sepharose columns. An attempt was made to discover whether removal of the stimulus to release (i.e. collagen) was causing platelets to re-incorporate released serotonin or whether the Sepharose itself was in some way causing this apparent loss. So a platelet-collagen mixture (62.5µg insoluble collagen $(10^8 \text{ platelets})^{-1}$ in platelet buffer A) was incubated for ten minutes then 1ml samples were taken and treated in different ways; one aliquot was passed through a Sepharose column (this took approximately 20 minutes), one was incubated for 20 minutes with 0.6g Sepharose (in 0.5ml platelet buffer A) and one was incubated for 20 minutes after removing the solid mass of fibrous collagen which had formed during the incubation period with platelets.

Table 21. Effects of different experimental methods on serotonin release

Incubation with insoluble collagen	Treatment after incubation with collagen	% serotonin release
yes	-	28.2
yes	Sepharose column	0
yes	Incubation with Sepharose	17.7
yes	Incubation after removal of solid collagen	25.1
no	Sepharose column	1.2
no	Incubation with Sepharose	3.4

Negligible release was induced by Sepharose in the absence of collagen (table 21). Released serotonin was completely "lost" on

the Sepharose column but the amount only decreased by about 40% when the platelet-collagen mixture was incubated with Sepharose. However, this decrease may have been due to dilution of the sample by extra buffer trapped in the Sepharose rather than any direct effect of the Sepharose. Removal of the solid lump of collagen had little effect on release, presumably because either the platelets had released the maximum amount of serotonin possible or there was still sufficient collagen left (as small fibres) to maintain the release reaction.

3.4. ^{14}C-serotonin release from platelets

Amounts of serotonin released never approached those obtained by Brass et al. (1976) except when collagen was used without Sepharose. It is possible that exchange of tritium with hydrogen was occurring during passage of serotonin through the Sepharose columns, so ^{14}C-serotonin was used in later experiments instead of ^{3}H-serotonin.

3.4.1. Effect of (collagen-) Sepharose columns on platelets with or without prior incubation with collagen

The effect of Sepharose on serotonin release found in previous experiments was investigated further in order to explain and possibly overcome this effect.

Platelets were incubated with different concentrations of serotonin in these initial experiments in case this had an effect on the measurement of serotonin release. Aliquots of platelets were passed through Sepharose or collagen-Sepharose in order to determine whether the columns would induce any serotonin release. Further aliquots of platelets were incubated with insoluble collagen (to induce serotonin release) before being passed through Sepharose or collagen-Sepharose columns. A 1/11 dilution of the platelet-collagen mixture was also taken in order to imitate the dilution occurring when platelets were washed through a column (table 22).

Table 22. Effect of Sepharose columns and incubation with collagen on serotonin release from platelets

Incubation with collagen*	Type of column	µg collagen (g Sepharose)$^{-1}$	% serotonin release (animal no.) 1	2	3	4	5	6
no	Sepharose	-	25.7	8.9	4.1	5.1	6.5	12.7
no	collagen-Sepharose	860	-	-	13.5	10.9	14.2	21.7
yes	-	-	27.9	36.9	40.2	31.9	52.3	57.0
yes(^{1}/11dil)**	-	-	-	-	14.6	6.4	19.6	24.3
yes	Sepharose	-	32.5	6.6	11.1	6.4	12.2	21.0
yes	collagen-Sepharose	860	-	-	31.4	22.6	30.0	41.7
µg serotonin (ml platelets)$^{-1}$ (initial conc.)			0.13	0.13	0.13	1.5	2.5	2.5

* Incubation: 0.25 ml insoluble collagen (1 mgml^{-1} in 1mM acetic acid) added to 1 ml platelets (4 x 10^{8} ml^{-1}) and incubated for ten minutes at 37°C.

** ^{1}/11 dilution sampled; results corrected for dilution.

Collagen-Sepharose induced more serotonin release from platelets than did Sepharose (table 22). Dilution of the incubated platelet-collagen mixture caused a decrease in released serotonin (results had been corrected for the dilution) possibly due to unreacted platelets, when diluted, taking up extra (released) serotonin. When the platelet-collagen mixture was passed through a Sepharose column, released serotonin was "lost" (possibly due to the dilution effect described above) but collagen-Sepharose caused a relatively small decrease in released serotonin. This may be because platelets which had not reacted with the collagen of the incubation reacted with the collagen attached to the Sepharose.

Thus after being passed through the collagen-Sepharose column there were fewer unreacted platelets present to take up a greater amount of released serotonin (from incubation with collagen and from passage through collagen-Sepharose) than was the case with the Sepharose column (serotonin was released only during incubation with collagen). Consequently the net release of serotonin obtained after passing a platelet-collagen mixture through collagen-Sepharose, while smaller than that in the original mixture, was greater than the net release obtained after passing this mixture through Sepharose.

3.4.2. Incorporation of serotonin by platelets

Garattini (1965) noted that the concentration of serotonin in the medium is the critical factor in determining whether passive diffusion or active transport takes place. Experiments were therefore carried out to determine the optimum concentration of serotonin with which to incubate platelets and to discover the effect, if any, of diluting platelets by the same amount as those which had been passed through a column (i.e. 1/11). An attempt was also made to determine whether it was possible for platelets, after being incubated with ^{14}C-serotonin and washed, to incorporate more ^{14}C-serotonin during a second incubation.

a) Effect of varying serotonin concentration and constant platelet concentration on uptake of serotonin. Uptake of serotonin

by platelets depends on the concentration of serotonin present (Garattini, 1965). Platelets at the concentration normally used (4×10^8 ml^{-1}) were incubated with different concentrations of serotonin in order to determine the optimum concentration for incorporation.

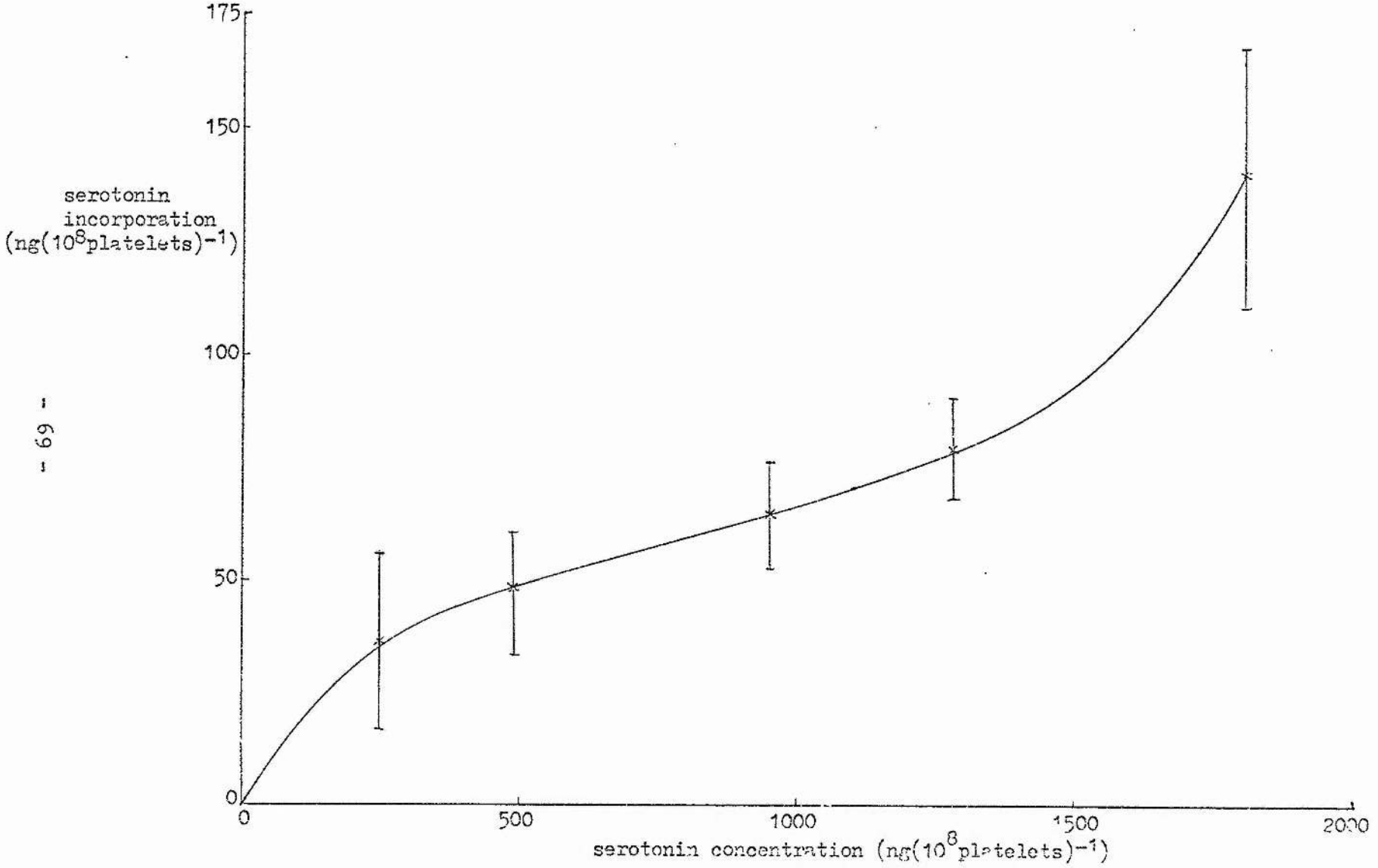
Table 23. Platelet concentration constant, varying serotonin concentration

