INVESTIGATIONS INTO CHANGES IN PLATELET FUNCTION IN EXPERIMENTAL MALARIA

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ABSTRACT

In this thesis the problem of haemostatic disorders with particular reference to platelet dysfunction in experimental malaria models has been examined. In the Swiss albino mouse model infected with Plasmodium berghei (P. berghei), regular thrombocytopaenia was associated with the parasitaemia. Mild leucocytosis due to the presence of red cells in the peritoneal space and partly due to the infaction was also observed. It was found that treatment of the infection with standard dose of chloroquine sulphate for the standard 4 days was not adequate to eradicate the parasites completely, and the platelet count did not return to preinfection levels in up to 27 days. On the other hand, treatment of the infection with the same dose of chloroquine for 7 days completely cleared the parasitaemia by the end of the treatment period and platelet counts returned to baseline values by the 27th post-infection day. Coagulation parameters, such as PT and APTT were significantly prolonged in the infected animals while clottable fibrinogen was reduced when compared to control animals. A negative correlation existed between clottable fibrinogen and percentage parasitaemia. It was concluded that disseminated intravascular coagulation (DIC) complicated acute malaria in Swiss albino mice.

Platelet hypersensitivity to only low concentrations of ADP (0.5uM) but not collagen, was manifested in the infected mice PRP, although this was not accompanied by any dense granule secretory defect when stimulated with either agonist.

In the suckling rat model infected with P. berghei, platelet survival studies using ^{51}Cr-labelled homologous platelets revealed a 52% reduction in mean survival time. There was also a significant

reduction in total platelet sialic acid content in the infected animals. The decrease in platelet survival in the infected rat was attributed to a drop in total platelet sialic acid content. The latter was shown to have a negative correlation with rise in parasitaemia. It was concluded from this study that the drop in total platelet sialic acid content was probably as a result of loss of membrane rather than granule sialic acid.

In the in vitro system designed in this study, washed human platelet suspension was allowed to interact with washed cultured *P. falciparum* infected human rbcs, and the post-interaction platelet rich suspensions (PRSi) tested for changes in platelet function. The control system comprised similarly treated sham cultured erythrocytes (PRSc). Platelet hypersensitivity to low ADP concentrations (0.25μM) was observed only in the PRSi samples. This result was attributed to synergism between ADP, TxA_2 and serotonin secreted from the stimulated platelet as well as an unidentified protein "factor" demonstrated in significant amount on SDS-PAGE gel. The latter factor was heat stable at 56°C for 1 hour and was still present after thrombin inactivation.

It was concluded that the in vitro model is a suitable system to use in investigating different aspects of platelet/malaria interactions. It was also concluded from these studies that platelet dysfunction is common in acute malaria infections. Some of the mechanisms involved have been identified and the direction of further investigations identified.

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- Ally -

CERTIFICATION

We certify that the work presented in this thesis was carried out by Mr. A. L. Inyang of the Department of Pharmacology and Therapeutics, University of Ibadan.

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DEDICATION

This thesis is dedicated to:

Jenny and "Hon", my parents,

for such superlative love

and sacrifice,

and also to,

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LIST OF COMMON ABBREVIATIONS

1. ACD = acid citrate dextrose
2. ADP = adenosine diphosphate
3. ATP = adenosine triphosphate
4. APTT = Activated partial thromboplastin time
5. cAMP = cyclic adenosine monophosphate
6. ^{14}C-5HT = carbon-14 labelled 5-hydroxytryptamine
7. CP/CPK = creatine phosphate/creatine phosphokinase
8. ^{51}Cr = chromium-51 radioisotope
9. D.I.C. = Disseminated intravascular coagulation
10. EDSc = control erythrocyte derived supernatant
11. EDSp = (P. falciparum) parasitized erythrocyte derived supernatant.
12. EDTA = Ethelene diamine tetracetic acid
13. GP as in GPI = glycoprotein I
14. 5-HT = 5-hydroxytryptamine
15. n = no. of experiments
16. NANA = N-acetyl neuraminic acid
17. NS = not statistically significant
18. P = student t-test
19. P. as in P. berghei = Plasmodium berghei
20. P.C. = platelet count
21. PG as in PGH_2 = prostaglandin H_2
22. PK/PEP = pyruvate kinase/phosphoenulpyruvate
23. PPP = Platelet poor plasma
24. PRP = Platelet rich plasma

25. PRSc = shear-cultured-(control)-erythrocyte interacted platelet rich suspension

26. PRSi = (P. falciparum)-infected-erythrocyte-interacted-platelet rich suspension.

27. PT = Prothrombin time

28. r = correlation coefficient

29. SA = sialic acid (same as NANA)

30. SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis

31. $t_{1/2}$ = platelet half-life

32. Tx as in TxA_2 = thromboxane A_2

33. WPS = washed platelet suspensions

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CHAPTER ONE

INTRODUCTION

HISTORICAL REVIEW

Platelets are non-nucleated blood cells of 1.5 - 3.0um in diameter. The circulating platelet is discoid in shape and was thought to be a simple cell basically comprising the granulomere (granule concentrated area) and the hyalomere (a slightly basophilic area enclosed by the plasma membrane (Kubisz 1975, Ulutin 1976). It is now known to be a very complex structure like other blood cells.

Historically, platelets were the last of the cellular elements in the blood to be discovered. Its discovery, development of knowledge about it, and its role in haemostasis and thrombosis were closely linked with the development of the optical microscope by Van Leuwenhoek (Robb-Smith 1967, Spaet 1980) and subsequently the compound microscope (Spaet 1980). This made it possible to resolve objects at 1.0um diameter and platelets have a diameter of 1.5 - 3um. Alfred Donne (1842), a French clinical pathologist, first decribed the platelets which he called "globulins of chyle". Other contributors to this early discovery of the platelet include two English Medical Practitioners, George Gulliver (1842) and William Addison (1842) who working independently made the first drawings of the platelet, Franz Simon (1842) Friedrich Arnold (1845) Gustav Zimmerman (1846) William Osler (1874) George Hayem (1878). The latter believed that they were red cell precursors.

The culmination of these early studies was in 1882 when Julius Bizzozero, a noted Italian Haematologist distinguished the platelets from erythrocytes and leucocytes, taking pains to point out the specialized role of platelets in haemostasis and clot

formation. It was Bizzozero who gave the name "Platchen" (platelets) to these new cells (Spaet 1980). Eberth and Schimmelbusch (1885) later pointed out the relationship of blood platelets to blood clotting and described morphological changes that occur when platelets come in contact with a foreign surface or damaged vessels, mentioning that such changes enhanced their stickiness. Wright (1906) using his famous stains for blood cells, demonstrated that the platelets were produced from megakaryocyte fragmentation. This settled the issue of the platelet being different from the erythrocytes.

Although the role of the blood platelets in haemostasis and thrombosis, had been suggested by these early workers, detailed ultrastructural studies were initiated only recently by Wolpers and Rusta (1939) as this had to await the development of the more sophisticated electron microscope. With the improved resolution power of the microscope, better fixation techniques and a more sophisticated isolation procedure, the picture of the platelet ultrastructure offers a befitting explanation for its role in haemostasis and coagulation.

1.2. PLATELET STRUCTURE

The circulating platelet is a lentiform shaped cell showing no villous projections like the leucocytes but having a smooth surface contour. It has random indentitions on its surface which make up the open canalicular system (OCS, also called the surface connecting canalicular system SCCS) a communication system linking the exterior of the cell with the interior. The detailed platelet ultrastructure is described in relation to its function and includes

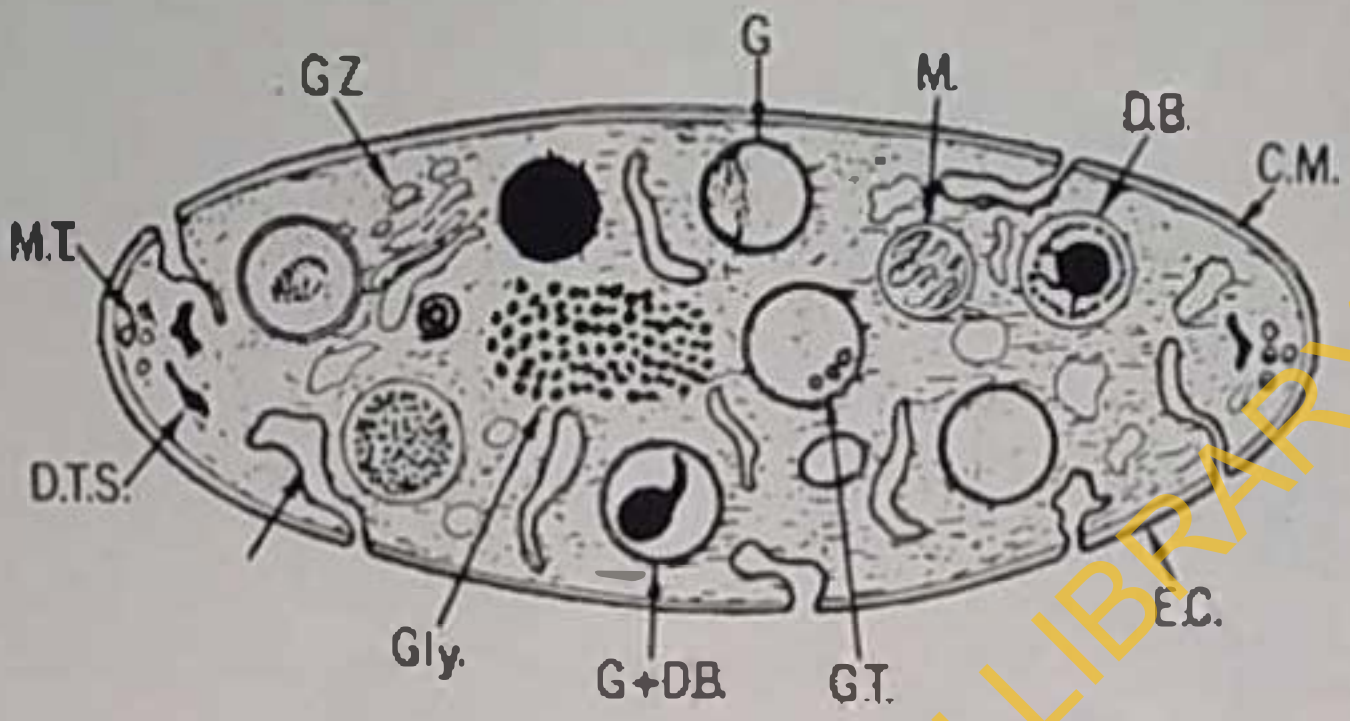
the peripheral zone, the sol-gel zone, the organelle zone and the membrane systems. (See Fig. 1 and 2).

1.2.1. THE PERIPHERAL ZONE:

This is basically involved with adhesion and consists mainly of the exterior coat, the unit membrane which is the cell wall, and the SCCS which are continuous with the cell wall.

1.2.1.1. Exterior Coat: The exterior coat or glycocalyx (White and Krivit 1965) is rich in protein antigens, enzymes and glycoproteins, the latter being considered as a component of platelet receptors. Such receptors, when triggered by stimuli, initiate platelet activation which release substrates for adhesion-aggregation reactions (Nurden and Caen 1974, 1975 and 1978). The glycocalyx is formed from a material rich in acid mucopolysaccharides and glycoproteins capable of adsorbing plasmatic proteins and cations. Recent biochemical resolution of the platelet glycocalyx, by polyacrylamide gel electrophoresis has revealed a minimum of three major glycoproteins (Glycoprotein I, Glycoprotein II and Glycoprotein III; each having subunits a and b : Phillip and Poh Agin 1977, Nurden and Caen 1978). Some of these are extremely important for platelet adhesion and aggregation (Nurden and Caen 1975). The chemical composition of this coat, is responsible for the negative charge on the platelet surface, with sialic acid and phosphates contributing a greater percentage of this negative charge.

1.2.1.2. Platelet Unit Membrane: The platelet membrane like any other unit membrane comprises a lipid bilayer rich in assymetrically distributed phospholipids and transmembrane proteins. It forms a physicochemical separation between intracellular and extracellular



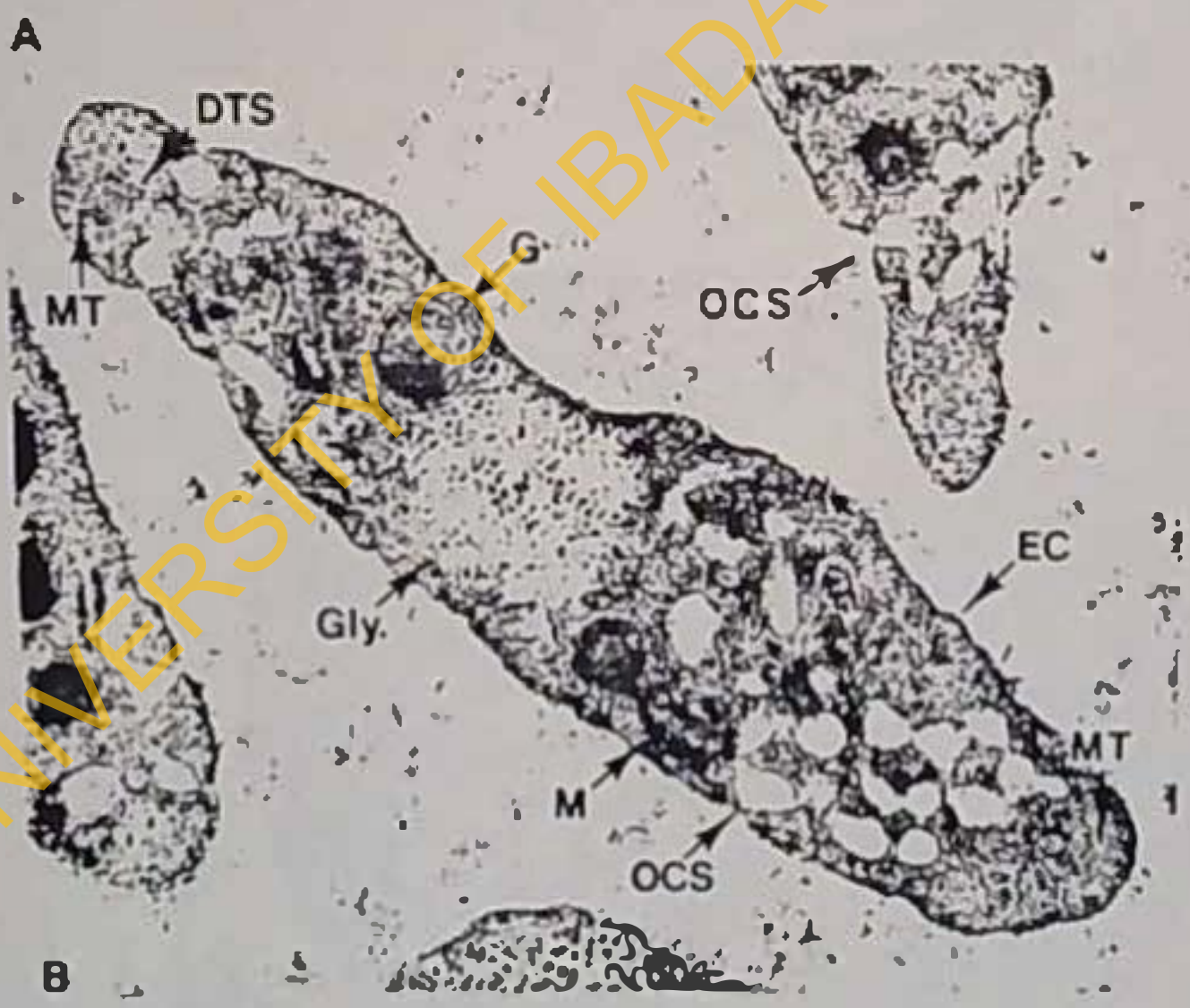


FIG. 1. Discoid platelets. The diagram summarizes ultrastructural features observed in thin sections of discoid platelets cut in cross section. A. Components of the peripheral zone include the exterior coat (*EC*), trilaminar unit membrane (*CM*), and submembrane area containing specialized filaments (*SMF*) which form the wall of the platelet and line channels of the surface-connected canalicular system (*CS*). The mass of the platelet interior is the sol-gel zone containing actin microfilaments, structural filaments, the circumferential band of microtubules (*MT*), and glycogen (*Gly*). Formed elements embedded in the sol-gel zone include mitochondria (*M*), granules (*G*), and dense bodies (*DB*). Collectively these constitute the organelle zone. The membrane systems include the surface-connected canalicular system (*CS*) and the dense tubular system (*DTS*) which serves as the platelet sarcoplasmic recticulum. (after White, J.G. and Gerrard, J.M. 1982).