ng serotonin $(10^8$ platelets$)^{-1}$* in incubation	incorporated
250	37.1 ± 19.4**
490	47.8 ± 13.9
950	64.9 ± 11.4
1280	79.6 ± 11.0
1800	140.1 ± 29.0

* 4×10^8 platelets ml^{-1}.
** mean ± standard deviation (3 experiments).

Active transport of serotonin into platelets occurred at low serotonin concentrations and passive diffusion occurred at high concentrations (table 23, graph 8). There is a plateau region on graph 8, between about 250 - 1250ng serotonin $(10^8$ platelets$)^{-1}$, where serotonin incorporation varied very little.

b) Effect on serotonin uptake of keeping platelets and serotonin in constant proportions. When platelets were passed through a Sepharose column, they were washed through (and hence diluted) with platelet buffer A. Any non-adherent platelets and any serotonin released were diluted in the collection tube by the column effluent. The effect of this dilution on determination of serotonin release was investigated by adding serotonin to PRP for incubation as usual, but diluting aliquots with PPP in varying amounts before incubating them. Platelets and serotonin were therefore in the same



Graph 3. Incorporation of serotonin by platelets incubated with different concentrations of serotonin

proportions in each aliquot.

Low concentrations of platelets and serotonin led to greater serotonin incorporation (table 24, graph 9) than high concentrations of both. Increasing the concentration of platelets and serotonin by eight times decreased the amount of serotonin incorporated threefold (per 10^8 platelets). Results from the two experiments were however very different at low concentrations and should be treated with caution.

c) Effect of varying platelet concentration and constant serotonin concentration on uptake of serotonin. If platelets adhere to collagen-Sepharose and release serotonin, the platelet concentration will decrease but the concentration of serotonin (which is washed through the column) may reach a relatively constant level. So varying concentrations of platelets were incubated with a constant serotonin concentration and the uptake of serotonin by platelets was measured.

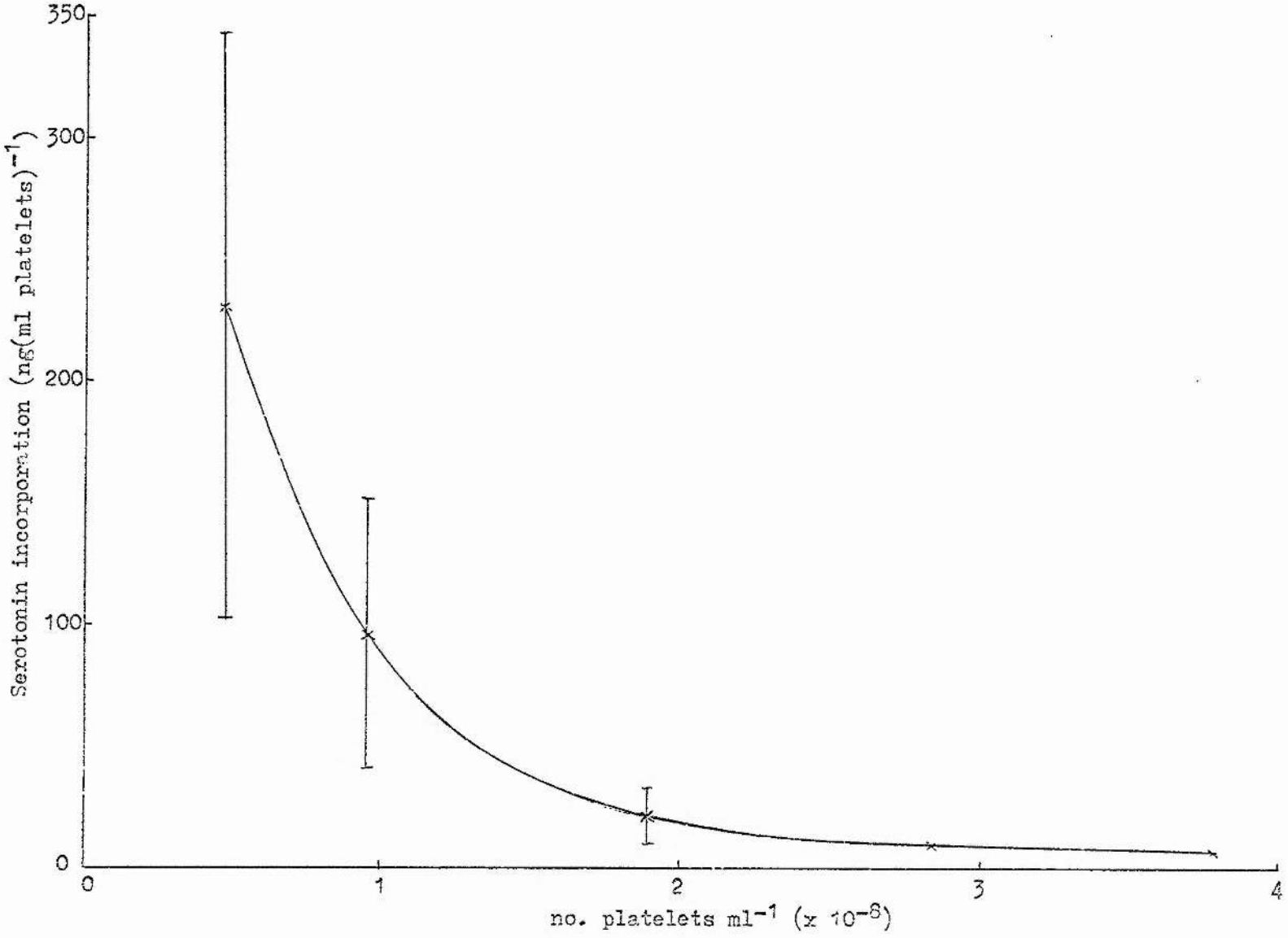
Non-adherent platelets which had passed through a Sepharose column and been collected in a tube were exposed to more serotonin which had been released by adherent platelets. So an aliquot of platelets at the concentration normally used (4×10^8 ml^{-1}) was incubated with serotonin, washed to remove the excess, then incubated with more serotonin at a concentration which might have been released by platelets (assuming 140ng serotonin (ml platelets)$^{-1}$ and 50% release). A 1/11 dilution (as though passed through a Sepharose column) was also incubated with serotonin for a second time.

High concentrations of platelets incorporated more serotonin than low platelet concentrations (table 25, graph 10). But at low platelet concentrations there was more serotonin available per platelet and individual platelets were therefore able to take up more than individual platelets at higher concentrations, although the total amount of serotonin incorporated by platelets at high concentrations was still greater. Platelets incubated with serotonin for a second time took up extra serotonin. This means that non-adherent platelets could take up serotonin released by adherent

Table 24. Platelets and serotonin in constant proportions

No. platelets $ml^{-1}(x\ 10^{-8})$	Serotonin ($ngml^{-1}$)	Serotonin incorporated* $ng(10^{-8}$ platelets$)^{-1}$	$ng(ml\ platelets)^{-1}$
0.473	296	105.5 ± 56.9	223.0 ± 120.3
0.947	592	90.8 ± 52.9	95.9 ± 55.9
1.894	1184	41.1 ± 22.3	21.7 ± 11.8
2.841	1776	31.1 ± 3.4	10.9 ± 1.2
3.788	2367	33.5 ± 1.9	8.8 ± 0.5

* Results from two experiments

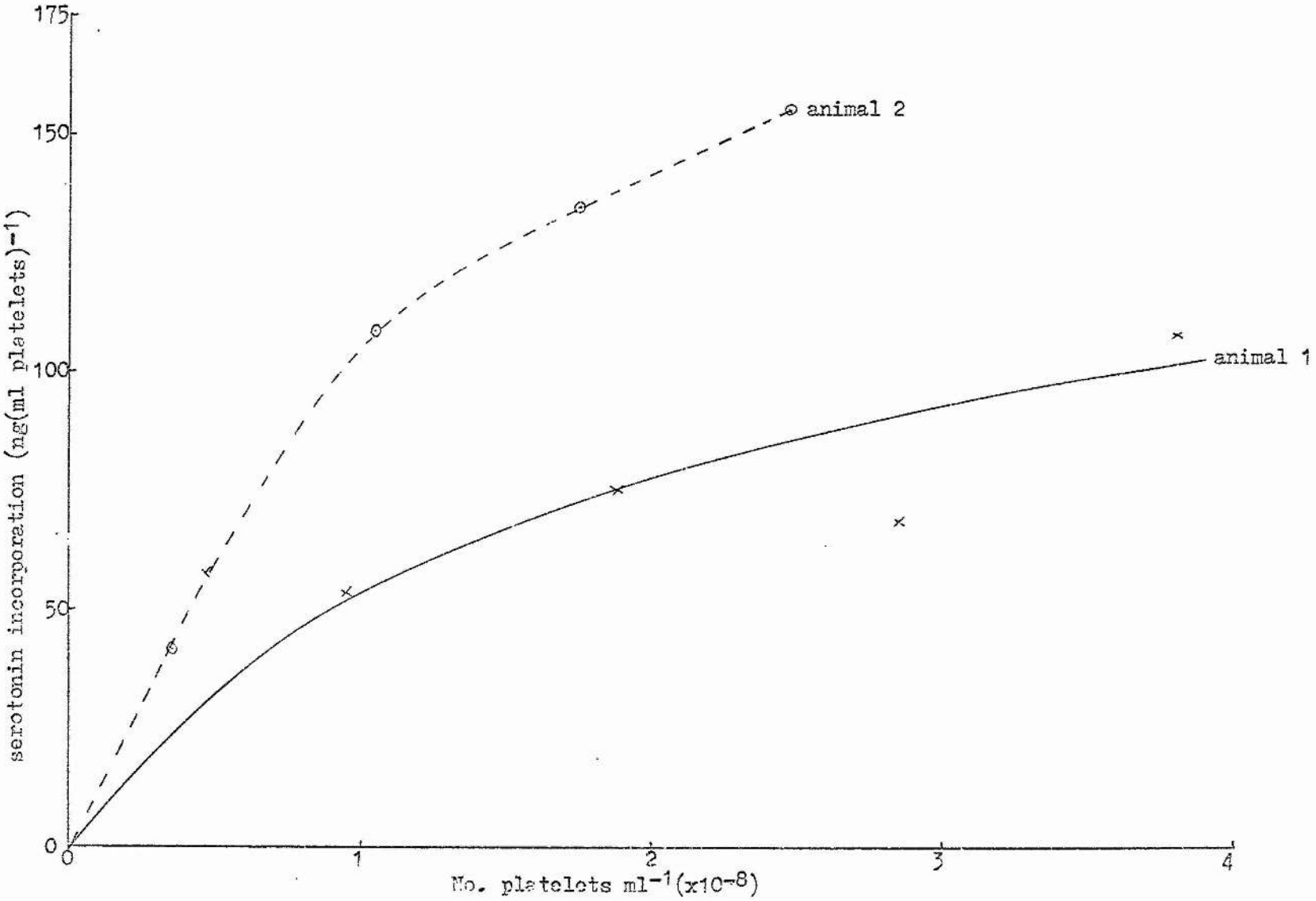


Graph 9. Incorporation of serotonin by platelets at constant proportions of both

Table 25. Varying platelet concentration, constant serotonin concentration

	No. platelets $ml^{-1}(x10^{-8})$	Serotonin incorporated ng $(10^8$ platelets$)^{-1}$	ng (ml platelets)$^{-1}$
Animal 1			
1st incubation	0.473	123.7	58.6
	0.947	55.7	52.8
	1.893	39.7	75.3
	2.840	24.0	68.2
	3.787	28.8	109.1
2nd incubation	0.344	5.4	6.9
	3.787	1.8	0.5
Animal 2			
1st incubation	0.350	116.7	40.9
	1.051	103.8	109.1
	1.752	76.9	134.8
	2.453	63.1	154.6
2nd incubation	0.223	10.5	2.3
	2.453	4.9	12.0

1st incubation : 2.42 µg serotonin (ml platelets)$^{-1}$
2nd incubation : 69.3 ng serotonin (ml platelets)$^{-1}$



Graph 10. Incorporation of serotonin by different concentrations of platelets

platelets. It is possible however that this apparent second incorporation of serotonin was in fact exchange of ^{14}C-serotonin from solution with unlabelled serotonin from inside the platelets.

3.4.3. Choice of incubation conditions

A concentration of 625ng serotonin $(10^8$ platelets$)^{-1}$ was chosen for incubation with platelets since this was in the middle of the plateau region in graph 8. At this concentration active transport was at a maximum but passive diffusion was not taking place.

Tables 24 and 25 are apparently contradictory for incorporation of serotonin per millilitre of platelets, but results in table 24 are very variable whereas those in table 25 are more consistent. A platelet concentration of 4×10^8 ml^{-1} was chosen for use in further experiments because of greater serotonin incorporation and because more platelets would be available for adhesion to collagen-Sepharose.

For a concentration of 4×10^8 platelets ml^{-1}, the serotonin concentration was therefore 2.5μg ml^{-1}.

3.5. Effect on platelets of modifications to collagen-Sepharose

Having determined the optimum concentrations of platelets and serotonin, the effects of modifying the collagen attached to the Sepharose were investigated, specifically the ε-amino groups of lysine and hydroxylysine.

3.5.1. Succinylation of collagen-Sepharose

The ε-amino groups of soluble collagen-Sepharose were blocked by succinylation, the amino groups being replaced by carboxyl groups. Platelets were passed through three types of Sepharose column (untreated Sepharose, collagen-Sepharose and succinylated collagen-Sepharose) in order to compare the amount of serotonin

release induced (table 26).