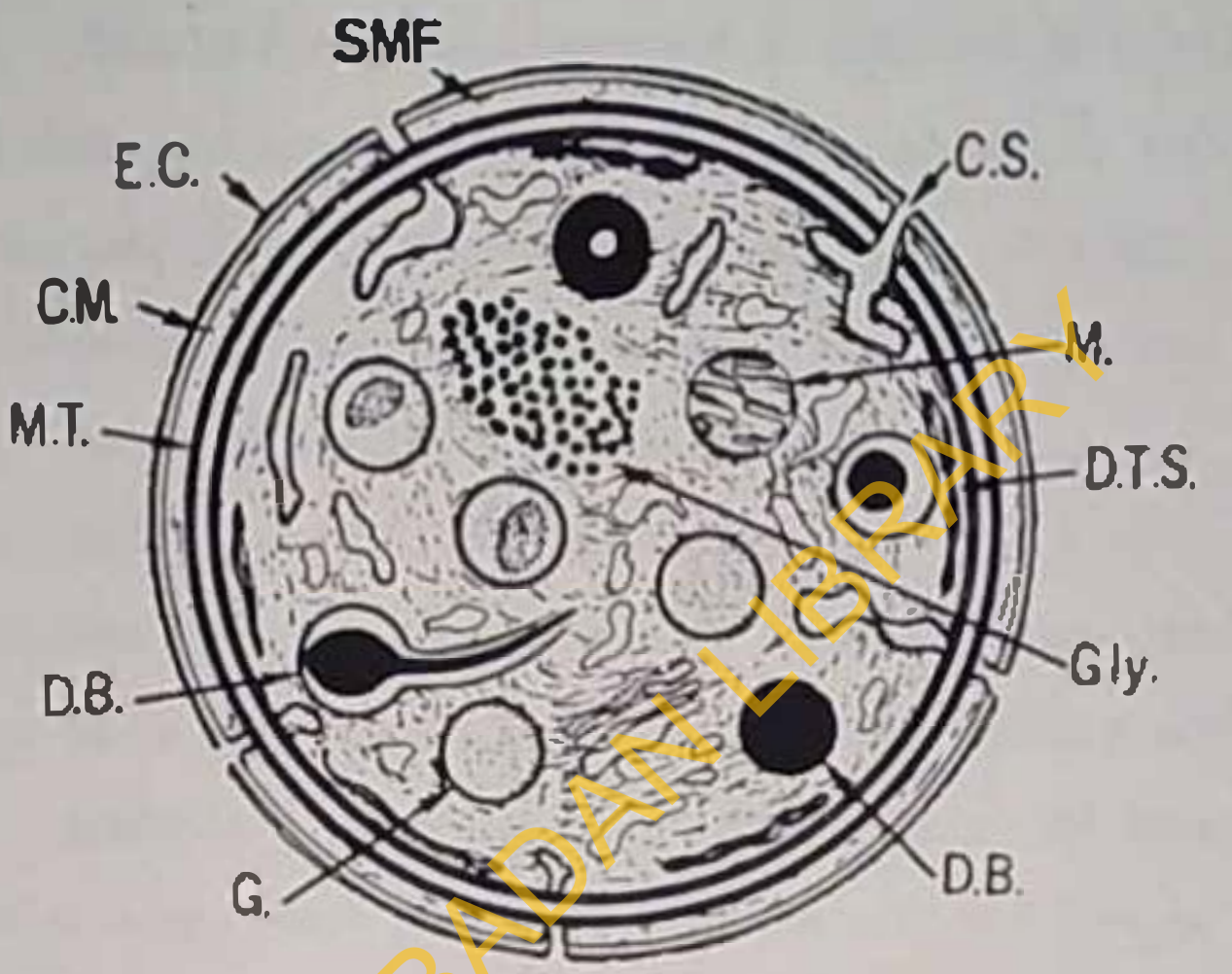


Fig. 2. A platelet sectioned in the equatorial plane which reveals most of the structures indicated on the diagram. The membrane complex (MC) is a specialized association of the *DTS* and *CS* (original magnification 2 × 30,000). *C* & *D* Discoid platelets. The diagram summarizes the structures observed in platelets sectioned in the equatorial plane and *D* demonstrates a coated pit (cp) and coated vesicle (cv). The designations for the structural features are presented above (original magnification 4 × 32,000).

(After White, J.G. and Gerrard, J.M. 1982).

constituents, and is believed to provide the essential surface for interaction with coagulant proteins such as Factor Va and Xa (Walsh 1974). The platelet plasma membrane is composed of 35-40% protein, 50% lipid and about 10% carbohydrates which are in the form of glycolipids or glycoproteins (Phillips 1983). The main platelet membrane lipids include phospholipids, glycolipids and cholesterol. The phospholipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) are particularly important, being the source of platelet arachidonic acid (AA) the fatty acid precursor of prostaglandins (Marcus 1978). Hematoside, a platelet glycosphingolipid contains 6% of the total platelet sialic acid. Most platelet proteins are in the form of glycoproteins except for the contractile proteins actin and myosin. The glycoproteins exist as peripheral proteins (which are easily isolated) or integral proteins (which cannot be easily removed) with the latter predominating and forming the transmembrane protein. The platelet plasma membrane is studied with a variety of receptors which aid in its response to physiological stimuli by such agents as prostaglandins, thrombin, collagen, arachidonic acid and adrenaline. This membrane is particularly important during haemostasis as it releases arachidonic acid (Bell et al 1979), Platelet - activating factor (Vargaftig et al 1981) and platelet - factor 3, the latter being an essential membrane activity that accelerates aggregation and coagulation (Marcus and Zucker-Franklin 1964, Hardisty and Hutton 1966).

1.2.1.3. The Submembrane Region: This is the area which lies just inside the unit membrane and is closely linked to the latter, translating

signals received on the outside surface into chemical messages and physical alterations required for platelet activation (White 1983). The submembrane region is distinct from the sol-gel zone but contains filamentous elements in a relatively regular arrangement (White 1969). The submembrane filaments aid in pseudopod formation: the maintenance of platelet shape through their interaction with circumferential microtubules (see below) and other elements of the platelet contractile system. These interact to induce clot retraction and platelet aggregation.

1.2.2. SOL-GEL ZONE:

The platelet interior presents a structureless appearance except for a few granules hence the name hyaloplasm given to this zone. Electron microscopy has revealed this zone to be basically composed of the fibrous systems including microtubules, microfilaments (and the submembrane filaments), the most prominent structure being the circumferential microtubules (Behnke 1970). The platelet cytoskeletal system is linked with the proteins of the contractile system. The microfilaments (or actin-containing filaments) and the microtubules (the tubulin containing filaments) form the platelet cytoskeleton (Nachmias 1983). The location of the circumferential band of microtubules in the equatorial plane just under the cell wall in discoid platelets suggests their participation in cytoskeletal support. This view is supported by the fact that the circumferential band of microtubules disappears when the platelet is exposed to brief chill periods (an effect that converts the platelet to an irregular shape) and reappears when the platelets are warmed to 37°C (White and Krivit 1967).

Platelet microtubules are not involved in delivering the contractile force during platelet contraction or clot retraction as their depolarization with colchicine or vinca alkaloids does not affect these activities (White 1968, 1969). Platelets have about 50% of their protein content as contractile proteins, actin and myosin forming the main blocks of the contractile apparatus. These are surrounded by "satelite" proteins which are necessary for the efficiency of the contractile process involved in the diversified function of the platelets. Platelet actin (or thrombosthenin) is the most abundant protein in the cell, accounting for 15-20% of the total platelet protein (Bettex-Galland and Luscher 1961), and exists in a molar ratio of 100 : 1 with respect to myosin (Pollard 1975). It exists in a state of dynamic equilibrium between its two forms, the monomeric G - (Globular) actin and the polymerized form the F - (Fibrillar) actin (Korn 1978). These states are regulated by proteins such as profilin (which stabilizes G-actin and is attached to this form in the resting state and dissociates on activation, Markey et al 1981) and the alpha-actin and actin-binding proteins (which enhances polymerization by detaching the profilin from the G-actin). (Schollmeyer, Rao and White 1976; Markey and Lindberg 1978).

Platelet myosin represents only 2-5% of cell protein and has a similar structure as skeletal muscle myosin. Platelet myosin contains six polypeptides, two M_r = 200,000 heavy chains, two with M_r = 20,000 light chains and another two with M_r = 16,000 light chains (Fox and Phillips 1983).

Phosphorylation of the polypeptide of M_r = 20,000 during platelet activation produces substantial activation of the actin-stimulated-ATPase-activity of the myosin heavy-chain head, which is necessary to drive the contractile process (Adelstein and Conti 1975). Phosophorylation of the myosin light chain is catalysed by the myosin-light-chain Kinase (MLCK) which contains calmodulin as part of its structure. It is activated by micromolar amounts of Ca^{++}.

Two other proteins, Tropomyosin and Troponin have been identified in the platelets. As in skeletal muscle, tropomyosin masks the reactive site on the myosin head in the resting cell but during activation, this is uncovered by a configuration change by the troponin/Ca^{++} complex leading to actin binding.

Platelet activation results in an increase of cytoplasmic Ca^{++}-levels, polymerization of actin from G-actin to F-actin, structural reorganisation of actin filaments mediated by actin-binding proteins, Ca^{2+} mediated phosphorylation of myosin chain by MCLK, further reorganisation of filaments following the gelsolin induced severing action on filaments and the deliverance of a contractile force (Fox and Phillips 1983).

Platelet actin filaments, as in the red blood cells, where they are bound to the cytoplasmic face of the membrane through an actin-spectrin - ankyrin band 3 linkage (Lux 1979), demonstrates a similar interaction with the membrane (Zucker-Franklin and Grusky 1972). However the presence of several other proteins that may be involved in linking filaments to the membrane such as ankyrin (Bennett 1979), vinculin and alpha-actin (formerly thought to be glycoprotein IIIa, Connelis and Hachmias 1984), has been demonstrated (Langer et al 1982).

Alpha-actinin has been identified in platelet plasma membrane and immunofluorescence studies suggest that it is concentrated here. A further point for actin attachment to the membrane has been suggested. This is the glycoprotein IIa-IIIb complex which serves as a binding site for fibrinogen and also to connect fibrin clots on the external surface of the platelets to actin filaments in the cytoplasm. The attachment of actin filaments to this glycoprotein complex would result in the retraction of fibrin clots; a recent study has provided morphological evidence that pseudopodial retraction occurs during clot retraction making the latter process dependent on platelet contractile proteins (Cohen, Gerrard and White 1982).

The relationship between platelet cytoskeleton and the platelet contractile mechanism is observed during platelet stimulation. While the centralization of organelles results from a constriction of the circumferential microtubules (White and Gerrard 1978), the final contractile wave results from the specialized orientation of interdigitating network of actin and myosin filaments, to produce the appropriate squeezing motion which pushes the granules to the center of the platelet, thus giving an appropriate organelle orientation for platelet secretion (White 1968).

The relative plasticity of the hyaloplasm in the unaltered discoid platelet and the rapidity with which the matrix - mainly formed by microtubules and microfilaments with their associated contractile proteins - can flow into pseudopia effecting platelet shape change, suggests a highly efficient and dynamic system capable of responding instantaneously to stimulation and ensuring a return to normalcy afterwards.

The importance of this component of the cell structure has been appropriately summarized by White and Gerrard 1982.

1.2.3. ORGANELLE ZONE:

Electron microscopy and cytochemical techniques have shown that several organelles like mitochondria, alpha-granules, dense granules, peroxisomes, liposomal granules and Golgi body-like granules are embedded in this zone. In addition, glycogen granules not bound by any membrane, are scattered throughout the matrix. Experiments using biochemical, ultrastructural and cytochemical techniques have confirmed the heterogeneicity of the alpha-granules (Breton-Gorius et al 1975, Bentfield and Bainton 1975). While peroxidase activity has been detected only on the membrane of some of these granules, others contain acid phosphatase and arylsulphatase. The majority of the dense granules do not however manifest these activities.

Experiments using platelets from Gray platelet syndrome patients have permitted characterization of specific chemical substances associated with alpha granules (Gerrard et al 1980, Packham and Mustard 1984). The chemical constituents of the alpha-dense and lysosomal granules are shown in Table 1. The other major granule content of the platelets are the dense granules. These electron dense granules which are relatively fewer in number, form the most readily releasable source of platelet secretable substances and thus are regarded as the primary secretory organelle of the platelet. Mg^{2+} and Ca^{2+} are secreted during platelet secretion (Holmsen et al 1969). It has been suggested that at the high divalent cation and nucleotide concentrations within the granules, the metals

Table 1 - Platelet granule types and their contents.

GRANULE	CHEMICAL CONSTITUENTS
ALPHA-GRANULE	Platelet derived growth factor (PDGF), Platelet factor 4 (PF4), Beta-thromboglobulin (BTG), Factor V, Factor VIII-related antigen, albumin, thrombospodin, fibrinogen, fibronectin antiplasin, alpha - antitrypsin, α_2 - macroglobulin, permeability factor, and glycosaminoglycans.
LYSOSOMAL GRANULES	Acid hydrolases, acid phosphatases, and cathepsin.
DENSE-GRANULES	Serotonin, pyrophosphate, catecholamines, Mg^{2+}, Ca^{++}, non-metabolic ADP-ATP pool, guanosine di - and tri-phosphates.

and nucleotides are present in one compartment as high-molecular weight metal - nucleotide complexes (Holmsen 1982). Hermansky - Pudlak syndrome, a storage-pool disease, is marked by the absence of dense granules in the platelet of these patients (White and Gerrard 1976) and associated with defective aggregation in vitro and haemorrhagic symptoms in vivo.

Platelet mitochondria, as in other cells are energy centres of the cell, where oxidative phosphorylation proceeds to support cell processes by contributing the platelet ATP pool. The observation that the number of mitochondria is relatively low when compared with ATP content in platelets led to the discovery that the metabolic ATP pool including that of the mitochondria and the cytosol are in dynamic equilibrium. Being a highly active cell, the platelet energy metabolic pathway is highly efficient with glucose and glycogen being the main energy sources (Akkerman 1979). This explains the presence of glycogen granules in the platelets.

1.2.4. MEMBRANE SYSTEMS:

These consist of the dense tubular system (DTS) and the open canalicular system (OCS). The OCS, as revealed by improved fixation techniques, consists of tortuous invaginations of the cell wall tunneling throughout the cytoplasm in a serpentine fashion. White (1971) has demonstrated that the open canalicular system remains patent in the activated platelets suggesting that besides serving as an increased surface area for membrane dependent reactions, OCS might also act as conduit for extruded substances during platelet release reactions.

The dense tubular system (DTS) and the OCS are randomly dispersed in the platelet cytoplasm in close association with the circumferential band of microtubules. It has been suggested that the DTS represents the smooth endoplasmic reticulum of the precursor cell and plays a role in the stabilization of the circumferential band of microtubules which supports and maintains the normal resting platelet discoid shape. The two membrane systems: the OCS and the DTS are in very close apposition although no connecting ducts link them up (White 1983). The DTS is the site for Calcium sequestration.

The importance of the membrane systems in platelet function becomes clearer when seen in relation to platelet contractile system. The OCS and the DTS play the role similar to that of the skeletal muscle's transverse tubular system and the sarcotubular systems respectively in muscles. For instance the OCS in platelet conducts signals from the platelet exterior to the interior. This triggers the release of calcium from the DTS to the cytoplasm, and the subsequent activation of the platelet contractile system. Platelet Ca^{++} - fluxes are regulated by Ca^{++}/Mg^{++} ATPase localized in the DTS (Cutler et al 1978). The activity of this calcium pump is essential in maintaining a low cytoplasmic calcium level in order to keep the microtubules polymerized such that cell shape is thus maintained. Most inhibitors of platelet function act by raising intra-platelet cyclic-adenosine monophosphate (CAMP) levels which in turn induces the sequestration of cytosolic Ca^{++} in the DTS, thus causing a reduction of platelet Ca^{++} level.

The platelet membrane systems provide an internal base for membrane-located enzymes such as peroxidases (Breton-Gorius and Guichard 1972). Studies on platelet subcellular fractions have

shown that enzymes for prostaglandin synthesis reside in both plasma membrane and the dense tubular systems (Gerrard et al 1976, Gerrard and White 1978).

In summary, although apparently simplistic in its appearance, the blood platelet has been structured to aid its essential roles in haemostasis, coagulation, and clot retraction. The presence of storage granules with various proaggregatory, procoagulant or bioactive agents (or factors), a complicated membrane network that permeates the whole cell, providing enzyme centres, receptors, egress routes for secreted substances and surfaces for membrane requiring activities, a readily activated contractile system, makes the platelet a highly efficient first line of response to vascular injury.

Some other functions of the blood platelets not directly involved with haemostasis includes growth promoting activities, its role as a scavenger, and its contribution to fighting bacterial infection. Platelet growth promoting activities involves the participation of the platelet derived growth factor (PDGF) which is released from the alpha-granules during clotting. PDGF may serve to promote wound healing since it is the most potent mitogen in serum for cells of the mesenchymal origin including fibroblast, glial cells and smooth muscle (Deuel and Huang 1984). Platelets act as scavengers in vivo, phagocytosing several kinds of particles thus removing these from the blood stream (Gordon and Milner 1976). Platelets from some animal species secrete substances with some high bacteriocidal activity from their lysosomal granules ensuring the concentration of this activity at sites of tissue damage (Gordon and Milner 1976).

1.3. PLATELET PRODUCTION

Platelets are formed when mature megakaryocytes shed their cytoplasm (Wright 1906). The mature megakaryocytes are formed in the bone marrow from megakaryoblasts which undergo maturation stages from the basophilic, granular to the thrombocytic stages (Bernard et al 1972). This process is mediated by the formation of microvesicles which coalesce to form platelet demarcation membrane system (DMS) (Behnke 1968). Within each DMS, a prospective platelet area, contains mitochondria and cytoplasmic granules. During shedding megakaryocytes align near a macrosinus, sending out cytoplasmic processses which carry the DMS. The latter undergoes attenuation and develops constrictions to release platelets into the marrow from where it gets into the blood circulation (Radley et al 1980).

Unlike in rats and rabbits where complete sequence of megakaryocyte maturation takes only about 3 days, it takes 4-5 days in man. A number of factors regulate platelet production. For instance, platelet production is increased by thrombocytopaenia and reduced in thrombocytosis. These changes are regulated by thrombopoietin, a humoral substance that regulates platelet production by megakaryocytes (Levin and Evatt 1979) through a mechanism that is yet unknown. (The levels of thrombopoetin increases in rabbits and man 2 hours after induction of acute thrombocytopaenia, Levin 1982). Platelet production has been determined using ^{51}chromium label or ^{32}P-diisoprophylfluorophosphate ($DF^{32}P$). From such experiments, platelet prooduction has been given as $20-40 \times 10^3/mm^3$/day (Ebbe 1968). Each megakaryocyte produces about 4000 platelets (Williams et al 1980).

1.4.1. **PLATELET SURVIVAL**

Studies on the distribution, lifespan and fate of human and animal platelets have been done using radioactive labels. Platelets are known to be pooled in the spleen leaving only two-thirds in general circulation (Aster, 1966). This suggests that the spleen plays a regulatory role in platelet count in peripheral blood.