Table 26. Effect of succinylation of collagen-Sepharose on serotonin release by platelets

Type of column	% serotonin release*	μg collagen (g Sepharcse)$^{-1}$
Sepharose	11.7 ± 4.7	-
soluble collagen-Sepharose	22.4 ± 5.1	825 ± 88
succinylated collagen-Sepharose	5.8 ± 1.3	797 ± 184

* Results from eight experiments.

Collagen-Sepharose induced significantly greater serotonin release from platelets (table 26) than did either Sepharose ($p < 0.01$) or succinylated collagen-Sepharose ($p < 0.001$) and the latter induced significantly less serotonin release than untreated Sepharose ($p < 0.02$).

Collagen-Sepharose had 710μmoles free ε-NH_2 groups per gram of collagen whereas succinylation led to complete blockage of these groups (Sepharose also had none).

3.5.2. Trinitrophenylation of collagen-Sepharose

The ε-amino groups of collagen-Sepharose were next modified by the addition of trinitrophenol (TNP) groups. Platelets were passed through the resultant TNP-collagen-Sepharose and serotonin release was compared with that induced by Sepharose and collagen-Sepharose (table 27).

Table 27. Effect of trinitrophenylation of collagen-Sepharose on serotonin release by platelets

Type of column	% serotonin release*	μg collagen (g Sepharose)$^{-1}$
Sepharose	9.5 ± 5.6	-
soluble collagen-Sepharose	19.0 ± 4.7	745 ± 13
TNP-collagen-Sepharose	48.2 ± 6.8	677 ± 7

* Results from eight experiments.

Again (table 27), collagen-Sepharose induced significantly greater serotonin release than did Sepharose ($p < 0.02$), but trinitrophenylated collagen-Sepharose induced significantly greater release than did either Sepharose ($p < 0.001$) or collagen-Sepharose ($p < 0.001$).

3.5.3. Dinitrophenylation of collagen-Sepharose

Finally the ε-amino groups of collagen-Sepharose were modified by dinitrophenylation to give dinitrophenyl (DNP)-collagen-Sepharose. The effect of this modification on serotonin release by platelets was determined and compared with serotonin release induced by Sepharose and collagen-Sepharose (table 28).

The difference between serotonin release induced by Sepharose and by collagen-Sepharose (table 28) was again significant ($p < 0.01$) and dinitrophenylated collagen-Sepharose, like TNP-collagen-Sepharose, induced significantly greater release than did either Sepharose ($p < 0.001$) or collagen-Sepharose ($p < 0.001$).

Table 28. Effect of dinitrophenylation of collagen-Sepharose on serotonin release by platelets

Type of column	% serotonin release*	μg collagen (g Sepharose)$^{-1}$
Sepharose	6.4 ± 1.8	-
soluble collagen-Sepharose	19.1 ± 6.7	795 ± 76
DNP-collagen-Sepharose	41.4 ± 4.2	879 ± 57

* Results from eight experiments.

3.5.4. Comparison of effects on serotonin release of modifications to collagen-Sepharose

In each set of experiments collagen-Sepharose induced greater serotonin release than did Sepharose. Succinylation of collagen-Sepharose induced less release than that induced by Sepharose whereas trinitro- and dinitrophenylation induced greater release than did collagen-Sepharose. There was no significant difference ($p > 0.2$) in the figures for release induced by Sepharose in the latter two sets of experiments (tables 27, 28), nor in the release induced by collagen-Sepharose ($p > 0.9$). But there was a highly significant difference in release between TNP- and DNP-collagen-Sepharose ($p < 0.05$), the latter inducing less serotonin release than the former, although more collagen was present in the DNP-collagen-Sepharose than in the TNP-collagen-Sepharose.

3.5.5. Comparison of extent of modification of TNP- and DNP-collagen Sepharose

It is possible that the difference in serotonin release between TNP- and DNP-collagen-Sepharose was due to differing extents of

modification of collagen-Sepharose. The extent of modification of each preparation was determined by amino acid analysis and comparison of the amount of lysine and hydroxylysine present with respect to unmodified collagen-Sepharose (table 29).

The extent of modification varied, a smaller percentage of collagen being dinitrophenylated than trinitrophenylated (table 29). Modification of collagen in both these ways induced more serotonin release than unmodified collagen (tables 27 and 28), but although there was more DNP-collagen than TNP-collagen present on Sepharose, more serotonin was released when passed through TNP-collagen-Sepharose. This difference in serotonin release is likely to be due to the groups added rather than the extent of modification, but more samples are needed for a statistical analysis.

Table 29. Extent of modification of TNP- and DNP-collagen-Sepharose

Type of collagen-Sepharose	µg collagen (g Sepharose)$^{-1}$	% modification	µg modified collagen (g Sepharose)$^{-1}$	% serotonin release*
TNP	670	95.3	639	50.3 ± 4.6
TNP	684	73.9	505	46.0 ± 8.7
DNP	825	60.9	502	43.3 ± 4.8
DNP	932	79.7	743	39.6 ± 3.2

* Results from four experiments

4. DISCUSSION

4.1. Choice of analytical method for collagen determination

The amount of collagen in contact with platelets affects the extent of the platelet reaction so it is important to know how much collagen is present. This is especially important when modifying collagen attached to an insoluble support because it is possible that some of the collagen present might be removed during the modification procedure. Any decrease in platelet reaction might then be attributed to the modification rather than to the decrease in the amount of collagen present.

When determining the amount of collagen present on an insoluble support, the support material must not interfere with the collagen determination, or if it does, the extent of interference must be constant. Four different methods of determining collagen concentration were used, all of which were affected to some extent by the presence of activated Sepharose.

The first method, using hydroxyproline determination (Stegemann, 1958), proved to be unreliable even for collagen alone. There are several possible reasons for this: instability of the Chloramine-T and p-dimethylaminobenzaldehyde, incomplete destruction of Chloramine-T by perchloric acid, varying evaporation of the samples while being heated, low colour yield and interference from the colour of the hydrolysed collagen.

Problems in colour stability and the extent of the reaction made the second method of hydroxyproline determination (Serafini-Cessi & Cessi, 1964) unreliable too. When using this method, an attempt was made to determine the optimum time interval between incubation of the hydrolysed collagen with the hydrogen peroxide and distillation, and between distillation and measuring the absorbance, in order to make the assay more reproducible and therefore practical. But the variation in results was such that another

method of collagen determination was tried.

Ninhydrin may be used to determine amino acid concentrations (from hydrolysed protein) and so some ninhydrin solution was incubated with samples of activated Sepharose with and without collagen. But the activated Sepharose gave such a great reaction with the ninhydrin that the presence of collagen made insufficient difference for the assay to be reliable.

The fourth method of collagen determination employed an amino acid analyser. This also used the reaction of ninhydrin with hydrolysed protein, but in this case the cation exchange resin separated the reaction products of ninhydrin and activated Sepharose from the amino acid-ninhydrin products. The amount of collagen present was then determined from the size of the glycine peak. Activated Sepharose still had some effect on collagen determination because collagen and activated Sepharose plus collagen gave different results. However, this difference was consistent so the amino acid analyser method was chosen for determination of collagen. The use of the amino acid analyser also had the advantage that the extent of di- or trinitrophenylation of collagen-Sepharose could be calculated from the results obtained for collagen determination.