Platelet survival studies have been carried out with various radioactive labels such as radioactive chromium (^{51}Cr-chromate), ^{32}P-Diisopropylfluorophosphate (DF^{32}P), radioactive carbon label of serotonin (^{14}C-5 hydroxytryptamine) and ^{111}In-indium oxime. Besides the radioactive method, Stuart et al (1975) introduced a method that uses the degree of inhibition of malondialdehyde (MDA) formation by aspirin-treated platelets to measure the proportion of circulating platelets.

These studies have given a mean value of 8-12 days as the life span of human platelet (Aster and Jandl 1964, Bittel et al 1967, Harker 1978, Heyns et al 1980). Corresponding values include 3-5 days in rats (Ginsburg and Aster 1966, Aster 1967, Winocour et al 1982, 1983) and 3-4 days in rabbits (Ebbe et al 1967, Winocour et al 1982, 1983).

Mustard (1978) recommended the use of ^{51}Cr - for platelet survival studies as it has a longer half-life (28 days) and therefore a more practicable use in studies although ^{111}In - provides a much higher platelet specific radioactivity. However questions raised concerning the use of ^{51}Cr include, cohort labelling, elution of label in vivo, red cell labelling and pH of labelling which might affect platelet activity.

Mustard (1978) showed that four major factors affect platelet survival. These include :-

(a) Vessel injury in which Ross and Harker (1976) showed that removal of endothelium and its regeneration correlated closely with platelet survival changes although it had earlier been shown that balloon catheter injury to rabbit aorta did not result in any reduction in platelet survival. Platelet survival is reduced in patients with artificial heart valves and prosthetic arterial grafts.

(b) Alteration of platelet surfaces such as partial removal of sialic acid (Greenberg et al 1975) by proteolytic enzymes plasmin, chymotrypsin and trypsin (Packham et al 1977 a, b) or by viruses such as influenza (Terada et al 1966, Scott et al 1978). These agents shorten platelet survival thus suggesting a life-span determining role for sialic acid.

(c) Platelet age, size and density have been associated with its sialic acid content, Platelet factor 4 (PF4) levels and platelet life-span. It has been shown that young platelets with higher survival are usually larger and more dense than old ones and these findings were attributed to the higher sialic acid content and PF4 level of young platelets (Greenberg et al 1977). This subsequently led to the hypothesis that loss of sialic acid could be a factor in platelet aging and eventual removal from circulation. However, some workers suggest that platelet density and size have no relation to its age (Paulus 1975). Differences in results could arise from the use of different radioisotopes or method analysis of the data obtained.

1.4.2. PLATELET SURVIVAL IN PATHOLOGICAL STUDIES:

Platelet survival determinations in a number of pathological conditions have yielded useful information on platelet activities and life span in such states. In thromboembolic and vascular diseases for instance, there is a significantly shortened platelet survival time which is indicative of increased platelet activation and platelet consumption in vivo (Harker and Slitcher 1972, Ritchie and Harker 1977, Fuster et al 1983). Shortened platelet survival has also been reported in patients following insertion of artificial heart valves (Weiley et al 1974), in some congenital disorders such as Wiscott-Aldrich syndrome, Bernard-Soulier syndrome and in thromboembolic complications of sickle cell crisis and hepatic cirrhosis (Mustard 1978). In the latter condition, reduced platelet life-span has been attributed to degradation of membrane glycoproteins (Abrahamsen 1966, Ordinas et al 1978). In immune thrombocytopaenic purpura, immune processes such as binding of antiplatelet antibodies, C3 component of complement, to platelets may be involved in reducing platelet survival in these conditions (Myers et al 1982). Smoking and fatty diets predisposes to reduced platelet survival (Mustard 1978).

1.4.3. DRUGS AND PLATELET SURVIVAL:

Extensive studies have shown that some drugs prolong shortened platelet survival in some cases, particularly those associated with vascular injury and thrombosis. These drugs include dipyridamole, sulphinpyrazone, the combination of dipyridamole and aspirin (not aspirin alone) clofibrate, ticlopidine and heparin, the latter being commonly used in the management of venous thrombosis (Packham and Mustard 1980, 1984).

E-aminocaproic acid (E-ACA) has been shown to prolong platelet survival in experimental animals fitted with indwelling aortic catheters presumably because it inhibits the generation of plasmin and hence prevents its effects on platelets (Mustard et al 1982).

1.5. PLATELET FUNCTION

Haemostasis, the process that leads to the cessation of bleeding after injury to blood vessel is a basic platelet function. Besides this, the blood platelet plays an active role in the provision of coagulant activity for blood coagulation. It also participates in the maintenance of vascular endothelial cell integrity and plays a role in inflammation and immunological responses and the response of tissues to injury (Gordon and Milner 1976). Despite its widespread involvement in these other biological responses, the ability of the platelet to respond to vascular injury is supported by its ability to adhere to damaged endothelium, change its shape, aggregate, retract clot and secrete stored biogenic materials that will support haemostasis and coagulation.

Hence, platelet function can be conveniently reviewed in terms of the following processes :-

(a) adhesion (the interaction of platelets with blood vessel wall or other foreign surfaces)

(b) aggregation (the interaction of activated platelets with one another) and

(c) the release reaction (a platelet secretory process that mobilizes more platelets into the formed aggregate)

(d) coagulation.

1.5.1. PLATELET SHAPE CHANGE:

Morphologically, the first response of the platelet to an aggregating agent such as ADP or collagen is shape change. This involves the loss of the discoid shape followed by extrusion of numerous pseudopods and centralization of granule contents. The platelet now assumes a spherical shape with multiple spiky and bulky shaped pseudopods extruding out from the cell. The long thin pseudopods contain parallel arrays of microfilaments which arise from the rapid polymerization of F-actin by actin-binding proteins. The microfilaments are attached to the membrane at the barbed end attachment sites formed by alpha-actinin or vinculin (Fox and Phillips 1983). The pointed ends of the microfilaments are attached to myosin, thus the actin-myosin interactions would pull these filaments into the central region of the platelet, contributing eventually to clot retraction. This highly regulated and specific response is essential to physiologic expression of platelet function in haemostasis (White and Gerrard 1978).

Platelet shape change occurs rapidly - within 2 seconds - after stimulation and involves, besides an increase in platelet surface area an increase in the number of exposed sialic acid residues (Motamed et al 1976). This phenomenon of shape change is independent of fibrinogen and external Ca^{++}, but may require intracellular Ca^{++} shifts (Michal and Born 1971) and activation of contractile proteins with rearrangement of circumferential band of microtubules (Shattil and Cooper, 1976). Platelet shape change can be inhibited by cyclic-adenosine monophosphate (c-AMP). The increase in platelet surface area associated with shape change involves the recruitment of new

membrane from intracellular sources such as the surface connected canalicular system (SCCS) and partial evagination of the canaliculi (Cohen 1982).

1.5.2.1. PLATELET ADHESION:

In blood circulation, the intact endothelium is basically "inert" to circulating platelets. Platelet contact with denuded endothelial surface (exposed subendothelial collagen, basement membrane, other constituents of subendothelium or damaged endothelial cell), results in its activation and this is a major trigger for in vivo platelet aggregation (Baumgartner and Muggli 1976, Packham and Mustard 1984). The mechanisms responsible for the fact that normal platelets do not interact with healthy vascular endothelium, are not completely understood. Among the mechanisms suggested are the capacity of endothelial cells to synthesize prostacyclin (from arachidonic acid (AA) through prostaglandin endoperoxide PGH2), a potent inhibitor of platelet adhesion and aggregation (Moncada et al 1976). Gimbrone (1976) also demonstrated that cultured endothelial cells synthesize heparin-like mucopolysaccharide which functioned like an anticoagulant. However, erythrocytes because of their rotational and translational movement in the blood stream (Goldsmith 1972, Goldsmith and Marino 1977) and their non uniform distribution near the vessel boundary, enhance the diffusion of the smaller platelets towards the wall, a process that may be influenced by RBC size and deformability, leading to an increased platelet concentration in the boundary layer (platelet diffusitivity). This in turn may increase the likelihood of platelet-surface/vessel wall interaction and conceivably platelet-platelet interaction. But in normal blood vessels, the reactivity of the platelets to the vessel wall is reduced

by the presence of PGI_2 and its heparin-like activity previously mentioned.

These mechanisms may account for the inertness of the vascular endothelium to blood cells. Platelet adhesion to subendothelial structures such as elastin is almost negligible except to collagen fibrils where it is most marked.

Platelet contact with collagen, basement membrane and microfibrils of exposed subendothelium induces morphological changes such as a change from its normal lentiform shape to a spherical shape and centralization of platelet organelles. The shape change is rapidly followed by protrusion of pseudopodia which makes the platelet spread in a bid to cover a wider surface area. This is associated with secretion of its granule contents into the micro-environment. In vivo, platelet adhesion is rapidly accompanied by aggregation of other platelets in the microenvironment of the adherent platelets with the formation of a haemostatic plug. Only platelets associated with the subendothelium release their alpha-granule contents (Baumgartner and Muggli 1976).

Until recently, in vitro platelet adhesion test were based on its ability to adhere to non-physiological surfaces such as glass beads in packed columns or polymer surfaces.

While the results may have some relationship to thrombosis in vivo on biomaterial surfaces, the link between such experiments and platelet adhesion to damaged vascular surfaces was uncertain. This led to the construction of an annular perfusion chamber containing blood vessel surfaces denuded of endothelial cells and perfused at a constant, though variable flow rate, for platelet adhesion studies (Cazenave et al 1973, Baumgartner and

1.5.2.2. FACTORS AFFECTING ADHESION:

Platelet adhesion is influenced by physical and biochemical factors. These include blood and fluid composition, the haemodynamics of blood circulation such as blood flow and shear rate (Baumgartner and Muggli 1976), Turitto and Baumgartner 1982). It has been demonstrated that platelet adhesion increases with shear rates (a parameter of blood flow rate). This is typically so in the large vein and arteries, although at higher flow rates as exist in the capillaries and arterioles, adhesion becomes independent of shear rates (Turitto and Baumgartner 1982). Also platelet diffusion, another physical factor that influences platelet collision with vessel wall, is dependent on haematocrit and shear rates. The influence of higher haematocrit can account for the higher adhesion observed in whole blood than platelet-rich plasma on subendothelium or glass bead columns (Hellem 1960). This effect, previously accounted for as due to the release of ADP by red blood cells, has since been recognised as a physical, as red cell ghosts added to PRP gave similar results as whole blood (Zucker et al 1972). However at higher shear rates (above 800 s^{-1}) and haematocrit (above 40%), a biochemical influence becomes involved (Turitto, Muggli and Baumgartner 1977).

Unlike platelet aggregation, adhesion to vascular endothelium is independent of metabolic energy as incubation of platelets with antimetabolite (2-deoxyglucose or antimycin) did not reduce platelet binding to collagen (Lyman et al 1971).

Several biochemical factors influence platelet adhesion. The observation that there is reduced platelet adhesion in Bernard Soulier syndrome patients has led to the finding of a deficient glycoprotein Ib

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and accounts for the resultant bleeding tendency (Hurden, Dupuis, Kunicki and Caen 1981). Also in von Willebrand disease (vWd), the poor platelet adhesion to subendothelium observed has been attributed to a reduced level of the plasma factor VIII: von Willebrand factor (vWF) (Tschopp, Weiss and Baumgartner 1974). However, this is only observed under conditions of high shear rates as such as occur in the microcirculation (Baumgartner, Tschopp and Meyer 1980). Jaffe et al's (1974) demonstration that Factor VIII: vWF activity is produced in the endothelium, explains the observation that adhesion to the subendothelium is dependent upon the concentration of this factor bound to the subendothelial surface (Sakariassen et al 1979).

Calcium is involved in platelet adhesion to collagen or the subendothelium as platelet adhesion in the presence of calcium chelators, e.g. ethylene diamine tetraacetic acid, is greatly reduced although not completely prevented (Kinlough-Rathbone et al 1980). It is also worth noting that aspirin and other non-steroidal anti-inflammatory drugs (NSAID) did not interfere with platelet adhesion to collagen, neither do they inhibit platelet release reaction from those platelets directly attached to collagen fibrils (Kinlough-Rathbone et al 1980).

1.5.2.3. MECHANISMS OF PLATELET ADHESION:

The current concept of the mechanism of platelet adhesion is that fibronectin, a high molecular weight glycoprotein found in the plasma and alpha-granules of platelets (Benausan et al 1978, Zucker et al 1979) is involved in the process. Two forms of fibronectin have been identified

i) the cold-insoluble globulin (C.I.G.) of plasma; and

ii) the large external transformation sensitive (LETS) type, which is membrane bound.

The CIG fibronectin has been implicated in the adherence of platelets to collagen (Bensusan et al 1978) as it was found to be the most abundant protein attached to the collagen fibrils after disruption, by sonification, of the adherent platelets. The binding of fibronectin to the cell surface may occur through gangliosides on the cell surface.

1.5.3. PLATELET AGGREGATION:

Aggregation is one of the principal responses of platelets to vascular injury. The haemostatic role of the blood platelet is largely dependent on its ability to adhere to exposed subendothelium and to one another (i.e. aggregate) in the event of vessel wall damage. Platelets also respond to specific in vivo aggregation-inducing stimuli such as adenosine diphosphate, thrombin, prostaglandin endoperoxides (PGG2 and PGH2), thromboxane A_2 (TxA_2), platelet activating factor (PAF), and adrenaline. These substances act at defined receptor sites on platelet membrane (Mills and MacFarlane, 1976). Platelet aggregation is greatly enhanced by the release of proaggregatory substances stored in its dense - and alpha-granules. Platelet surface is studded with a number of glycoproteins that form receptors for these aggregatory agents. The platelet membrane therefore plays an important role in the reception of these external stimuli, the transmission of the impulses generated into the cell interior and in the execution of the platelet response which involves shape change, adhesion and aggregation.

Primary platelet aggregation follows within seconds of the shape change. It involves the response to stimulus through a cascade of events that requires metabolic energy, platelet membrane changes that lead to exposure of fibrinogen receptors, calcium fluxes and

biochemical synthesis, all of which lead to increased platelet stickiness. Platelet aggregation, differs from agglutination - a physical process - as the latter does not require metabolic energy or divalent cations (Kinlough-Rathbone et al 1983). Ristocetin, an antibiotic, agglutinates platelets provided vWF is present and this may be through the formation of electrostatic bridges between platelets (Howard 1975). Ristocetin induced agglutination may be followed by secondary aggregation due to ADP, PGG_2 and/PGH_2 and thromboxane A2 synthesis and release.

1.5.3.1. MEASUREMENT OF AGGREGATION:

Born (1962) designed an optical method for the measurement aggregation of platelet-rich plasma (PRP) or suspensions of isolated platelets in artificial media (O'Brien 1962; Mustard and Packham 1970). This is a technique that measures changes in light transmission (optical density) of PRP or washed platelet suspensions (WPS) on addition of an appropriate stimulus. The equipment, the aggregometer has a recorder that gives a typical platelet aggregation curve as shown in Fig. 3. The platelets are stirred rapidly and maintained at 37°C (for human platelets) for optimal aggregation. Following addition of an aggregation agent, aggregation starts, resulting in a decrease in the number of free platelets, hence an increase in light transmission occurs and is recorded. Depending on the strength of the stimulus, aggregation may reach maximum as indicated by no further increase in light transmission and remains in this position for an indefinite period. If a weak agonist is added to the suspension or PRP, aggregation will be followed by a decrease in light transmission as platelet aggregates break up (Fig. 3, 1) thus

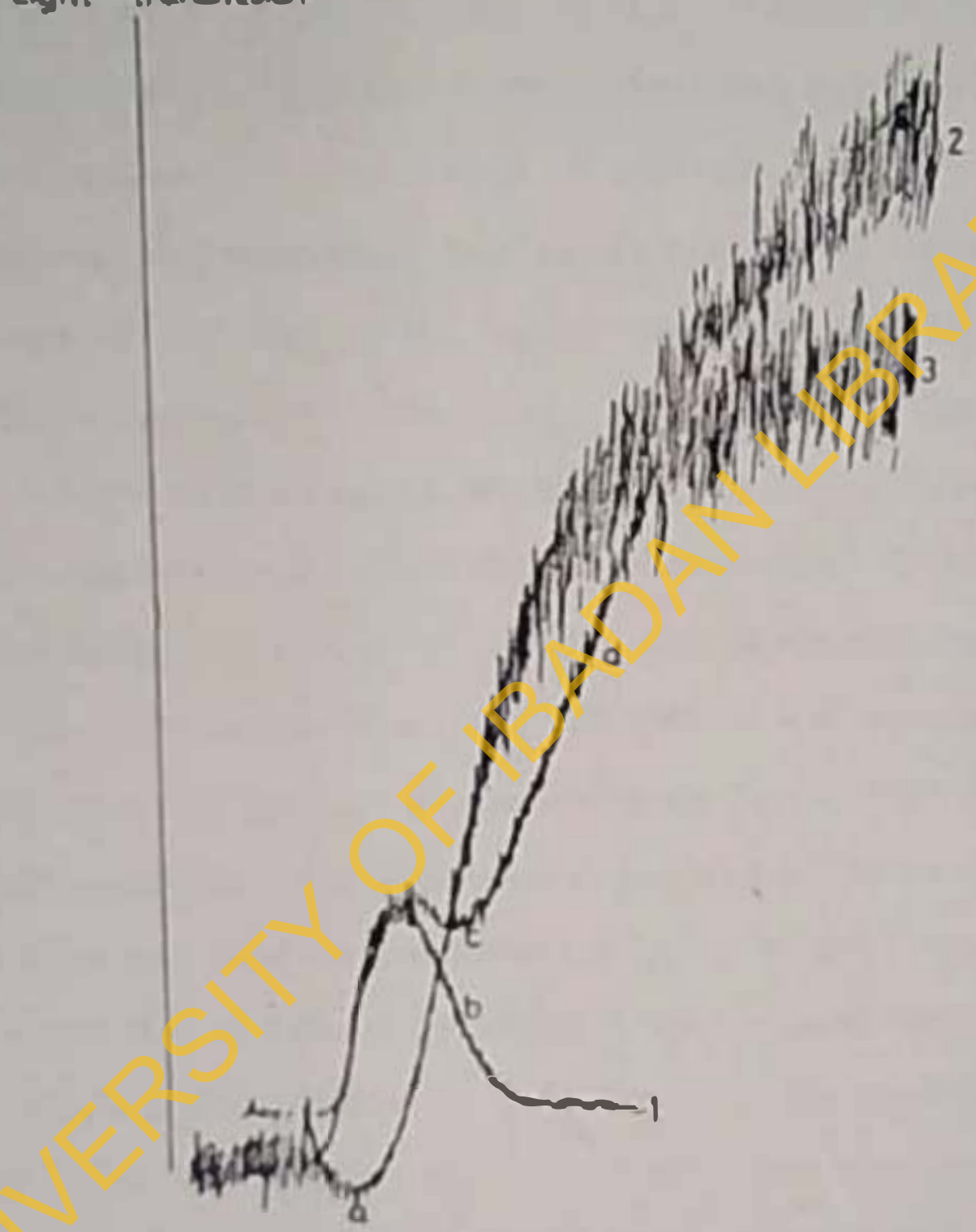

Fig. 3. Typical platelet aggregation curves
(see text for details)

increasing the number of free platelets in suspension. The first wave is called the primary aggregation wave.