4.2. Use of collagen-strips and different investigation methods

4.2.1. Effect of different investigation methods on determination of platelet-collagen interactions

A method was developed for immobilising collagen on to strips of polyamide sheet in order that the collagen conformation would remain constant during its interaction with platelets (section 2.3.1). The platelet-collagen interaction was investigated using three different methods.

a) Aggregometer method. Collagen-strips were incubated with platelet-rich plasma in an aggregometer (section 2.6.1.) and the absorbance at 600nm was recorded as a measure of aggregation. No

platelet aggregation was induced by the collagen-strips (section 3.1.2.).

b) Sivertson's whole blood method. Collagen-strips were incubated with whole blood (section 2.6.2.) and the number of unreacted platelets was counted (after fixing with formaldehyde). This indicated the amount of adhesion plus aggregation which had been induced. Collagen-strips induced significantly more platelet adhesion plus aggregation than did strips without collagen (section 3.1.3.).

c) ^{3}H-serotonin release. Collagen-strips were incubated with platelets which had previously been incubated with ^{3}H-serotonin (section 2.6.3.). Release of ^{3}H-serotonin was measured and found to be marginally greater when platelets were incubated with collagen-strips than with activated strips (section 3.1.4.). However, results were very variable, possibly due to variations in the individual animals from which the blood was taken.

4.2.2. Comparison of results obtained using the aggregometer and Sivertson's whole blood method

There are several possible explanations as to why collagen-strips did not induce platelet aggregation in the aggregometer while inducing adhesion plus aggregation in Sivertson's whole blood method.

a) Wrong collagen conformation. It is possible that the conformation of the collagen fibrils formed on the polyamide strips was not suitable for inducing platelet aggregation. But adhesion and aggregation were induced by collagen-strips when Sivertson's whole blood method was used, so the collagen fibril conformation must have been suitable for recognition by platelets.

b) Importance of ε-amino groups. The attachment of collagen to polyamide was via the free (ε) amino groups of collagen. These groups have been thought to be important in the recognition of collagen by platelets (Wilner et al., 1968b), so the lack of

aggregation in the aggregometer might be because these groups are indeed important. But again this theory is contradicted by the adhesion and aggregation measured using Sivertson's whole blood method.

c) Insufficient collagen. The most probable explanation of these results is that the amount of collagen exposed to the platelets in the aggregometer was insufficient to induce aggregation. Although Kronick and Jimenez (1979) maintained that the minimum amount of collagen required to induce platelet aggregation in an aggregometer is 0.35μg (10^9 platelets)$^{-1}$ (i.e. 0.17μg collagen would be required for each aggregometer cuvette in these experiments), Burns (1976) found that minimal aggregation was induced by 20μg collagen in the system used here.

When collagen is immobilised on to polyamide strips, only the outer layer is available for interaction with platelets. If it is assumed that this outer layer of collagen is arranged as a monolayer, a 4cm^2 strip covered on both sides with collagen would have 0.96μg collagen available to platelets. This would be insufficient to induce platelet aggregation in this system even if all the collagen was in the optimum conformation. At least 100μg collagen would be required since modifications to the collagen might induce decreased aggregation which should still be measured. Nylon beads and tubing were also calculated to have a surface area for collagen attachment that would be insufficient for the volume of platelets to which they could be exposed.

Platelet adhesion and aggregation induced by collagen-strips was measureable using Sivertson's whole blood method, so although there was insufficient collagen present to induce measureable aggregation in the aggregometer there was sufficient for this method. Sivertson's method is more sensitive than the aggregometer since adhesion is measured as well as aggregation and because individual platelets are counted. This means that even if small aggregates are formed, the number of platelets counted decreases and an effect of collagen-strips is noticed. A greater extent of aggregation is necessary for detection using the aggregometer.

4.2.3. ^{3}H-serotonin release

If the difference between results obtained using the aggregometer and Sivertson's whole blood method was indeed due to insufficient collagen being present for aggregation to be induced and detected in the aggregometer, while there was sufficient present for adhesion and aggregation to be induced and detected using the more sensitive Sivertson's method, then a greater amount of ^{3}H-serotonin release would be expected than that obtained. But other factors affected the measurement of ^{3}H-serotonin release induced by collagen-strips.

Later experiments (section 3.4.2.c) showed that unreacted platelets could take up serotonin released from adherent platelets. The amount of collagen on a strip of polyamide available for platelet adhesion is not great, as previously shown, so the amount of ^{3}H-serotonin released is not likely to be very great either. If released serotonin is taken up by non-adherent platelets, the effect on released serotonin detected will be greater when there is little serotonin release (and hence many free platelets to take it up) than when more serotonin has been released.

When using treated polyamide strips for ^{3}H-serotonin release experiments it was necessary to cut the strips into smaller pieces in order that the platelets would be in contact with the entire surface. The strips were cut into 1cm^{2} pieces or into approximately 0.2cm^{2} pieces. Strips cut into 0.2cm^{2} pieces induced slightly more serotonin release than those cut into 1cm^{2} pieces (section 3.1.4.e), but more serotonin appeared to adsorb to the smaller pieces than to the larger (section 3.1.4.d). Both of these effects were probably due to the smaller pieces having more cut surfaces which were available for adsorption of serotonin or for interaction with platelets.

The method of stopping the release reaction of platelets, i.e. cooling the tubes in an ice bath, was slow because the tubes were plastic. The addition of ice-cold resuspension solution to the reacted platelets, followed by immediate centrifugation was tried

(section 3.4.1.d) but little difference was noticed. So reaction tubes were centrifuged as soon as possible after the reaction period. It would have been preferable to have used a refrigerated centrifuge in order to cool the samples, but no suitable centrifuge was available. Other possible methods of stopping the reaction between platelets and collagen have been described. Fauvel et al. (1976) used gel filtration on Sepharose 2B and centrifugation in Ficoll, both methods using 0.5ml samples. Ardlie and Han (1974) used an Eppendorf centrifuge, centrifuging the samples for one minute at 15 000g.

The adhesion induced by collagen-strips is apparently reversible (section 3.1.3.). Since the reaction time was longer in the ^{3}H-serotonin release experiments and the method of stopping the reaction was much slower than in Sivertson's method, some adherent platelets may have come off the collagen-strips and taken up released serotonin, thus decreasing the amount of ^{3}H-serotonin detected.

So there are many factors which may decrease the amount of released ^{3}H-serotonin which is detected after incubation of platelets with collagen-strips.

4.2.4. Use of other stimulants of platelet reaction

Sivertson's method and the ^{3}H-serotonin release method were both tried out with ADP and with collagen to ensure that the reaction of platelets could be determined as expected. ADP-induced platelet aggregation requires Ca^{2+} (Ardlie & Han, 1974; Zucker & Grant, 1978), but this was chelated by the EDTA present in the resuspension solution (^{3}H-serotonin release experiments). When $CaCl_2$ was added to the platelet suspension at a concentration sufficient to overcome the EDTA, ADP-induced aggregation occured and release of ^{3}H-serotonin could be measured (section 3.1.4.b). In agreement with Spaet and Lejnieks (1969), Ca^{2+} was unnecessary for collagen-induced serotonin release (section 3.1.4.c) although Kinlough-Rathbone et al. (1977) maintained that it is required for collagen-induced platelet aggregation. As expected, when platelets

were incubated with greater amounts of collagen, more serotonin was released. In experiments using Sivertson's whole blood method, ADP induced secondary, irreversible, aggregation whereas collagen tended to induce primary, reversible, aggregation.

4.3. Use of collagen-Sepharose and the serotonin release reaction

When platelets were stored between preparation and use there was a slow release or leakage of serotonin from them. This would lead to deceptively high figures for release unless separate control samples were taken at the same time as each column was run. This phenomenon was not mentioned by Brass et al. (1976).

Platelets have been incubated with various amounts of serotonin in different reports (Ardlie & Han, 1974; Brass & Bensusan, 1974; Brass et al., 1976), so experiments were carried out to determine the optimum serotonin concentration (section 3.4.2.). This was found to be between 1 and 5μg ml^{-1} serotonin, agreeing with Born and Gillson (1959) who found maximum uptake to occur above about 0.6μg ml^{-1} in experiments using up to 3μg ml^{-1} serotonin. So a concentration of 2.5μg ml^{-1} (being the centre of the plateau region of the graph of serotonin concentration against incorporation) was used. Below 1μg ml^{-1} the amount of serotonin incorporated by platelets increased with concentration and active transport of serotonin into the dense bodies was occurring; above 5μg ml^{-1} passive diffusion took place. This latter figure agrees with the finding of Grant and Zucker (1979) that passive diffusion occurs above a serotonin concentration of approximately 10μM (i.e. approximately 4μg ml^{-1}).