Aggregation can be followed by a second wave (Fig. 3,2) indicated by a further increase in light transmission which reaches a second plateau (Packham, Kinlough-Rathbone and Mustard 1976). This often results from release of platelet dense granule proaggregatory substances such as ADP and 5 - HT (Mustard and Packham 1970). This is the "second phase" or secondary wave of platelet aggregation (Born 1962). It is normally irreversible.

Other methods have been described by Wu and Hoak (1974) in which aggregation in whole blood is determined. In one of these, blood (0.5ml) is collected directly into a syringe containing 2ml of EDTA + Formaldehyde solution (0.77%EDTA + 4% Formalin) which fixes any circulating platelet aggregate immediately and another equal volume of blood into syringe containing EDTA solution only (0.077M EDTA made up in phosphate buffered saline). This method assumes that platelet aggregates in vivo break up on standing or as a result of manipulation. In a formalin/EDTA solution the aggregate are fixed immediately. Blood smear made from both samples are used in counting aggregates microscopically. Platelets may also be counted by a Coulter or other electronic counter. The platelet aggregate ratio is estimated thus:

$$\text{Platelet aggregate ratio} = \frac{\text{Platelet count in EDTA-Formalin-PRP}}{\text{Platelet count in EDTA PRP}}$$

Where this ratio is less than unity it is implied that platelet aggregates were present in vivo.

Recently, Velaskar and Chitre (1982) described a method of measuring platelet aggregation in whole blood using the light microscope without ignoring the hyperactive platelets which aggregate either in vivo or during blood collection. The new test takes into account circulating platelet aggregates and measures the response of total platelet population to an aggregating agent at a specific time interval determined after fixing the whole blood and calculating the ratio of aggregates to free platelets from a thin smear made from it.

Another relatively recent method, uses the whole blood aggregation module that measures platelet aggregation in whole blood (Cardinal and Flower 1980). The aggregation patterns of this method correlates with that of PRP. The principle of whole blood aggregation is based on the impedance approach which depends upon changes in electrical conductivity once a monolayer of platelets coats the electrode. These changes in conductivity is recorded on a paper. This method has the added advantage of quick testing as preparation of PRP is eliminated thus allowing a total platelet population aggregation activity to be observed in its natural environment. However, the impedance tracing gives no information on "shape change".

1.5.3.2. ANALYSIS OF AGGREGATION CURVE:

Fig. 3 shows typical platelet aggregation curves obtained from the aggregometer that employs the changes in optical density principle. Curve 1 illustrates a typical aggregation curve obtained when a low concentration of ADP (0.5uM) is used as the agonist. Random oscillations are recorded prior to platelet stimulation and are attributed to normal discoid plateletmovement when stirred.

Immediately after the addition of aggregating agent, there is a fall in light transmission (see section (a) of Curve 3) and a decrease in amplitude of oscillations. This fall in light transmission is due to platelet shape change from discoid to a spherical form with pseudopod formation (Adrenaline causes a less extensive shape change; Packham and Mustard 1984). This causes a decrease in the transmission of incident light.

Primary aggregation normally observed with low ADP concentration is accompanied by deaggregation (see section b of Curve 1) and is represented on the tracing as a fall in transmission. This is due to the dissociation of fibrinogen from the binding sites which were unmasked by the aggregating agent (Kinlough-Rathbone et al 1983).

With slightly higher or moderate concentration of ADP (e.g. 1-2uM) type 2 curve is obtained. Here the primary aggregation wave plateaus at (c) and is followed by a secondary wave (d) which has a higher amplitude of oscillation and proceeds to maximum. The continued increase in light transmission and amplitude of oscillation recorded is due not only to large aggregates formed but also to contraction of these aggregates through clot retraction. Type 2 curve is associated with close contact activation observed in PRP prepared from citrated-blood (Packham and Mustard 1984).

The type 3 curve (see Fig. 3) is obtained when higher concentration of ADP(5-10uM), thrombin 4U/ml or collagen (5 ug) is used as the agonist. The inflexion at point c on curve 2, disappears as the primary and secondary curves blend to give a single curve. This portion of the curve is associated with a sharp rise in oxygen uptake from the surrounding medium. This "oxygen burst" is utilized for the synthesis of prostaglandin endoperoxides and thromboxane A_2 (TxA2) all of which enhance platelet aggregation (Hamberg et al 1974, Marcus 1978, 1983).

1.5.2.5. MECHANISM OF PLATELET AGGREGATION:

Platelet aggregation appears to occur through three mechanisms that are basically interrelated. These are the ADP mechanism, thromboxane mechanism, and a third as yet undefined mechanism (Vargaftig et al 1981, Kinlough-Rathbone et al 1977, Packham and Mustard 1984). Detwiler, Charo and Feinman (1978) suggested that all agonists seem to act via a common pathway of calcium-mobilization from dense tubular system to the cytoplasm (see Fig. 4 a,b). Calcium mobilization from intracellular stores are enhanced by products of prostaglandin endoperoxides and thromboxane A2 which act as calcium ionophores (Gerrard and White 1981).

Vargaftig, Chignard and Benveniste (1981) have summerized that platelet aggregation occurs through three major pathways named above. The strength of an aggregating agent reflects on which pathway it is capeble of stimulating. Weak stimuli such as adrenaline, serotonin and low ADP levels (0.5uM ADP) normally cause only the primary wave of aggregation through the ADP pathway. Collagen (2.5ug or 5ug/ml) and low concentration of thrombin (1-2U/ml) can induce release of ADP and synthesis of prostaglandin endoperoxides/ thromboxane A2. High concentration of thrombin (4-8U/ml) and A23187 stimulate all three pathway.

(a) ADP - Pathway: Since the discovery of ADP as a factor present in erythrocytes that can cause platelet aggregation by Gaarder et al 1961, this substance has been implicated in the main platelet aggregatory pathway. The source of platelet ADP used in aggregation has been shown by Day and Holmsen (1971) to be released from

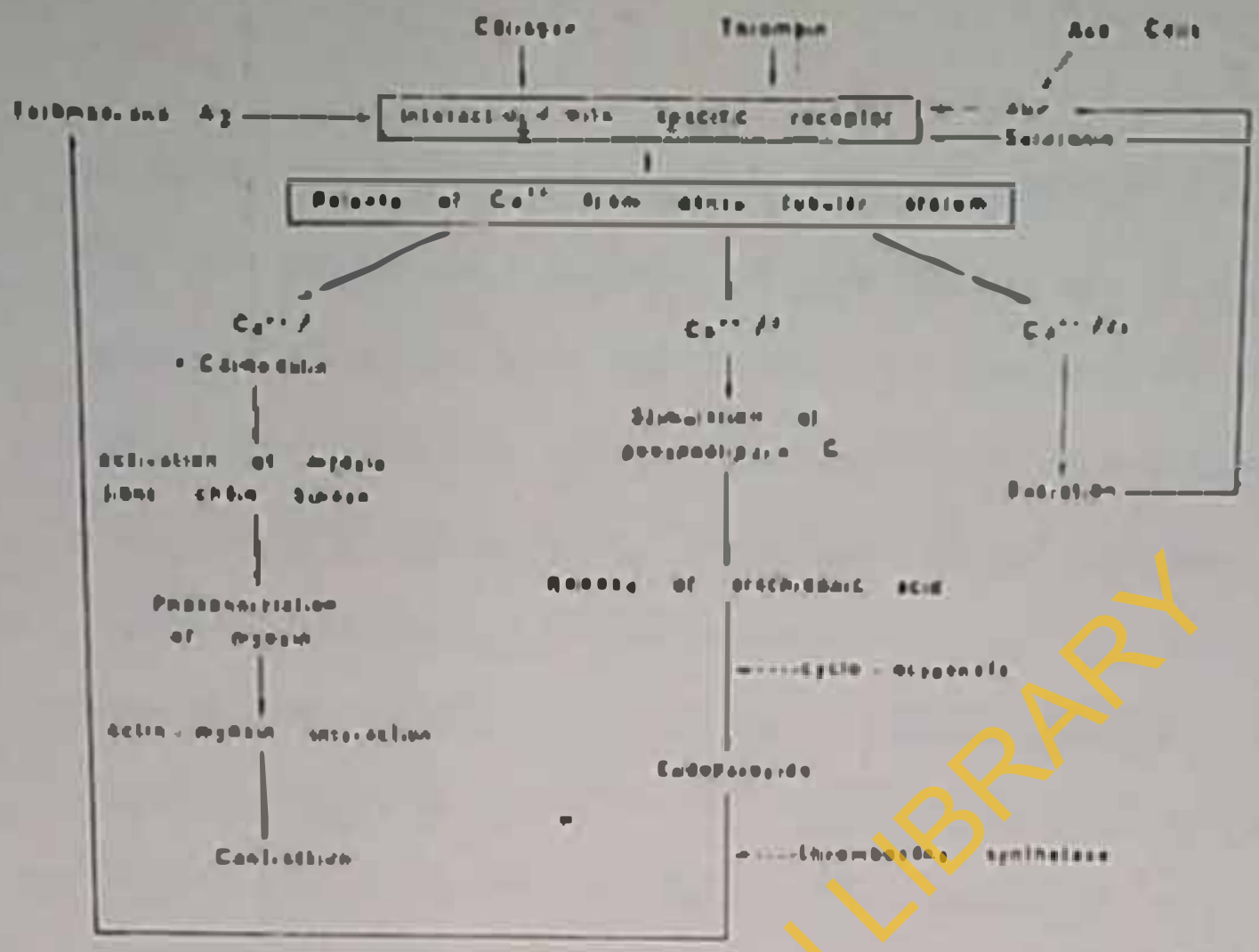

Fig. 4a. A simplified scheme of platelet activation (After Vermylen et al 1982).

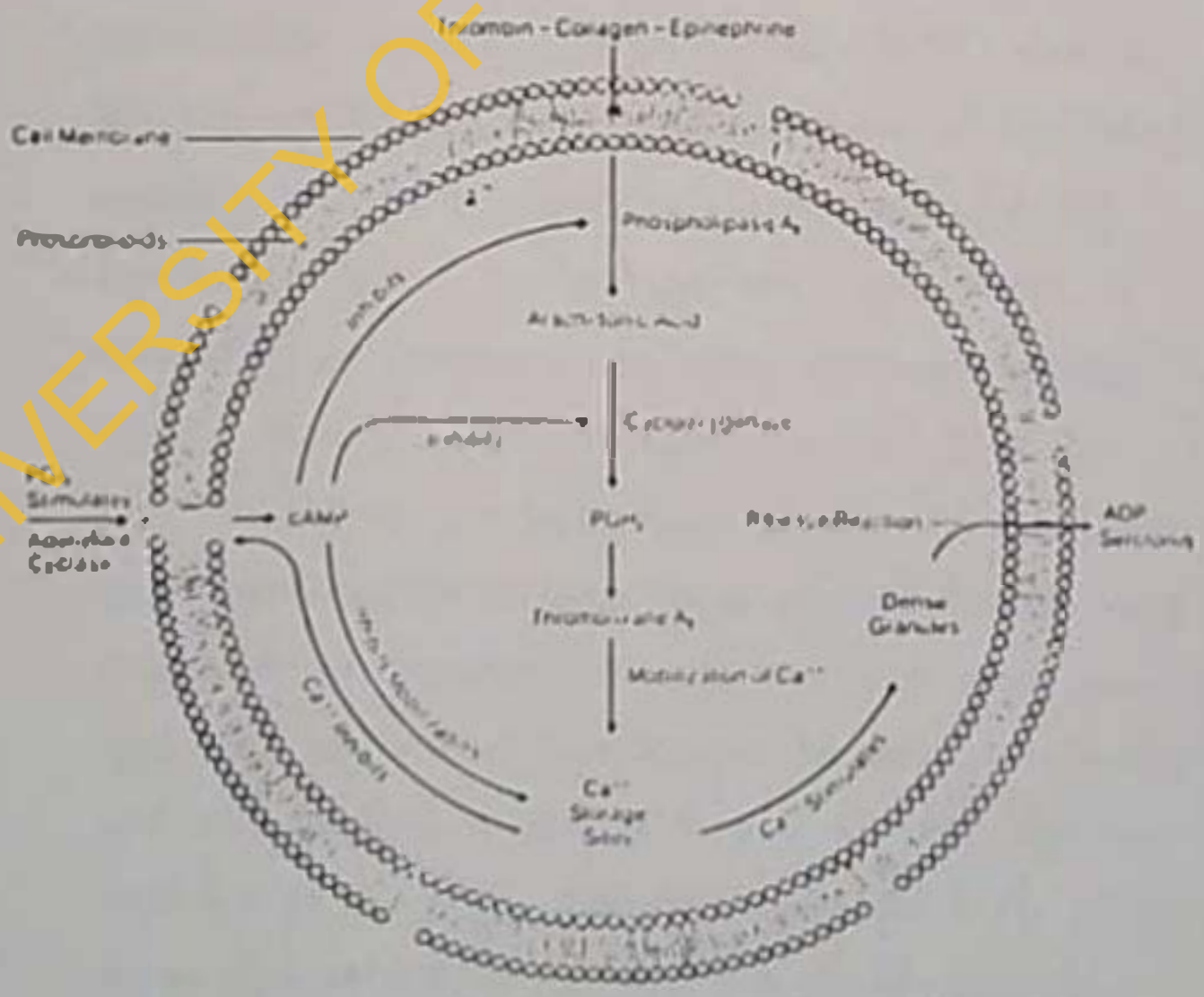

Fig. 4b

platelet dense granules together with ATP and 5-HT.

The role of ADP as a mediator of aggregation due to other agents such as collagen and thrombin is supported by its (ADP) dose dependent release during platelet stimulation (Grette 1962, Hovig 1963). Scavengers of ADP such as creatine phosphate/creatine phosphokinase (CP/CPK) and Phosphoenulpyruvate/pyruvate kinase (PEP/PK) reduce platelet aggregation due to ADP (Haslam 1964, Packham et al 1977). Other workers have shown that thrombin and vasopressin platelet stimulation also produce a secondary wave of aggregation with ADP secretion (Mills of al 1968, Haslam and Rosson 1972). However, evidence for other aggregatory pathways stemmed from observations that nonsteroidal antiinflammatory agents (NSAIA) such as aspirin could inhibit the secondary wave of ADP-induced aggregation, the accompanying release reaction and aggregation due to low collagen and low thrombin concentration (Packham et al 1977, Kinlough-Rathbone et al 1977). Several lines of evidence support this view.

Platelets that lack releasable ADP (either those that have been depleted of their dense granule content by prior treatment with thrombin or those that lack dense granules) aggregate in response to collagen, thrombin, arachidonate, and the ionophore A23187 (Reimers et al 1976, Kinlough-Rathbone et al 1977). Also Macfarlene and Mills (1975) have shown that adrenaline -, vasopressin - or serotonin-induced primary wave of aggregation was not inhibited by ATP, an agent that inhibits ADP-induced - aggregation.

The observed effects of ADP on platelets are mediated through ADP receptors(Haslam and Cusack 1981). Characterization of ADP receptors mediating platelet activation have been studied by Colman and Figures (1984). Haslam and Cusack 1981) had earlier shown that the ADP receptor that mediates aggregation is different from that which inhibits raised CAMP levels produced by prostaglandin E_1, D_2 and I_2. However a correlative evidence suggest that the same receptor which mediates aggregation initiates the metabolism of phosphatidylinositol and Ca^{2+} mobilization on stimulation (Haslam and Cusack 1981). Coleman and Figures (1984) suggest a structural and functional coupling of the ADP-receptor to the contractile process. They also implicated a single protein with M_r = 10,000 as the ADP receptor.

(b) The Arachidonate Pathway: Several observations have led to the implication of prostaglandin endoperoxides (PGG_2 and PGH_2) and thromboxane A_2 (TxA_2) as mediators of platelet aggregation. These include the finding by Smith and Willis (1972) that prostaglandins (PGs) are generated during platelet aggregation. In platelets that adhere to collagen or interact with thrombin (or other release inducing agent) phospholipase A_2 and C are activated. While phospholipase A_2 releases arachidonic acid from platelet membrane phospholipids (Phosphatidylinositol (PI) phosphatidylcholine (PC) and phosphatidylethanolamine (PE): Blackwell et al 1977, Marcus 1978, Brockman, Ward and Marcus 1980), the combined activity of phospholipase C, diglyceride- and monoglyceride

lipase cleaves arachidonate mainly from PI (Bell, Kennerly, Standford and Majerus 1979, Lapetina and Cuatrecasas 1979). The freed arachidonate is converted by cycloxygenase to PGG_2 and PGH_2 which are then converted to thromboxane A_2 by throoboxane synthetase (see Fig. 4a and 4b).