Increasing the platelet concentration (section 3.4.2.) caused more serotonin to be taken up per millilitre of platelets, although each platelet took up less than at lower platelet concentrations. Hardeman and Heynens (1974a) have reported that increasing the platelet concentration increases the initial rate of serotonin uptake, but they did not note the total amount of serotonin taken up.

When both serotonin and platelet concentrations were increased, the serotonin incorporation was very variable, especially at low concentrations, but the trend was for less serotonin to be incorporated at high concentrations than at low. This contradicts the experiments previously discussed, but due to the smaller variation in results, the previous results (i.e. serotonin incorporation increases with increasing serotonin or platelet concentration) are held to be more accurate. These experiments should be repeated to verify the relationship between serotonin and platelet concentrations and serotonin incorporation by platelets.

The amount of collagen present in a collagen-Sepharose column was between about 550μg and 650μg and this is in the optimum range found by Brass et al. (1976). Since serotonin release from platelets passed through such a column was much less than that obtained by Brass et al. (1976) (20% compared with 70%), platelets were incubated with collagen (sections 3.3.5. and 3.4.1.) to determine whether they were capable of releasing larger quantities of serotonin. Then an aliquot of the platelet-collagen mixture was passed through a column of either Sepharose or collagen-Sepharose in order to ascertain whether the relative lack of serotonin release detected was in some way due to the columns.

Large amounts of serotonin were released by platelets in contact with collagen, but when an aliquot of this suspension was passed through a Sepharose column the amount of released serotonin decreased markedly. Collagen-Sepharose also caused a decrease in serotonin release, but to a lesser extent. So the released serotonin was being lost somewhere between applying the platelet-collagen suspension to the columns and sampling the effluent. ^{14}C-serotonin was used instead of ^{3}H-serotonin in later experiments in case exchange of tritium with hydrogen was occurring during passage through the Sepharose column. Three other theories as to why released serotonin disappeared in the Sepharose columns were also tested.

a) Serotonin adsorption to or entrappment in Sepharose. When serotonin was passed through columns of Sepharose and collagen-

Sepharose (section 3.3.3.) it did not adsorb to either type of column. The unlikely possibility that serotonin was being trapped in the Sepharose in some other way was also tested by incubating a sample of a platelet-collagen suspension with Sepharose (section 3.3.5.) and determining the amount of serotonin released before and after incubation with the Sepharose. The amount of released serotonin decreased on incubation with Sepharose, but was still greater than when the platelet-collagen suspension was passed through a Sepharose column (this small decrease on incubation was probably due to buffer trapped in the Sepharose). Released serotonin was not therefore being trapped in the columns.

b) Reincorporation of serotonin. Another explanation for the decreased serotonin release is that the removal of collagen from the unreacted platelets by the filtering action of the Sepharose caused reincorporation of released serotonin. Less serotonin was "lost" when a collagen-Sepharose column was used (section 3.3.3.c), presumably because previously unreacted platelets adhered to the collagen attached to the Sepharose and then released serotonin. So the net decrease in serotonin detected was smaller than when the Sepharose column was used. An attempt was made to remove visible collagen from the platelet-collagen suspension, but it was not possible to remove all the smallest fibrils, and serotonin release was unaffected.

c) Secondary incorporation of serotonin. The final theory to be tested was that the dilution of the platelet-collagen mixture by the buffer washing the column caused released serotonin to be taken up by non-adherent platelets. So a sample of the platelet-collagen mixture was diluted with platelet buffer A (section 3.4.1.), as though it had been passed through a column, and this did indeed cause a decrease in the amount of released serotonin. It had originally seemed unlikely that platelets would take up more serotonin during a second incubation (i.e. in the collection tube after some serotonin had been released after passage through collagen-Sepharose) since such a large amount of serotonin remained in solution after the first incubation (approximately 80% remained). The possibility of secondary incorporation of serotonin was

invested (section 3.4.2.c) using a concentration of serotonin similar to that which might be present in the collection tube after collagen-Sepharose-induced serotonin release. The platelets did indeed incorporate more serotonin.

It is probable therefore that platelets passing through a collagen-Sepharose column without adhering will take up serotonin released by adherent platelets, so the measured release of serotonin is lower than the actual release. Salzman et al. (1977) have noted that in their investigation reuptake occurred, but they could not distinguish whether it was by the same platelets that had released the serotonin or by non-reacted ones. Reimers et al. (1975) also recorded that deaggregated platelets could reincorporate serotonin they had previously released. This effect on the determination of serotonin release will be greater when few platelets adhere and serotonin release is small than when many platelets adhere and serotonin release is great.

An attempt was made to separate the non-adherent platelets from the released serotonin and thus prevent secondary incorporation (section 3.3.4.), by collecting the column effluent in separate fractions. But the platelets and serotonin were not separated sufficiently by the Sepharose and were eluted from the column at approximately the same time. Another way of preventing this secondary incorporation might be to add imipramine to the platelet suspension after incubation with ^{14}C-serotonin or the platelet buffer A. There have been several reports (Costa et al., 1977; Da Prada & Pletscher, 1968; Packham et al., 1977) that imipramine prevents reuptake of serotonin by acting on the cell membrane while not significantly influencing endogenous serotonin (Da Prada & Pletscher, 1968) or by acting on the vesicle membrane (Costa et al., 1977), but although it causes no loss of serotonin (Costa et al., 1977), it is not obvious from the reports whether collagen-induced release is affected.

As stated above, serotonin release when using collagen-Sepharose columns was much lower (approximately 20%) than that obtained by Brass et al. (1976) (approximately 70%). Release of 30-40% and

occasionally more was obtained using both soluble and insoluble collagen (less collagen induced less serotonin release). Collagen-Sepharose columns using both types of collagen were also used (sections 3.3.2. and 3.3.5.), each type inducing less serotonin release than the relevant collagen preparation. It is possible that the collagen attached to the Sepharose was in the wrong conformation to induce adhesion and release, but when insoluble collagen was used the method of attachment employed was the same as that used by Brass et al. (1976). There is no apparent reason why the conformation of insoluble (bovine achilles tendon) collagen from Sigma should be different from insoluble (bovine achilles tendon) collagen from Ethicon when attached to Sepharose by the same method.

Cazenave et al. (1973) have shown that EDTA inhibits collagen-induced adhesion of platelets and will therefore also inhibit the release reaction and Kinlough-Rathbone and Mustard (1971) found that no release was induced by thrombin in the absence of Ca^{2+} or Mg^{2+}. If the small concentration of EDTA (0.3mM) present in platelet buffer A inhibits adhesion and release, this would explain the low figures obtained in these experiments for serotonin release. But if this concentration of EDTA does inhibit these reactions, Brass et al. (1976) should also have obtained lower figures for adhesion and release.

The difference in results is possibly due to slight differences in the type of collagen present in the commercial preparations used: Nyman (1977) has reported that collagen from different suppliers had different platelet-aggregating properties. Different types of platelets were also used: Brass et al. (1976) used human platelets whereas bovine platelets were used here.

4.4. Modifications to collagen-Sepharose

The ε-amino groups of collagen were modified in three different ways while attached to Sepharose and the effect of these modifications on serotonin release from platelets was investigated.

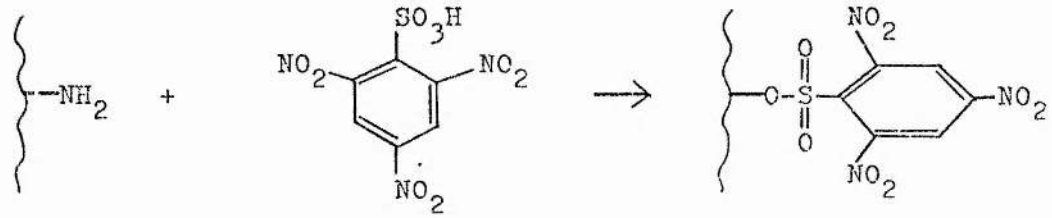
4.4.1. Succinylation

collagen succinic anhydride succinylated collagen

The ε-amino group, when succinylated, is replaced by a carboxyl group and so the charge at pH 7.4 is changed from positive to negative. This change in charge will cause aggregates to break up if they were held together by ionic bonds, so it is necessary to know the amount of collagen present in case some is lost during succinylation. In fact there was little difference in the amount of collagen attached to Sepharose before and after succinylation. This method caused complete blockage of the ε-amino groups (section 3.5.1.).

When platelets were passed through a column of succinylated collagen-Sepharose, release of serotonin was inhibited, in fact less serotonin was released than when platelets were passed through untreated Sepharose (section 3.5.1.).

4.4.2. Trinitrophenylation



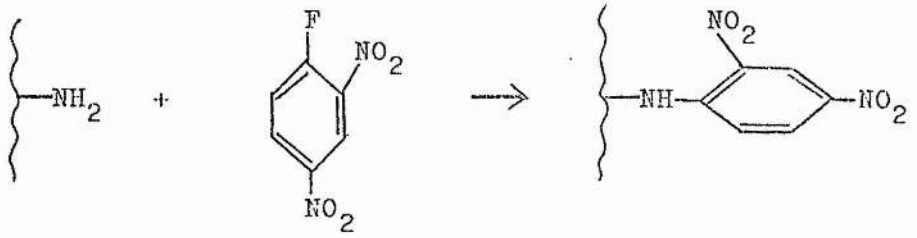
collagen TNBS TNP-collagen

TNP-collagen-Sepharose, at pH 7.4, has the positive charge of the ionised amino group replaced by the neutral trinitrophenyl group. Over 70% of the lysine and hydroxylysine residues of collagen were modified by the trinitrophenyl group (section 3.3.5.). The unmodified groups were probably either attached to the Sepharose or

buried in the fibres and hence inaccessible to the TNBS.

Collagen-Sepharose treated in this way induced enhanced release of serotonin by platelets (section 3.5.2.).

4.4.3. Dinitrophenylation



collagen DNFB DNP-collagen

When collagen is dinitrophenylated, the positive charge on the amino group at pH 7.4 is replaced by a neutral dinitrophenyl group. Dinitrophenylation modified 60-80% of the lysine and hydroxylysine residues, unmodified residues probably being inaccessible to the DNFB (section 3.5.5.).

DNP-collagen-Sepharose induced enhanced release of serotonin by platelets (section 3.5.3.), but to a lesser extent than that induced by TNP-collagen-Sepharose (section 3.5.4.).

4.4.4. Comparison of effects of modifications of collagen-Sepharose

The above results show that the ε-amino groups of collagen are not in themselves important as far as recognition by platelets is concerned, since when they were blocked by di- or trinitrophenyl groups, serotonin release was enhanced.

When the ε-amino groups were replaced by carboxyl groups, thus changing the charge from positive to negative, any ionic bonds involving the ε-amino groups would have been broken and instead of an attraction between them and neighbouring groups, repulsion would have occurred. This would have pushed the fibrils apart and it is

probable that the resulting disruption would have made it impossible for platelets to recognise the collagen.

But with both DNP- and TNP-collagen the ε-amino groups were modified in such a way as to neutralise the positive charges, so although the ionic bonds would have been broken, no repulsion and subsequent disruption of the collagen fibrils would have occurred. It is conceivable that some ionic bonds might have formed in the "wrong" places during immobilisation on to Sepharose and that the breaking of these bonds enabled the fibrils to become realigned, leading to the presence of more sites on the collagen which could be recognised by platelets. This would explain the increased serotonin release from platelets in contact with DNP- and TNP-collagen-Sepharose when compared with those in contact with unmodified collagen-Sepharose.

TNP-collagen-Sepharose induced greater serotonin release than did DNP-collagen-Sepharose. The extent of modification of the latter was lower than that of the former, but there was more DNP-collagen present than TNP-collagen and the amount of modified collagen was similar in both cases (section 3.5.5.). The difference in serotonin release is likely, therefore, to be due to the type of modification. Platelets should be passed through more preparations of TNP- and DNP-collagen-Sepharose in order that a statistical analysis may be carried out to verify this theory. It would also be interesting to modify collagen-Sepharose by adding nitrophenyl groups in order to discover whether serotonin release was slightly less than that obtained when using DNP-collagen-Sepharose.

If the difference in induced serotonin release is indeed due to the different types of nitrophenyl group, a possible explanation is that the trinitrophenyl group is stable, having nitro groups in all three possible positions, whereas the dinitrophenyl group will readily accept the introduction of an electrophilic group into the ortho position. Such a group may become attached from the buffer or, if the collagen fibrils do become realigned, an inter- or intra-fibril bond may be formed and cause misalignment of the fibrils. If the latter occurs, platelets would not recognise these misaligned

sections of collagen fibrils and hence the release of serotonin would be decreased in proportion to the degree of inter- or intra-fibril bonding to dinitrophenyl groups. This would explain the slightly lower serotonin release induced by DNP-collagen-Sepharose when compared with TNP-collagen-Sepharose.

The results obtained in this investigation indicate that, contrary to the reports by Wilner et al. (1968b) and Mohammad et al. (1977), the ε-amino groups are not necessary for platelet recognition of and interaction with collagen, since elevated serotonin release was induced by DNP- and TNP-collagen-Sepharose. This agrees with the findings of Wang et al. (1978a) and Whitin and Simons (1977). In the report by Wilner et al. (1971) it was noted that, when using insoluble collagen and heparinised PRP, TNP-collagen induced decreased aggregation whereas succinylated collagen had a similar platelet-aggregating activity to unmodified collagen, results in opposition to those found here. But experimental conditions were completely different, and while Wilner et al. measured aggregation, the investigation described here measured the release reaction, which would have been inhibited by the presence of heparin as the anticoagulant (Ardlie & Han, 1974).

If the theory proposed above, i.e. that succinylation of collagen in this way caused disruption of the fibrils and that di- and trinitrophenylation allowed realignment of fibrils, is correct, then these results agree with Brass and Bensusan (1974), Jaffe and Deykin (1974,1975), Muggli and Baumgartner (1973), Simons et al. (1975) and Wang et al. (1978), that the correct quaternary structure of collagen is required for recognition by platelets.

Collagen was immobilised with the intention that, being immobilised, any modification would affect only the outer groups and leave the fibrils in their original conformation. It is possible that modification caused disruption of the fibrils (and in some cases realignment) and hence the altered effect on platelets was probably due to this rather than to the modified groups themselves.

It is suggested, therefore, that the immobilised collagen should be fixed before being modified, in order to make the fibrils stable. If fixing involved crosslinking the free ε-amino groups, then when modification of such groups was carried out, only the non-crosslinked external ε-amino groups could be modified, causing no disruption or realignment of the fibrils. If other groups were crosslinked, then modification of ε-amino groups should not cause any disruption. Possible methods of fixing collagen are described by Wang et al. (1978), using ultraviolet irradiation which causes photopolymerisation and inhibition of fibril formation (Fujimori, 1965), or treatment with glutaraldehyde.

If succinylation of fixed, immobilised collagen still induced virtually no serotonin release from platelets, it would be worth investigating this further, in the field of polymer implants, with the aim of introducing implants which would be accepted by the body as "normal tissue". Polymers are used for tissue implantation and for replacement of parts of the cardiovascular system (Bagnall, 1978) and care must be taken that the implant is not rejected and that it does not cause thrombosis to occur.