Lipoxygenase could also convert the arachidonate to HPETE and HETE (Mugteren 1975: see Fig. 5). The PGG_2, PGH_2 and TxA_2 are the agents responsible for platelet aggregation when this pathway is stimulated (Hamberg et al 1974, 1975) and their synthesis can be inhibited by agents that inhibits platelet synthesis such as NSAIA.

Other products formed in platelets from prostaglandin endoperoxides are PGD_2, PGE_2, $PGF_{2\alpha}$, Malondialdehyde (MDA) and 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) (see Fig. 5). PGD_2 in some species are strong inhibitors of platelet aggregation and release reactions as they stimulate adenylate cyclase thus elevating CAMP levels (Smith, Silver, Ingerman and Kocsis 1974).

The activation of PG synthesis by high strength aggregating agents such as collagen, thrombin, or A23187 suggested a role for these substances (PG) in platelet aggregation. However, observation that aggregation inhibition using cycloxygenase inhibitors such as aspirin, can be overcome by increasing the dose of thrombin or A23187, suggests the involvement of yet another pathway in platelet aggregation (Kinlough-Rathbone et al 1977, Packham et al 1977). Packham, Puccione, Greenberg, Kinlough-Rathbone and Mustard (1977) demonstrated that the combination of enzymatic systems

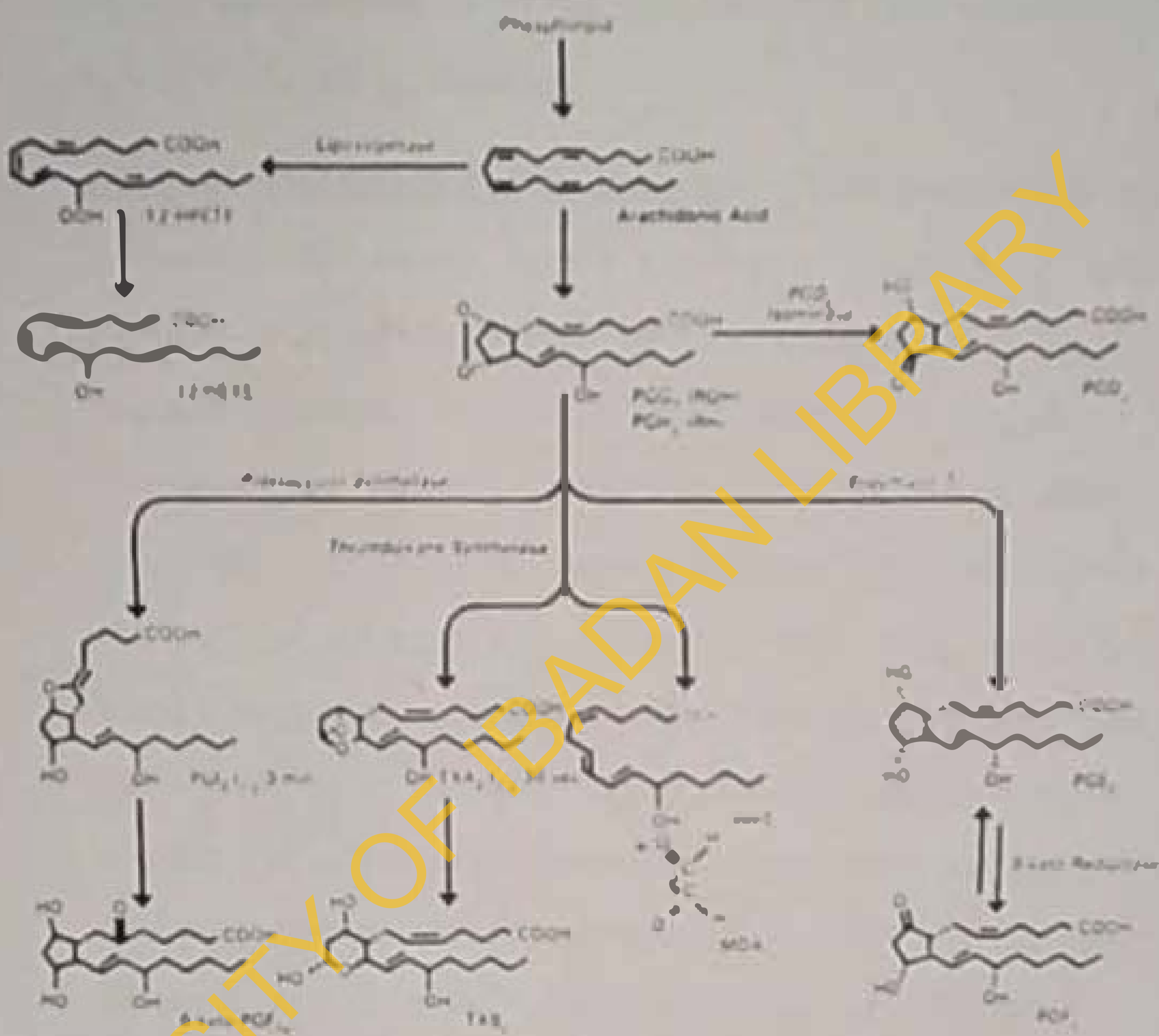

Fig. 5. Pathways of arachidonic acid metabolism. In platelets arachidonic acid is metabolized by two different enzymatic mechanisms. The lipoxygenase step results in formation of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 12-hydroxyeicosatetraenoic acid (12-HETE). The lipoxygenase can be inhibited by 5,8,11,14-eicosatetraynoic acid (ETYA) but not by non-steroidal anti-inflammatory compounds such as aspirin. The platelet cyclo-oxygenase forms endoperoxide PGH2 from free arachidonic acid. The endoperoxide is then converted to TXA2 and the various other products shown. Normally platelets produce very little of the 'classical' prostaglandins (PGF2α and PGE2). PGD2 is produced in brain tissue. In endothelial cells the lipoxygenase pathway is not very active but large quantities of PGI2 are produced. Endothelial cells also synthesize considerable quantities of PGF2α, PGE2, PGD2. Human endothelial cells do not produce thromboxane.

(After Marcus, A.J. 1983).

which destroy ADP and aspirin or indomethacin also failed to inhibit aggregation to high concentration of thrombin. This suggested that besides the ADP- and TxA_2 pathways, a third one still existed.

(c) Platelet-Activating Factor Pathway: This phospholipid (1-O-alkyl-2-O acetyl -2sn - glyceryl - 3 phosphorylcholine, platelet activating factor (PAF) is a component of the platelet membrane (Vargaftig et al 1981). It is known to be released from neutrophils and basophils following immune challenge. Pinckard and his co-workers (1982) elucidated its biology and chemistry and showed that it is a highly stable substance. Several workers have demonstrated that PAF - acether is released during platelet aggregation by thrombin or A23187 (Chignard et al 1979, Namm and High 1980, Benveniste et al 1982). It induces platelet aggregation by mobilization of Ca^{++} from its stores. It has been implicated as being the third pathway for platelet activation by strong stimuli (Vargaftig et al 1981).

1.5.2.6. ROLES OF CALCIUM, FIBRINOGEN MEMBRANE GLYCOPROTEINS AND PHOSPHOLIPID CHANGES IN AGGREGATION:

(a) Role of Calcium in Platelet Aggregation: Both external (plasma) and internal (platelet cytosol) changes in Calcium levels alter platelet response to stimuli (Packham and Mustard 1984). In the plasma, where the concentration of calcium is 3mM and that of free ionized calcium is about 1mM, calcium chelators such as EDTA or EGTA in high enough concentration to remove all divalent cations, inhibits

platelet aggregation although platelet shape change and release reactions could still be induced with agents such as A23187 (Charo, Feinman and Detwiler 1977). Mustard and his group (1978) observed that fibrinogen receptors are not unmasked when external calcium has been chelated. However it remains unknown whether calcium is directly involved in the binding of fibrinogen to platelets, although it is involved in the formation of the glycoprotein IIb-IIIa complex that forms the fibrinogen receptor on platelet stimulation. Calcium is also involved in the binding of thrombospondin, a platelet endogenous lectin involved in platelet surface (Phillips, Jenning and Prasana 1980; Jaffe et al 1982). Lowered plasma calcium levels result in enhanced platelet response to stimuli and the consequent stimulation of the arachidonate pathway that follows close contact activation (Mustard, Perry, Kinlough-Rathbone and Packham 1975).

The concept that calcium acts as an intracellular second messenger is supported by the observation that platelet responses of shape change, aggregation and release of granule contents are all dependent upon mobilization of calcium from their sequestration sites into the cytosol. This normally produces a hundred-fold increase in cytosolar calcium ion concentration (Holmsen 1974; Gerrard and White 1978; Rink, Smith and Tsien 1982). The finding that calcium ionophores cause platelet shape change, aggregation and release of granule content provides strong support for this view.

Further support is that most enzymes such as phospholipase A_2 and C, glycogen phosphorylase b, and myosin light chain kinase (which phosphorylates the 20,000 dalton light chain myosin) are all activated by micromolar calcium. Platelet contractile functions are also known to be regulated by calcium fluxes (Packham and Mustard 1984).

Various workers have demonstrated that agents which inhibit calcium mobilization either by affecting calcium binding activity of calmodulin (such as trifluperazine, a local anaesthetic) or those that elevate intracellular cyclic AMP levels (such as prostaglangins I_2, D_2 or E_1 Fig. 4b) are all strong inhibitors of platelet aggregation. However, platelet dense granule calcium are released to the extracellular medium during platelet secretion.

(b) Role of Fibrinogen in Platelet Aggregation: Current evidence suggests that aggregating agents such as adrenaline, collagen, arachidonate or thrombin (Plow and Marguerie1980; Harfenist et al 1982) but not ADP may induce fibrinogen binding independently of released ADP. This suggests a broader role such as forming the link between adjacent platelets for fibrinogen during aggregation. The inhibition of thrombin induced aggregation by Fab fragments of an antibody to fibrinogen supports this view (Tollefsen and Majerus 1975). However, only the role of fibrinogen in ADP- induced aggregation has been extensively studied. It has been well established that prior to stimulation, the platelets do not bind fibrinogen, but have their fibrinogen receptors unmasked

after ADP stimulation (Mustard et al 1978). Platelet bound fibrinogen dissociates during deaggregation (Kinlough-Rathbone et al 1983). Machean and Leung (1980) have shown that a complex of glycoproteins IIb and IIIa and calcium forms the fibrinogen receptors on platelet membrane which can be inhibited by amino sugars such as glucosamine and galactose (Kinlough-Rathbone et al 1984). The poor aggregation or fibrinogen-binding observed in thrombasthenic patients results from the markedly reduced or totally absent glycoprotein IIb and IIIa in platelets of such patient. A similar effect is observed in afibrinogenaemia is a reflection of the reduced levels of fibrinogen in such instances (Packham and Mustard 1984), although other views have been expressed (Caen, J.P. Personal Communication).

(c) Role of Platelet Membrane Glycoproteins in Aggregation:

Platelet membrane glycoproteins play an essential role in platelet surface interaction such as occurs in haemostasis and blood coagulation. They are involved in adhesion, aggregation, binding of agonist to receptors on platelets and recently have been implicated as serving as anchor for fibrin - microfilament attachment during clot retraction (Fox and Phillips 1983). Their importance has been further strengthened by the characterization of certain diseases such as Glanzmann's thrombasthenia and Bernard Soulier syndrome where defective membrane glycoprotein is the underlying disorder (Phillips 1980).

George and Lewis (1976), using SDS- gel electrophoresis, characterized platelets membrane glycoproteins into three major classes. These are glycoprotein I (with subunits Ia, Ib,

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George and Lewis (1976), using SDS- gel electrophoresis, characterized platelets membrane glycoproteins into three major classes. These are glycoprotein I (with subunits Ia, Ib,

Ic), glycoprotein II (with subunits IIa and IIb) and glycoprotein III (with subunits IIIa and IIIb). Glycoprotein IIb and IIIa are transmembrane proteins and have been implicated by Mustard and his group (1978) as forming the receptor for fibrinogen when they form a complex with calcium. Glanzmann's thrombasthenia involves the absence or abnormalities of glycoprotein IIb and IIIa and such thrombasthenic platelets do not aggregate nor bind fibrinogen when stimulated with an aggregating agents. Deficiency of glycoprotein Ib is associated with Bernard Soulier (or giant platelet) syndrome (Murden and Caen 1981). The bleeding abnormality that accompanies this defect has been attributed to impaired adherence of the platelets to subendothelial constituents.

(d) Phospholipd Change that Accompany Platelet Activation:

Platelet response to stimulation involves changes in its phospholipid composition. This response can be summarized as:

(i) breakdown of PI (PE or PC) with accompanying increase in phosphatic acid (PA)

(ii) the release of arachidonic acid which can enter the cycloxygenase or lipoxygenase pathways and

(iii) the conversion of phosphatidynositol - 4, 5, 5-, biphosphate (PIP_2) to phosphatidylinositol-4 phosphate (PIP).

The breakdown of PI follows calcium mobilization and has been shown to result in PA and AA liberation from platelet phospholipids (Lapetina, Billah and Quatrecasas 1981).

The first enzyme in the PI- response induced by thrombin is phospholipase C and the response leads to accumulation of 1, 2, - diacylglycerol (DAG) and PA (Lapentina and Cuatrecasas 1979). With A23187 as the stimulating agent, the fall in PI level is primarily due to its hydrolysis by phospholipase A2. The fate of DAG thus formed remains controversial. While some workers believe that it is acted upon by diglyceride and monoglyceride lipases to yield glycerol and free fatty acid including AA (Rittenhouse-Simmons 1979, 1981, and Bell, et al 1979) others have presented evidence that the DAG is phosphorylated to PA by 1,2, diglycerol kinase and subsequent resynthesis of PI (Lapetina et al 1981).

The possible role of elevated intracellular PA as calcium ionophore in the initiation of aggregation and platelet granule content release has been suggested (Lapetina 1982).

The release of arachidonate from platelet phospholipids following stimulation, results from the actions of phospholipases C and A_2 on PI and PC, PE and PI respectively. The formation of PA, from phospholipase C action on PI, in the presence calcium stimulates phospholipase A_2.

1.5.3. PLATELET SECRETION

1.5.3.1. Platelet secretion is closely linked with platelet aggregation and other platelet responses to appropriate stimuli. This is an agonist-induced response which enhances platelet activation. Most of the secreted substances have a direct action on platelets themselves or are converted to physiologically active substances in the plasma or on the cell surface. Platelet granule contents have been listed in Table 1.

The physiologically active substances such as ADP and 5-HT secreted by the dense granules act as part of the positive feedback loop to enhance platelet stimulation (Fig. 4a). Stimulation of the platelet is accompanied by PGG_2 and PGH_2/TxA_2 synthesis; these substances are potent stimulating agents for platelet aggregation and secretion (Fig. 4) (Holmsen 1982). The released substances are responsible for the second wave of aggregation.

Platelet secretion was first suggested by Bizzozero (1882) and observed for the first time by Grette (1962), who termed it "platelet release reaction" to describe the release of fibrinogen, adenine nucleotides and serotonin from pig platelets following thrombin stimulation. Platelet secretion occurs by exocytosis and this accounts for selective release of only granular contents and not cytoplasmic substances (MacIntyre 1976). Holmsen et al (1968) demonstrated that only the granule ADP content is released as the cytoplasmic pool which is readily labelled by radioactive adenosine is not released.

Ultrastructural studies associated with platelet granule secretion have shown that with centralization of granules and organelles by contraction of the circumferential band of microtubules on platelet stimulation, there is granular fission with the surface connected canalicular systems (SCCS) shortly before secretion (White 1974). Also internal contraction initiated by the platelet contractile systems are essential to drive the extruded substances into the channels of the SCCS then out of the platelet into the surrounding medium.

Platelet secretion requires metabolic energy and internal calcium mobilization (Fig. 4). Holmsen and associates (1974) demonstrated that when ATP synthesis is blocked by the simultaneous addition of the metabolic inhibitors 2 - deoxyglucose and antimycin A to platelets, there is a progressive loss in the ability to undergo release reaction. Platelet secretion can be induced by A23187 in the presence of calcium-chelator EGTA or EDTA indicating that it is independent of external calcium although Ca^{++} is necessary for the secretion that accompanies aggregation (White, Rao and Gerrard 1974). It has been suggested that energy from ATP metabolism along with intracellular Ca^{++}-mobilization initiates the contraction of thrombasthenin bringing about the centralization of organelles and brings them into close contact with the open canalicular system.

Most agents that induce aggregation are capable of causing platelet secretion. Holmsen (1982) has identified three types of platelet secretions based on the compartment involved. These are:

(a) dense granule secretion

(b) alpha-granule secretion

(c) acid hydrolase secretion.

He postulated that the strength of each agonist depended on how much Ca^{++} was mobilized. Thus, very strong stimuli such as high concentrations of thrombin, collagen, PAF- acether and A23187 are capable of initiating secretions from all three compartments while ADP, adrenaline and serotonin can only initiate dense granule secretion. Thromboxane A_2 is of intermediate strength and can stimulate both alpha- and dense granule secretions. Packham and associates (1977) showed that for some agents such as collagen and A23187 but not low concentration of thrombin, platelet secretion

starts as early as during the shape change phase. It has been observed that acid hydrolase secretion requires higher concentration of agonist such as A23187, thrombin or PAF-acether and was more suceptible to metabolic inhibition than dense granule secretion. Close cell contact activation such as occurs during centrifugation, can induce platelet secretion which can be stopped by formaldehyde (Costa et al 1976).

1.5.3.2. Methods of Studying Platelet Secretion: In some disease conditions such as diabetes mellitus and atherosclerosis, measurements of platelet secretion have served as markers of extent of platelet activation in vivo. Determination of some constituents of dense granule and acid hydrolases have been used as diagnostic tests of platelet disorders such as storage pool deficiency in which contents of the dense granules are markedly reduced (Weiss 1976). However, since some of these platelet constituents (e.g. dense- and lysosome granule contents) are not as specific as the alpha-granule contents (BTG and PF4), their plasma concentration as indices of platelet consumption or activation in vivo should be interpreted with caution. PF4 which was initially thought to be a platelet specific protein has been reported in the mast cell (Mclaren et al 1977).

Among platelet secreted substances the most widely studied are serotonin (Holmsen et al 1969, 1972, Packham et al 1977) adenine nucleotides (Holmsen et al 1969, Feinman et al 1977) BTG and PF4 (Moore et al 1975, Niewiarowski et al 1976). The latter (BTG and PF4) are measured by radioimmunoassay (Bolton et al 1976 a,b) while determination of platelet 5-HT uptake and release are carried out by measuring the release from stimulated platelets of prelabelled

platelet 5-HT (Holmsen et al 1972) Platelet adenine nucleotides are estimated by the fire-fly luminescence method and the kinetics of secretion monitored directly with the lumiaggregometer (Feinman et al 1977). Dangelmaier and Holmsen (1983) have reviewed methods for measuring platelet calcium and acid hydrolases.

1.6. PLATELET COAGULANT ACTIVITY

Early morphological studies indicated that fibrin formation occurred in close apposition to platelet aggregates (Addison 1842, Gulliver 1842). Bizzozero (1882) suggested that platelets released a procoagulant substance into the plasma. Schimmelbusch and Eberth (1885) pointed out the relationship between platelets and blood clotting. Observations made by Woolridge (1883) indicated that lipids were important for blood clotting and could be a possible source of procoagulant activity. Chargaff et al (1933), van Creveld and Paulssen (1952) isolated some platelet lipid with procoagulant activity which were designated platelet factor 3. All attempts at identifying a single phospholipid active in blood coagulation failed (Biggs and Bidwell 1957) and so Marcus (1966) submitted the concept that platelet factor 3 is a function of platelet membrane phospholipoprotein complex rather than a single phospholipid.

Thus platelets participate in blood coagulation by promoting a phospholipoprotein surface upon which coagulant proteins are brought into contact with one another. Walsh (1974) proposed that platelets play a crucial role in all phases of blood coagulation reactions by providing activated platelet membranes for both specific and non-specific adsorption and receptors for coagulation

factors. Platelets thus concentrate and protect these factors from plasma inhibitors. In addition, thrombin formed by the coagulation cascade also activates platelets and thus serves as a positive feedback mechanism which promotes effective haemostasis (see Fig. 6a, b).

To aid platelet procoagulant function, fibrinogen (James, Ganguly and Jackson 1977), factor V (Kane et al 1980) Factor XIII (Schwartz et al 1973) and Factor VIII-related antigen (Slot et al 1978) are either found in platelet alpha-granule or cytoplasm. The alpha-granule coagulation factors are released during aggregation. Factor XI-like activity has been reported to be firmly bound to platelet membrane (Schiffman et al 1977).

It is clear from available data that platelets play a significant role in promoting blood coagulation. For instance, existence of binding sites for Factor Va and Factor Xa defines a role for platelets in the terminal stages of blood coagulation. Similarly the occurrence of receptors for Factor XIa on the surface of stimulated platelets suggests platelet involvement in contact activation.

1.7 PLATELET FUNCTION INHIBITORS

These agents fall into 3 major subgroups :-

(a) Those that increase cyclic AMP levels in platelet cytosol thus impeding Ca^{++} mobilization.

(b) Those that inhibit arachidonate pathway.

(c) Those that inhibit specific agonist and receptors.

These antagonists of platelet function affect mainly platelet adhesion, aggregation and release reaction although some of those

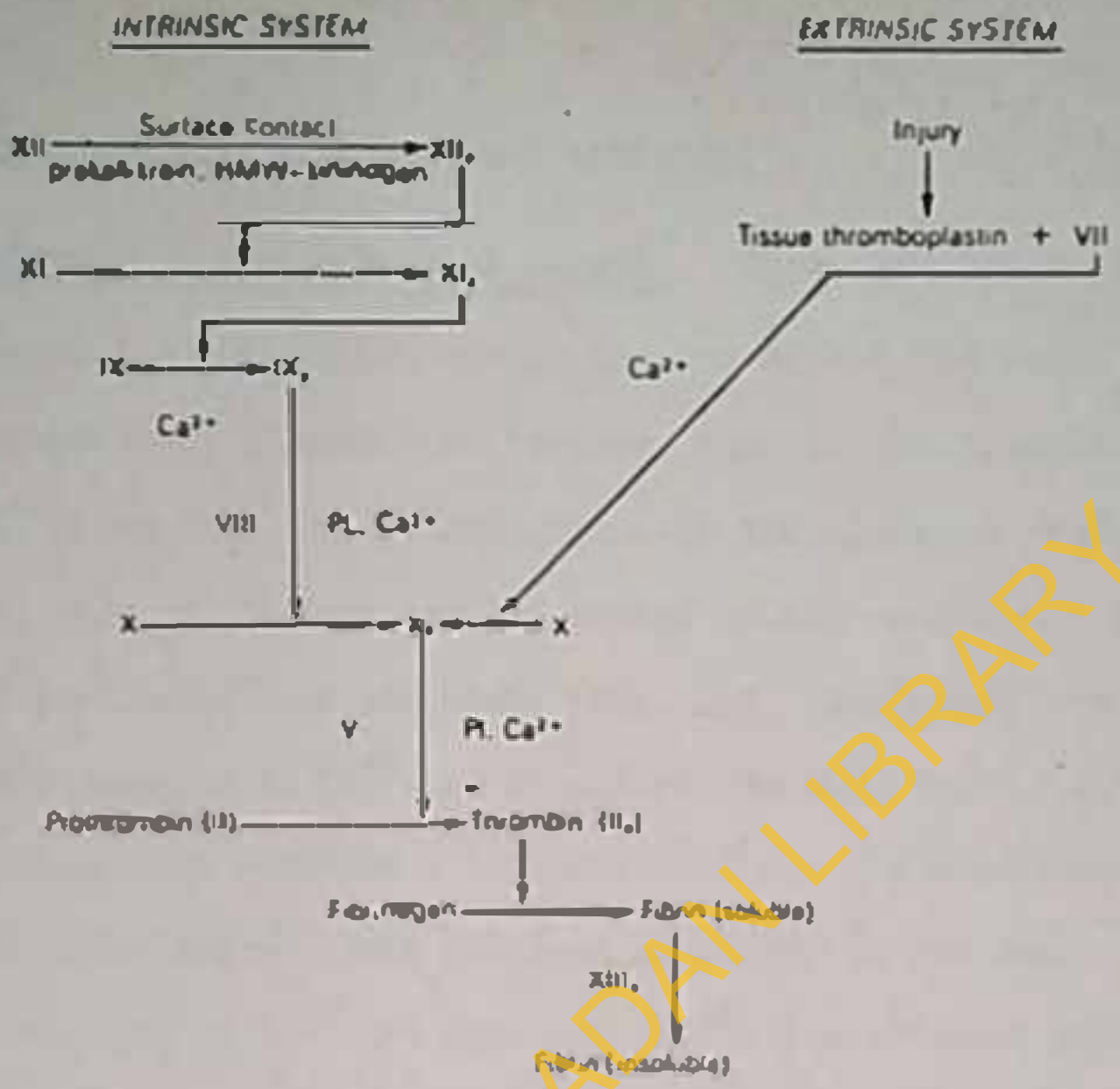


Fig. 6a. Factors II (prothrombin), VII (proconvertin), IX (Christmas factor), and X (Stuart-Prower factor) are vitamin K-dependent and occupy the core of the clotting scheme. Activation of all of these factors requires calcium ions. Factor V is accelerator globulin, factor VIII is antihemophilic globulin, factor XIII is fibrin-stabilizing factor, a transpeptidase. PL is phospholipid, factor, is active enzyme. (After Walsh, P.N. 1982)

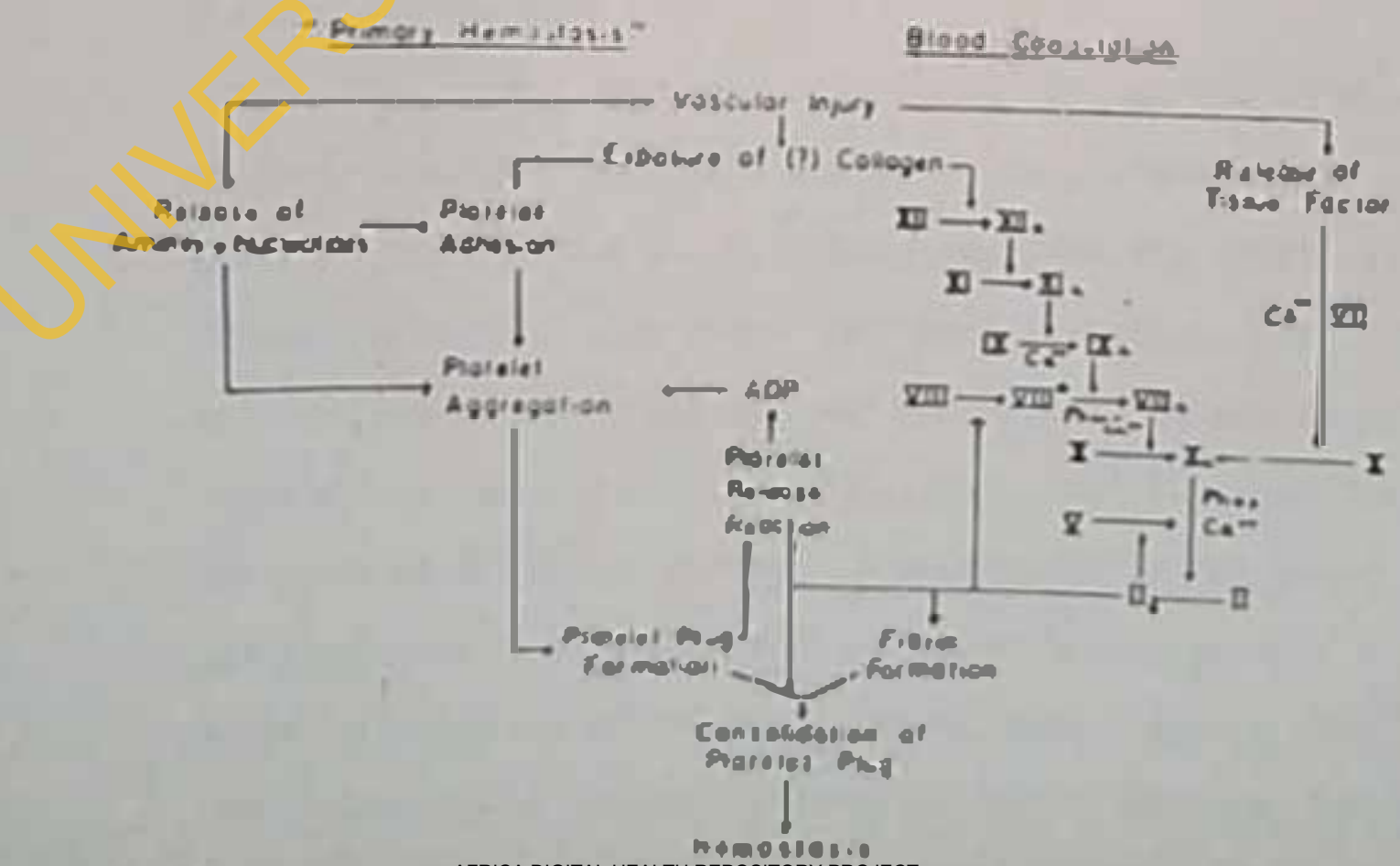


Fig. 6b [illegible] (After Greenspan, J.S. and Griffin, J.H. 1983).

may inhibit platelet procoagulant activity.

1.7.2. (a) Agents that Increase CAMP Levels: These are the most powerful antiplatelet agents as they affect the basic mechanism of platelet activation, that is mobilization of Ca^{++}-ions from its storage sites to the platelet cytosol. The role of CAMP is closely linked to the concentration of cytosolic Ca^{++} in platelets (Fig. 6b). Increased levels of CAMP results in Ca^{++}-ion sequestration into dense tubular system with subsequent inhibition of all platelet responses to stimulation. This has been suggested as the main mechanism of platelet function inhibition (Mustard and Packham 1978, Vermylen, Defreyn and Deckmyn 1982, Packham 1983).

Platelet CAMP is elevated by agents that stimulate adenylate cyclase and to a lesser extent by those that inhibit phosphodiesterase (an enzyme that breaks down CAMP: Mustard and Packham 1978).

PGI_2, PGD_2, PGE_1, and adenosine through the stimulation of their specific receptors activate adenylate cyclase (Packham 1983). Forskolin, a diterpene from the roots of Coleus Forskohlii, increases CAMP in platelets by direct stimulation of adenylate cyclase and appears to act in a synergistic fashion with the PGE_1, PGD_2, and PGI_2 and the phosphodiesterase inhibitors. Prostacyclin is the most potent platelet inhibitor and is formed mainly by the endothelial cells of the blood vessel wall. Agents like Dipyridamole, Ro 15-2041, papaverine and Cilostamide inhibit platelet phosphodiesterase. Dipyridamole has gained clinical application in thrombotic therapy.

Some agents interefere directly with Ca^{++} mobilization. These include Ca^{++}-blockers such as Verapamil (Mehta et al 1983), local anaesthetics such as tetracaine and antidepressants such as chlorpromazine, imipramine and amitryptyline (Holmsen 1978).

(b) Agents that inhibit Arachidonate Pathway: These agents inhibit the arachidonate pathway by blocking the enzymes involved in AA metabolism such as phospholipase, cycloxygenase and thromboxane synthetase. Drugs that inhibit the phospholipase include the steroidal antiinflammatory agents such as hydrocortisone 21-sodium succinate, methylprednisolone, sodium succinate, the antimalarial agent mepacrine, trifluperazine and bromophenacyl bromide. Others are propanolol, mepacrine and calmodulin-antagonist, phenothiazine (Blackwell and Flower 1983).

Agents that inhibit platelet cycloxygenase also cause a reduction in vascular wall synthesis of prostacyclin and so are clinically less effective than initially anticipated. Such agents will result in the blockade of PGG_2, PGH_2, PGI_2 and TxA_2 synthesis. They include the NSAIAs such as aspirin, indomethacin, ibuprofen and diflunisal (Packham and Mustard 1980, Mitchell 1983). Some of the cycloxygenase inhibitors such as aspirin irreversibly acetylates the enzyme rendering it inactive (Roth and Majerus 1975). Buchanan and his group (1982) have suggested that aspirin may inhibit platelet function by a mechanism independent of cycloxygenase acetylation, probably by inhibiting 12-HETE synthesis (12.HETE prevents deaggregation).

Currently, interest is centered around beneficial effects of thromboxane synthetase inhibitors, as they do not interfere with prostacyclin formation. Dazoxiben (UK37248) is the most useful of all thromboxane synthetase inhibitors. Vermylen, Defreyn and Deckmyn (1982) have tested the drug clinically and shown it to be superior to aspirin as an antithrombotic agent since it does not only inhibit TxA_2 but also enhances prostacyclin formation. However, "responders" and "non-responders" have been identified (Bertele et al 1981) and this has been related to the individual ability to form either PGE_2 or PGD_2 from accumulated PGG_2 during thromboxane synthetase inhibition (Bertele et al 1984b). However, the mechanism of action of Dazoxiben is related to stimulation of cAMP as SQ22536, an adenylate cyclase inhibitor, prevents its antiplatelet effect (Bertele et al 1984a).

(c) Drugs that Inhibit Specific Agonists and Receptors:

Thrombin receptors are blocked by some peptides such as D-Phe-Pro- Arg-H.H_2SO_4 and D-Phe-Pro-Arg-CH_3 Cl (Packham 1983). Adrenaline and serotonin induced aggregation can be blocked by alpha blockers (phentolamine, phenoxybenzamine or dibenzaline) and ketanserine (or cycloheptadine) respectively (Packham and Mustard 1971; Bevan and Heptinstall 1983).

1.8. MALARIA

1.8.1. General Introduction:

Malaria, a highly prevalent disease of man and other vertebrates, caused by a blood inhabiting protozoan parasite of the genus Plasmodium, is transmitted by the bite of infected female mosquitoes, a common one being the female anopheles species. Not only is it a severe health problem in the tropics and a major impediment to economic development, it is also responsible for the annual death of more than one million children and infants in tropical Africa (Trigg, 1978, Wernsdorfer 1980).

Of the four species which infect man (Plasmodium falciparum, P. ovale, P. vivax and P. malariae) P. falciparum has received most attention as a result of its often hyperacute and fatal clinical course. Besides these, a range of Plasmodium species which infect rodents (P. berghei, P. yoelii, P. chabaudi and P. vinckei), non-human primates (P. knowlesi, P. simium, P. coatneyi, and P. cynomolgi), birds (P. gallinaceum and P. lophurae) and reptiles (P. agamae) are also known (Wernsdorfer 1980). These non-human malariaes have lent themselves to complex experimental manipulations and studies which hitherto would have been impossible with the human plasmodia.

1.8.2. LIFE CYCLE:

As complex as its life cycle seems, the malaria parasite spends most of the time intracellularly. In the vertebrate host, most of its development takes place within the red cells, establishing a two way relationship in which the parasite modifies the antigenicity and composition of the erythrocyte, at the same

time equally depending on the host cell metabolism and constituents for its development and growth. (Fig. 7).