In normal wound repair, scar tissue of disordered collagen fibres is formed, through which severed capillaries may be regenerated. But implants are usually encapsulated by ordered collagen fibres (which do not adhere to the implant) and capillaries cannot be restored if the implant is large and impervious (Bagnall, 1978). If the polymer implant could be coated with collagen which was modified so as not to induce thrombosis, the body might accept it as normal collagenous tissue and normal scar tissue would be formed. Similarly for cardiovascular implants, if collagen-coated polymers were used (with modified collagen in order to prevent thrombosis), they might be accepted as "normal".

Another use for immobilised collagen is as an accurate method of testing platelets for normal activity. The glass bead column is a commonly-used method of determining platelet "adhesion", but in reality aggregation is also measured. Immobilised collagen (modified or native type) could be used as a more physiological

substrate to measure platelet adhesion and release, which are closer to the initial platelet-collagen interaction.

APPENDIX

Counting efficiency of liquid scintillation spectrometer

When using a liquid scintillation spectrometer, only the emissions in a certain energy spectrum are recorded. This spectrum is different for different emitters. The presence of even small amounts of water and other solvents and proteins etc. may cause quenching, in which the spectrum of emission is shifted causing some counts to be lost. A graph can be constructed to help calculate the counting efficiency for each sample so that counts per minute may be related to disintegrations per minute. The method used was the external standard method.

A ^{137}Cs source (the external standard) is placed adjacent to the sample, causing the generation of Compton electrons in the liquid scintillant. The energy spectrum is similar to that of a β emitter (e.g. ^{3}H or ^{14}C) and shifts with changes in quenching. This shift may be seen as a change in the ratio of counts in two channels, one a wide distribution of energy (or window) and the other a narrow one. First the sample is counted with the external standard present, then the external standard is removed and the sample recounted in the same two channels. The ratio of counts in the two channels (due to the external standard alone) is calculated.

When constructing a graph for calculating counting efficiency, various volumes of a quenching agent such as chloroform or ethanol are added to a series of vials containing liquid scintillant plus a known amount of radioactive source. Ratios of the two channels are plotted against counting efficiency and the counting efficiency of subsequent samples can be found using the channels ratio which is measured for each vial.

Method

^{14}C-hexadecane ($0.526\mu Ci$ ^{14}C (g hexadecane)$^{-1}$, $\rho = 0.773 g\ cm^{-3}$ at $20^{\circ}C$) was used as a standard. Ten vials were used, each

containing 10ml scintillant/ ^{14}C-hexadecane. Counts per minute were recorded for each, then various volumes of chloroform were added and the vials replaced in the scintillation spectrometer and recounted.

Efficiency = $\frac{\text{counts per minute recorded}}{\text{disintegrations per minute of standard (activity added)}}$

Channels ratio, $\frac{B}{A} = \frac{B_1 - B_2}{A_1 - A_2}$

where A_1 = counts in sample + external standard using narrow window.

B_1 = counts in sample + external standard using wide window.

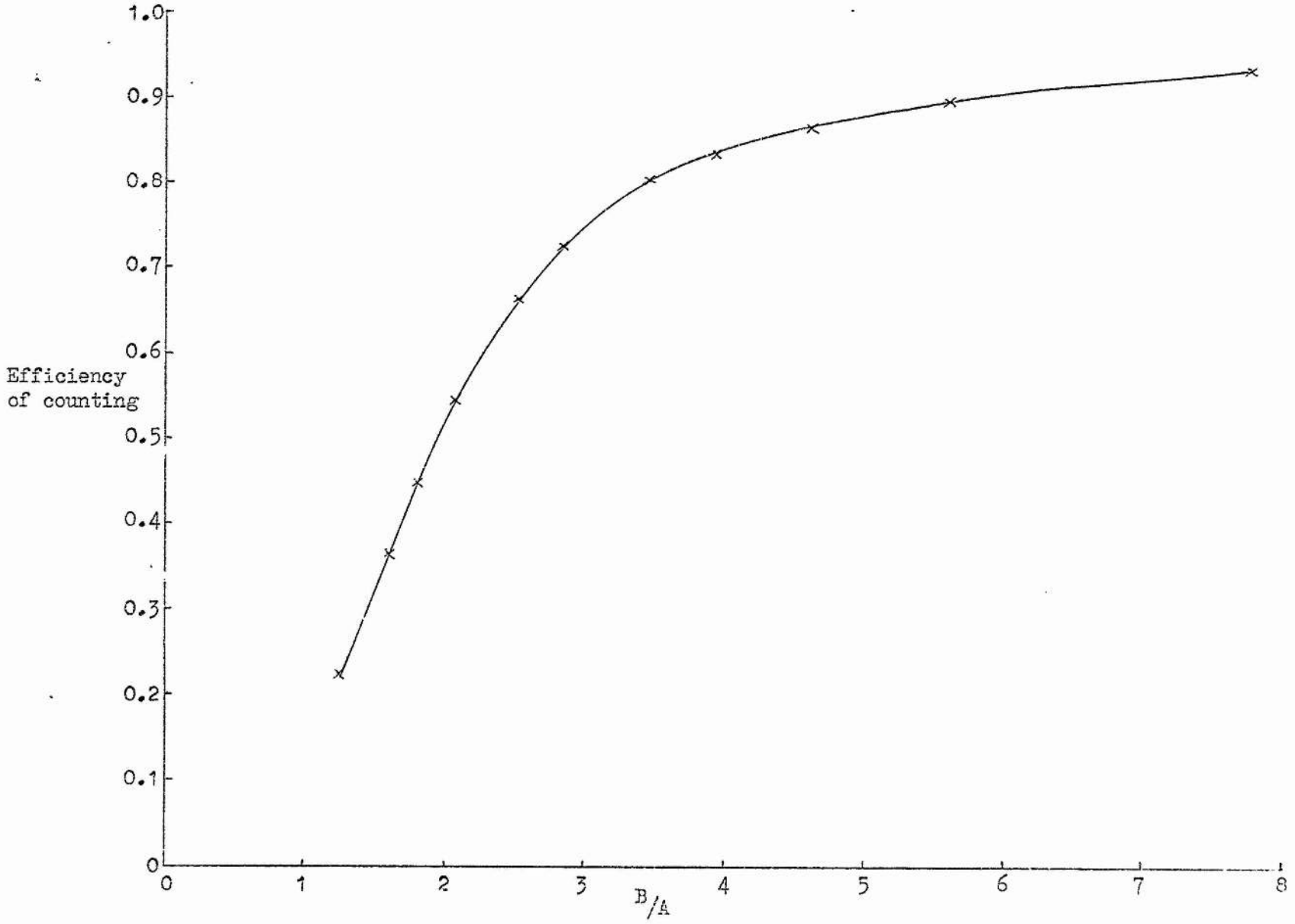
A_2 = counts in sample alone using narrow window.

B_2 = counts in sample alone using wide window.

Results and discussion

Table of counting efficiencies

ml $CHCl_3$	Efficiency	B/A
0	0.936	7.739
0.05	0.897	5.601
0.10	0.866	4.584
0.15	0.834	3.899
0.20	0.804	3.433
0.30	0.726	2.833
0.40	0.665	2.493
0.60	0.546	2.072
0.80	0.446	1.791
1.00	0.367	1.605
1.50	0.220	1.258



Efficiency curve for scintillation spectrometer

B/A was plotted against efficiency.

Experimental values for B/A for ^{14}C counting were between 6.1 and 6.3, which gave efficiencies of 91.5% - 91.7%. This was such a small difference that counts per minute rather than disintegrations per minute were used in calculations of per cent serotonin release.

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