(a) EXOERYTHROCYTIC STAGE: Normelly, the vertebrate stage begins with the transfer of sporozite from the salivary gland of an infected mosquito into the blood stream of the vertebrate host. They are cleared rapidly, usually within one hour, and effectively by the circulatory system to the tissues, leaving only a few sporozoites which find their way into the lymphatic tissues. In mammals, the sporozoites invade the parenchymal cells of the liver, either through the kpuffer cells then to the hepatocytes (Smith et al 1981) or directly into the hepatocytes shunting the kpuffer cells (Shortt 1948). Invasion through the endothelium has also been considered (Bray and Garnham 1982). Within 40 to 48 hours (for P. falciparum), these sporozoites, 3µM in diameter, mononucleated having only little cytoplasm, have almost completely invaded the hepatocytes (Krotoski et al 1981). Recent studies suggest that, the sporozoites attach to the free end of sugars like D-mannose and D-fructose molecules. The hepatocytes, having receptors for these sugars, recognise them and take them in together with the attached sporozoite. This is similar to the system described by Baenziger and Fiete (1980).

The intrahepatocytic forms develop into schizonts within 20 to 30 hours of invading the liver cells. During this phase which is known as the primary exoerythrocytic schizogony, each sporozoite develops into one schizont within each host tissue cell, and inside the cell are formed

FIG. THE LIFE CYCLE OF MALARIA PARASITES.

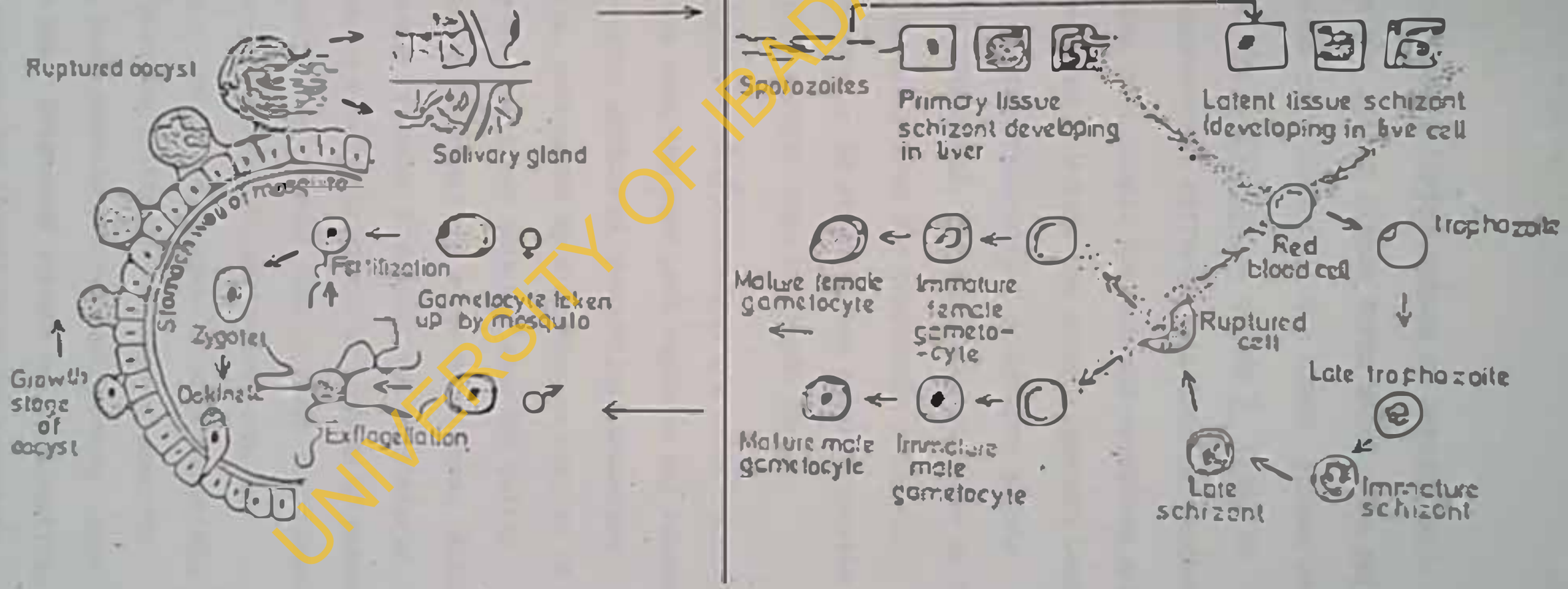
INVERTEBRATE PHASE (SEXUAL)-

Takes place in the female anopheline mosquito

VERTEBRATE PHASE (ASEXUAL)-

Takes place in man

Sprozoites picked by mosquito and injected into a suitable host.



(After Bruce-Chwatt, 1951)

numerous daughter cells (Cryptozoites or merozoites). Hypnozoites are the latent tissue forms which when released after clearance of parasites from the blood stream cause a true relapse. In P. falciparum unlike P. vivax or P. ovale, all sporozoites differentiate into merozoites, a form consisting of a single nucleus and cytoplasm and thus shows no true relapse. Unlike the avian plasmodia where a second round of exo-erythrocytic schizogony exists (metacryptozoic schizonts), the erythrocytic forms in the mammals arise directly from the cryptozoic schizonts (primary schizogony). In rodents, the exoerythrocytic forms mature much earlier than the human forms.

(b) ERYTHROCYTIC STAGE: The merozoites are released into the blood stream when the host cell ruptures and releases the exoerythrocytic schizonts, now called merozoites. The ovoid merozoite, surrounded by a pellicular complex of two membranes has an apical end that contains the paired organelles (the rhoptries) and a few micronemes. Although it has a lifespan of about 30 minutes extracellularly, the merozoite invades a red cell within 20 seconds of release, in a multistep fashion (Pasvol 1982). These steps involve a) recognition, b) attachment and orientation, and c) endocytosis. Merozoite recognition of a suceptible erythrocyte involves initial recognition of specific receptors on both parasite and red cell surface. Some proteins (or their fragments) present on the suceptible erythrocyte surface, are thought to be the receptors (or receptor units) for their specific merozoites.

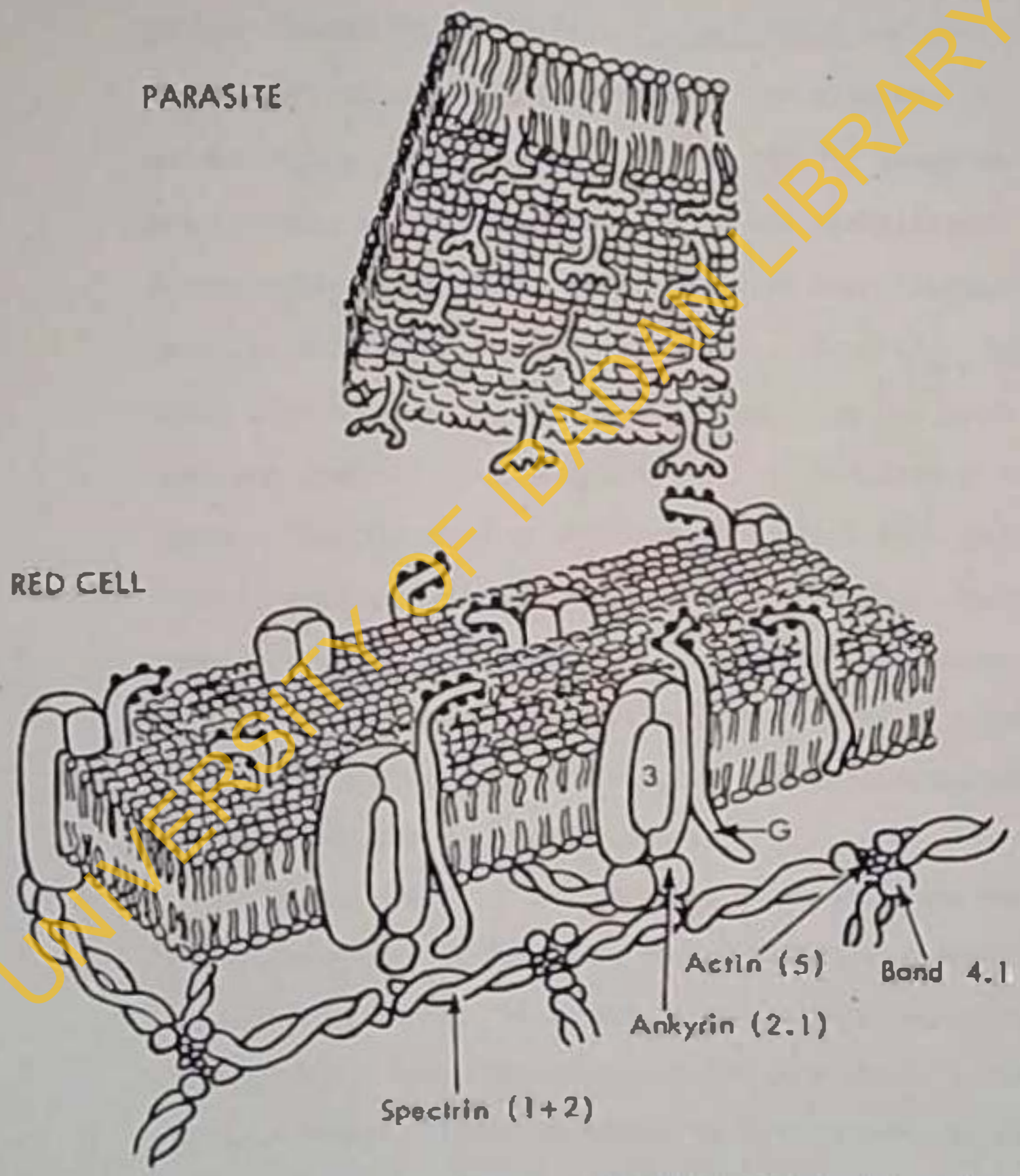
Some examples of such protein receptors include:

(i) Duffy antigen (Duffy determinants Fy^a and Fy^b) as a receptor for P. Knowlesi and P. vivax (Miller et al (1975), Miller et al (1976), Miller et al 1977, Miller et al (1978). These studies were performed with Duffy negative erythrocytes.

(ii) Glycophorins (A, B and C). This is a sialic acid-rich glycoprotein with the A and C types providing 75% of the erythrocyte surface sialic acid, which acts as receptor for P. Falciparum (Miller et al 1977, Perkins 1981, Pasvol et al 1982).

Erythrocyte Entry: Interference microscopy has revealed that merozoite attachment to the erythrocyte induces rapid, transient and marked deformation of the red cell membrane cytoskeleton (Aikawa et al 1978, Bannister et al 1975). This attachment following orientation of the apical region of the merozoite to make contact with red cell membrane, is aided by the histidine-rich protein (Kilejian 1976) of the rhoptries (Aikawa et al 1978). This complicated process also involves the formation of a parasitophorous vacuole by the erythrocyte in which the parasite eventually lies and movement of the junction (between erythrocyte and merozoite) around the merozoite to aid endocytosis through transient rearrangement of erythrocyte cytoskeleton (comprising of glycophorin, band 3 protein (anion channel) ankyrin and spectrin. See Fig. 8) (Dvorak et al 1975, Aikawa et al 1978). Eventually the erythrocyte is resealed after complete endocytosis.

FIG. 8 A schematic diagram of the interaction between the surface membrane of the human erythrocyte and the malarial parasite *P. falciparum*



"Bristle-like" structures on the parasite's surface coat interact with oligosaccharide moieties on the exposed *N*-terminal region of the integral membrane sialoglycoprotein, glycophorin (G). This protein is depicted in close association with the other main transmembrane glycoprotein, band 3 (3). The cytoskeletal network, consisting of spectrin, band 4.1 and actin, is linked to band 3 by ankyrin (After Pasvol, G. and Wilson, R.J.M. 1982).

Inside the erythrocyte, the merozoite enveloped by the parasitophorous vacuolar membrane ingests, 25 to 75% haemoglobin while growing from the ring form to the schizont. Certain erythrocyte membrane alterations such as Schuffner's dots and Maurer's cleft develop on the parasitized red cell surface (Aikawa et al 1975). P. falciparum induces formation of "knobby" structures (as seen with the electron microscope) on red cell surface (Langreith et al 1978). These structures are involved in the sequestration of the parasitized erythrocytes to capillary endothelium in deep tissues where parasite multiplication occurs in vivo. However, P. malariae which also induces the formation of knobs on red cell membrane does not bind to endothelial cells (Udeinya et al 1981). Modification in erythrocyte surface carbohydrate (sialic acid content and reduced lectin bindings) therefore results from infection with several species of plasmodium organisms. There are in addition, alterations in erythrocyte membrane fluidity, osmotic fragility and passive permeability. (Sherman 1979, Pasvol and Wilson 1982).

As the growth of the parasite progresses, the merozoite cytoplasm gradually displaces the vacuole, and haemozoin (a malaria pigment), is formed as a digestion product of haemoglobin. The trophozoite, as the parasite is called at this stage, divides asexually to form numerous erythrocytic schizonts which occupy the whole cell. The maturation of the schizonts and the release of merozoites occupy an interval of time characteristic of the parasite and these are generally in multiples of 24 hours. P. gallinaceum has

a 36 hour asexual cycle, P. berghei has a 24 hour cycle, while P. falciparum matures in 48 hours. Finally the mature schizont ruptures the red cell, liberating the individual merozoites which in mammalian species can only invade other erythrocytes although avian or reptilian plasmodia can also invade tissue cells. The periodic fever chills which characterise malarial infections are caused by synchronous asexual development and rupturing of erythrocytes.

(c) SEXUAL PHASE: Upon invading a new erythrocyte, the merozoite can either initiate renewed blood schizogony, or develop into a female (macrogametocyte) or a male (microgametocyte). The stimulus for this transformation is yet unknown.

The mature macrogametocyte has a compact nucleus juxtaposed with an accumulation of pigments. The microgametocyte has a larger nucleus but shows a spongy appearance. Both gametes are still surrounded by the host erythrocyte membrane. When taken up by a suitable arthropod, the gametocytes transform into gametes. The microgamete (fewer than the macrogamete) undergoes three extraordinary, rapid divisions (Sinden 1981) to form filamentous cells each having a flagellum with which on erythrocyte rupture, it goes in search of a mature macrogamete outside the red cell. The exflagellated microgametes move actively towards the macrogamete invading and fertilizing it. The fertilized macrogamete (zygote) now called the ookinete is elongated into an actively motile form. The ookinete penetrates the arthropod's intestinal epithelium to lie on the side of

coelomic cavity. There the ookinete is encysted to form the oocyst within which intensive nuclear division (i.e. cellular division) occurs. The duration of this sporogonic like division depends on environmental temperature. The sporozoites thus formed emerge from the ocyst into the haemolymph through small individual holes or through large opening, where the ocyst wall has been torn (Sinden 1975). The sporozoites migrate by chemotaxis to the acinal cells of the salivary glands. Here, they mature, penetrate and lodge in the salivary ducts where they are able to infect the next vertebrate when the mosquito bites, by flowing with the saliva into the wound.

1.8.3. EXPERIMENTAL MODELS:

Until recently the study of malaria parasites in the laboratory was mainly restricted to the use of animal models such as rodent species, primates and birds with their corresponding malaria parasites. However the successful in vitro cultivation of the erythrocytic stages of *P. falciparum* and *P. coatneyi* by Trager and Jensen (1976) has added another dimension to in vitro experimentation. This system, besides being a method which inevitably supplies information about the developmental requirements of the parasite, open up channels for difficult studies which hitherto could have only been speculated. In the studies reported here we have used both the rodent model (*P. berghei* in mice and rats) and in vitro *P. falciparum* erythrocyte cultures.

1.8.3.1. PLASMODIUM BERGHEI: This species belongs to the subgenus P. Vinckeia which is characterized by small erythrocytic schizonts and round gametocytes. Vinke and Lips (1948) described the first species of murine rodent malaria parasite P. berghei in its natural host Thamnomys Surdaster (tree rats).

The P. berghei has proved easily adaptable to laboratory rodents like rats, hamsters and mice, which are infected by blood passage and sporozoite infections. However a marked difference in suceptibility and course of infection has been observed in mice infected by blood passage from an infected one with P. berghei. This difference depends on the volume of inoculum and strain of P. berghei. The ease of cyclic transmission through blood passage and the rapidity with which high levels of parasitaemia are attained, have made P. berghei a widely used tool for biological, chemotherapeutic and immunologic research.

The prepatent period which parallels the development of the exoerythrocytic schizonts is quite rapid, lasting only about 24 hours and is used to identify this subgenus (P. berghei) except in P. girardi which infects the lower primate lemur with a prepatent period of 72 hours.

Pathologically, the mouse model has a 25-100% mortality rate with a bimodal mortality curve. Thrombocytopenia, haemoglobinuria and anaemia which leads to shock presents in the first week. If the animal survives this there is a high recticulocyte count, reticulocytes being preferentially attacked leading to further anaemia. The animal finally dies of anoxia if untreated. Kreier and Leste (1968) suggested that the anaemia was due to the action of both auto-antibodies and the destruction of parasitized erythrocytes.

During infection, at terminal stages, the animal is extremely pale, with brown skin pigmentation in the skin ducts due to hemozoin deposites. Hypothermia has also been recorded during P. berghei infections in mice (Kretschmar 1961).

Synchronicity is not shown and response to infection has been associated with sex, age and weight (Greenberg, Nadel and Coatneyi 1983). Spleenomegaly is not as pronounced in rodents as in humans (Servent and Poncet 1955).

1.8.3.2 MALARIA CULTURES: The earliest attempt, by Bass and Johns (1912), at in vitro short term cultivation of the erythrocytic stage of malaria parasite using whole defibrinated blood from malaria patients and which was supplemented with glucose, could sustain P. falciparum and P. vivax for only 2 cycles. Later short-term cultures were attempted using avian (P. lophurae) and primate (P. Knowlesi) (Trager 1947, McGhee and Trager 1950, Ballet et al 1945) using the "Trocker" technique. While Trager (1943) noticed the presence of viable male gametocytes of P. lophurae up to 16 days of incubation with a 3 fold increase in parasitaemia, Gieman and coworkers (1946) could sustain P. knowlesi for only 6 days despite its shorter cycle. Trigg and Shakespear (1976) explained the latter result in terms of its progressively fewer multiplication with each successive cycle.

These early experiments, revealed specific nutritional and environmental requirements which greatly helped in the final breakthrough in parasite in vitro cultivation. Trager (1943) demonstrated a Calcium pantothenate requirement for P. lophurae, while Bull (1946) showed that para-Amino benzoic acid (PABA) was a requirement for P. Knowlesi and P. lophurae (Maier and Riley 1942).

Other factors such as biotin, methionine, purines, stearate, and other essential nutritional requirements for parasite growth were also identified.

The first successful experiment of continuous parasite cultivation was by Anderson (1953) who used a medium containing chicken erythrocyte extract prepared in chicken serum. In this medium, he maintained P. gallinaceum for 10 days at the same level parasitaemia with twice daily dilutions with fresh blood samples. In 1971, Trager suggested that a settled layer of red cells with a slow flow of medium over them would mimic the deep organ endothelium sequestration of parasitized red cells of the P.coatneyi and P. falciparum. This led to the development of the flow-vial technique for malaria parasite culture.

Trager and Jensen (1976, 1978) later showed in an experiment using P. coatneyi and P. falciparum infected-red cells, kept in the leucocyte culture medium RPMI-1640 (Moore et al 1967), that the flow-vial technique could sustain parasite growth for up to 2 weeks. Other requirements for the successful cultivation were that the medium should be charged with 25mM HEPES buffer (N-2-hydroxy-ethylpiperzine-N-2-ethanesulfonic acid), 15% rhesus monkey serum in (2-5%) CO_2 and (5-16%) O_2. This later led to the development of two parasite cultivation methods: the very simple petri-dish candle jar method and the improved continuous flow method.

Other improvements on the method showed that human erythrocytes of ABO blood groups, with 10-15% compatible serum, even after preservation at $4^{o}C$ in either ACD or CPD, could sustain growth for up to 4 weeks. Such serum has to be separated from freshly collected blood and stored at $-20^{o}C$ or lyophillized. The gas mixture of 5%

CO_2 mixed with air, which is also required, helps to maintain the pH of the bicarbonate containing medium. The modified Ball and Gieman's medium and Dulbecco's-high glucose medium which were also tried were not as suitable as RPMI-1640.

1.8.3.3. CHANGES IN CULTURE: Antigenic changes that accompany long term in vitro cultivation of bacteria and viruses have been reported for P. faciparum cultures. This consists of the loss of erythrocytes surface protrusions - "Knobs" (Rudzinska and Trager 1968) which help the parasitized erythrocyte attach to the capillary endothelium (Luse et. al 1971). These knobs contain parasite-derived antigenic materials (Kilejian et al 1977) and its loss might have serious immunologic or pathological implications. At present , the infectivity of the knobless variant (K^-) is more significantly reduced than the "knobby" (K^+) red blood cells (Trager 1982). Nguyen-Dinh and Trager (1980) have also reported that there was reduced chloroquine sensitivity in some of the (K^-) clones studied, an observation that might be useful in studies dealing with plasmodium resistance to antimalarial agents.

1.8.4. PATHOLOGY OF MALARIA:

MecKel (1847) reported that there was brownish pigmentation which could be removed from blood during some febrile states. This observation was made before the discovery of the malaria parasite in blood in 1884. It was subsequently shown that changes observed in patients primarily accompany the presence of the erythrocyte as well as changes in the host immune responses. These changes (basically biochemical and histological) can be seen as affecting mainly the following systems or organs:

(a) the Haematologic system

(b) the Spleen

(c) the Liver

(d) the Circulatory system,

(e) the Nervous and other systems.

(a) Hematopathology: Since the red blood cell which provides a rich source of nutrients in an abundant, rapidly renewable tissue, is easily accessible to mosquitoes, and offers an intracellular environment that may help the parasite evade the host immune response, the erythrocyte remains the main target cell of the malarial parasite, although the parasite spends only a short part of its lifespan in the red blood cell. This parasite therefore remains a major hematotropic pathogen of man, birds, primates and rodents.

Besides, morphological changes such as crenation, as is observed in P. falciparum, enlargement of parasitized cells in P. vivax, these parasites show a preference for certain types of red cells. For instance P. berghei is reticulotropic, P. falciparum invade both old and young cells although recent evidence suggest that it prefers rectics, while P. malariae only old red cells (Seed and Kreier 1972).

The anaemia observed is induced by several mechanisms such as - erythrocyte rupture to release mature merozoites, splenic pooling of altered red cells and direct hemolysis resulting from host immune response (Weatherall and Abdalla 1982). There is also some degree of bone marrow failure.

Leucocytosis has been observed in P. berghei infected mice (Singer (1954), Wellde et al (1972). This was attributed mainly to a rise in peripheral monocytes (Singer 1954) who reported that the monocytosis correlated well with stimulation of recticuloendothelial system by the malaria pigments and accelerated destruction of infected and non infected red cells. Malaria parasite induced leucocytosis has also been linked with Burkitt's lymphoma (Okrusah et al 1979).

To date there are few studies on platelet changes during malaria infection. Thrombocytopaenia which increases with parasitemia (Voller et al 1969) has been recorded in rodents infected with P. berghei, Aotus monkey infected with P. Falciparum (Dennis et al 1966, 1967). Depletion in coagulation factors such as fibrinogen, factor VIII, increase in fibrinogen degradation products and prothrombin time have been observed (Dennis et al 1967, Abilgrasd et al 1975). These findings have been interpreted as being consistent with disseminated intravascular coagulation (Borochowitz et al 1970).

(b) The Spleen: Spleenomegaly is a frequent finding in human, nonhuman primate and rodents (Jervis et al 1972, Singer 1954). This occurs as a result of splenic congestion by red blood cells and proliferation of the white pulp was due to hyperplasia of the endothelial cells, macrophages and lymphoid elements. There is also accumulation of malaria pigments.

(c) The Liver: Hepatomegaly is a common finding in human malaria infections and results from blockage of sinusoidal spaces by parasitized red cells. This gives rise to sluggish circulation with resultant congestion and central necrosis due to portal hypertension (Jervis et al 1972). The liver changes colour progressively from pink to black due to deposition of haemozoin and haemosiderin. Tissue anoxia results from inadequate oxygen supply or by the inability of the tissue cells to utilize oxygen, a condition that leads to shock and subsequent death.

(d) The Circulatory System: The vascular pathology associated with malaria consists mainly of capillary occlusion due to masses of agglutinated infected erythrocytes. In P. falciparum this causes haemorrhage and necrosis in perivascular areas of the brain, myocardium and skin (Spitz 1946). The erythrocytes develop excrescences or "knobs" (seen under the Electron microscope) with which they anchor unto the capillary endothelium during schizogony which occurs in deep blood vessels (Wilson 1971). P. falciparum infected animals may also develop D.I.C. (Wellde et al 1972).

The discovery of inflammation exciting substances like bradykinin (Goodwin and Richards 1960) and histamine (Mesquita and Craveiro 1970) capable of causing increased vascular permeability in circulation would also account for circulatory pathology observed.

(e) Other Changes: Renal pathology observed during malaria has been attributed to deposition of immune complexes in the kidney. This induces renal malfunction as observed in P. malariae, P. falciparum and experimental malaria models such as in P. berghei and P. brasilianium infections (Aikawa, Suzuki and Gutierrez 1980).

Cerebral malaria - the condition where many parasitized red blood cells are deposited in the microcirculation of the brain - is marked with central nervous system changes. The cerebral capillaries are found occluded by erythrocyte mass and the presence of chronic inflammatory cells are also observed especially around the vessels. These latter changes often result in punctuate haemorrhages and thrombosis.

Examination of the placenta has revealed abundant parasite deposition in the maternal circulation and in the intervillous spaces of the placenta. This serves as a focus for fetal infection leading to cases of intrauterine-acquired malaria which may be associated with premature birth and increased foetal death (Archibald 1956).

1.8.5. MALARIA CHEMOTHERAPY:

The main drugs commonly used in the treatment of malaria are chloroquine, dihydrofoleate reductase inhibitors such as pyrimethamine, chloroguanide primaquine and quinine. Sulphonamides, sulphones and tetracyclines have also sometimes been used. These treatment agents can be classified into: Casual prophylaxis, agents for suppressive treatment or cure, clinical cure, radical cure and others for gametocidal therapy (Rollo et al 1975).

Some agents such as proguanil, Chlorogiuanide and pyrimethamine prevent demostrable infections by exerting a lethal effect on the malarial parasite during the exoerythrocytic stage. No true causal prophylactic agent has been found. Others such as chloroquine, quinine amodiaquine and mepacine used for suppressive treatment inhibit the erythrocytic stages of the parasite such that there is no clinical manifestation of the disease. The latter drugs are most widely used in malaria treatment. The 4-aminoquinolines interfere with intraerythrocytic schizogony and thus terminate clinical attacks. Primaquine, an 8-aminoquinoline derivative, exerts a radical cure by eradicating both the exoerythrocytic and erythrocytic stages of infection (See Fig. 7).

This section of the report will focus attention mainly on chloroquine as a drug of choice in malaria treatment and only briefly mention others where it is considered necessary.

Chloroquine is one of the most widely used and highly effective of the schizontocidal agents available in malaria treatment. It is very potent against asexual forms of P. berghei, P. vivax, and P. falciparum and has some gametocidal actions on P. vivax gametocytes. It controls parasitaemia in acute attacks and within 48-72 hours after administration can give negative blood films in man infected with P. falciparum.

Chloroquine, a 4-aminoquinoline derivative is readily soluble in water at acidic pH, to give a stable solution. Besides its antimalarial properties, it exhibits antiinflammatory properties as well. This is the basis of its use in the treatment of rheumatoid arthritis and other inflammatory states. Its mechanism of action as an antimalarial agent is mainly by

interference with parasite replication by blocking nuclei acid synthesis as it forms a molecular complex with DNA (Hahn et al 1966). This is achieved probably by intercalating between base pairs of the double helix, with resultant multiple misreading of the genetic code followed by abortive protein synthesis. In addition to its effect on the DNA, Chloroquine exhibits enzymatic inhibition of glycolytic enzymes and amino acid synthesizing enzymes (Ting et al 1966, Siu et al 1967); Polet and Barr (1968) have demonstrated that the therapeutic value of chloroquine is dependent on the ability of the parasite to concentrate the drug and the derangement of this selective toxity has been suggested as a basis for resistance.

Chloroquine is rapidly absorbed after oral administration reaching maximum plasma concentration in about 1-2 hours after ingestion. It also can be administered intramuscularly and intravenously although the latter route is rarely used. It is rapidly cleared from the plasma and is concentrated in tissues where active protein synthesis and cell multiplication are highest. Ten to twenty-five per cent of administered chloroquine is excreted unchanged in the urine. It is metabolized in the body into desethylchloroquine and bisdesethylchloroquine. In tissues where it is deposited such as the liver, kidney, lungs erythrocytes, platelets and leucocytes, its presence can still be detected for up to 5 years after administration.

Chloroquine is also used in the treatment of amoebiasis, bronchial asthma, cardiac arrythmias, epilepsy and it has a local anaesthetic effect at high concentrations. Its main side effects include itching, visual disturbances, neuromuscular effects and gastrointestinal discomforts.

Other antimalarial agents such as pyrimethamine, chloroguanide (both inhibitors of dihydrofoleate reductase) sulphonamides and sulphones (inhibitors of folic acid synthesis) act by interfering with parasites biochemical pathway of synthesis of folinic acid from paraamino benzoic acid (PABA). Thus they affect basically the parasites biosynthesis of purines, pyrimedines and certain amino acids.

1.9. PLATELET REACTIONS DURING ACUTE MALARIA INFECTIONS

There are few studies on changes in platelet function during malaria infections. Acute malaria infection causes hemostatic abnormalities in their host; these range from assymptomatic thrombocytopaenia to fulminant disseminated intravascular coagulation (DIC) (Devakul et al 1966, Dennis et al 1966 a,b; 1967 Voller et al 1969). While early investigators suggested that the major coagulation abnormality was DIC, recent findings reported that thrombocytopaenia was an early sign of acute malaria infections whereas DIC is rare (Bearle et al 1972).

This observation has been confirmed in animals and man alike. Abilgaard et al (1975) reported a slowly developing fall in platelet count with a late onset in simian monkeys infected with P. knowlesi. Voller and his group in 1969 observed that in all Aotus monkeys infected with P. falciparum, thrombocytopaenia increased with the degree of parasitaemia. Essien et al (1979) reported a mild depression of platelet counts in Nigerian children with acute falciparum malaria. In golden hamsters it was observed that P. berghei parasitemia was associated with progressive thrombocytopaenia and mild leucocytosis (Essien et al 1984).

In most of these instances, hematological studies indicated enhanced consumption of coagulation factors (Dennis et al 1967, Abildgraad et al 1970, Borochowitz et al 1970). However, Voller and coworkers (1969) explained that the normal fibrinogen values in P. falciparum-infected Aotus monkey was due to the unusually high levels of antithrombin present in the animal. These findings of depletion of coagulation factor and circulating levels of platelets are indicative of a hyper-coagulability state.

Other workers who have examined the mechanisms of thrombocytopaenia with acute infection failed to demonstrate any evidence of DIC in plasmodium induced thrombocytopaenias (Skudowitz et al 1973, Beale et al 1972, Srichaikul et al 1975, Horstman et al 1982, Kelton et al 1983). They interpreted decreased life-span of platelets as being antibody-mediated by showing that there was a rise in platelet related Immunoglubulins A, G and M (IgA, IgG and IgM) during early infection periods. Bearle et al (1972) suggested that immunologically altered platelets during malaria were removed from the blood stream at an enhanced rate by the recticuloendothelial system. In the study conducted by Skudowitz and his group (1973), ^{51}Cr-labelled platelets showed a decreased half-life and excess spleenic pooling but evidence of decreased platelet production was however not documented. The clearance of ^{125}I-fibrinogen from plasma of patients was faster than in controls but was not rapid enough to implicate DIC.

As occurs in erythrocytes, the presence of plasmodium in the platelets (Farjardo 1979) probably induced membrane functional and biochemical alterations. Platelet hypersensitivity to ADP and adrenaline during acute plasmodium infections in man has been reported by Essien and Edinoh (1981).

They later demonstrated increased platelet secretory activities in acute human P. falciparum infections by showing a higher plasma beta thromboglobulin (BTG) and Platelet Factor 4 (PF4) levels as well as lactic dehydrogenase (LDH) loss than in healthy control persons (Essien and Ebhota 1983). They concluded that the latter changes suggested in vivo platelet activation during plasmodium infection, at least in man and that the enhanced platelet lysis which the LDH loss suggested was probably an important mechanism of the reduced circulating platelet numbers reported in patients with the acute disease. The recent observation of enhanced thromboxane B_2 (TXB_2), a disintegration product of TXA_2 and 6 keto-prostaglandin $F_1\alpha$ (6 keto $PGF_1\alpha$), a quantitative breakdown product of prostacyclin (PGI_2), levels in P. berghei infected golden hamsters have been taken to indicate that haemostasis was tilted towards hypercoagulability state during acute malaria infections (Essien et al 1984).

This brief review of changes in platelet functions during acute malaria infections in man and in some rodents has revealed the need for further study of the problems which may lead to improvement of patient management strategy as well as help clarify some of the basic questions of biology of platelets which earlier observations raised.

1.10. RESEARCH OBJECTIVE

The general objective of the present study was to re-examine some aspects of platelet functional changes reported in acute malaria infections in both man and animals and to devise an in vitro model to aid investigations into the mechanism(s) involved in the disorders reported.

The study has the following specific objectives:

(i) To monitor thrombocytopaenia in *P. berghei* infected mice and to examine changes in hemostatic parameters and platelet function. Also to study the effect of chloroquine therapy on platelet levels in infected mice.

(ii) To study platelet survival parameters in the rat malaria model.

(iii) To devise an in vitro system that reproduces the platelet functional changes described in vivo as an aid to examine the mechanism(s) involved in such changes.

It is hoped that this study will add to the scanty body of knowledge on platelet-plasmodium interactions and contribute a better understanding of the mechanism(s) involved in such interactions.

CHAPTER TWO

MATERIALS AND METHODS

.1. ANIMALS

.1.1. Mice: Adult male and female inbred Swiss albino mice aged 7-10 weeks, each weighing between 22-30gms, and maintained at the Central Animal House, University College Hospital, Ibadan were used in these experiments.

2.1.2. Rats: Adult Wistar rats weighing 200-310gm were used in platelet survival studies and sialic acid determinations.

All animals were fed ad libidum with standard pellet diet and unrestricted water.

2.2. INFECTION:

Each adult mouse was infected intraperitoneally with 0.15ml of Plasmodium berghei berghei from infected mouse blood diluted 1 in 3 (v/v) with sterile saline (Essien et al 1984). The strain of P.berghei has been maintained for several years by blood passage in albino mice at the Central Animal House U.C.H., Ibadan. The mice erythrocytes were collected during acute rise in the infection to avoid possible antibody effect occuring during the experimental interval (Zucker and Yoeli 1954). It was calculated that an innoculum size within the range of $1\text{-}5x10^{6-8}$ parasitized red cells per ml of blood was administered to each mouse.

The suckling Wistar rats were infected for platelet survival studies and sialic acid estimation. The suckling rats were innoculated intraperitoneally as described for the mice and with the same dose of innoculum. This age of suckling rats was chosen as Zuckerman and Yoeli (1954) had earlier demonstrated that immunity increased with age as progressively wilder and lower mortality rates were recorded with advancing age. It was necessary

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to separate the suckling rats from their mother as indirect evidence had suggested that there was increased suppression of P.berghei infections in lactating mothers (Maegraith et al 1952) and this immunity could be transferred to the rats if left to breast-feed.

2.3. DETERMINATION OF PARASITE LEVELS

2.3.1. Leishmann Stains (Searle Diagnostics, Bucks, England) were prepared as described in Dacie and Lewis (1975) 1.0gm of powdered dye was dissolved in 1l of methanol in a conical flask. The solution was warmed up to 50°C for 20 minutes with occasional shaking. The dye was allowed to stand for 2 days before it was filtered and used.

2.3.2. Buffered Water: One tablet of phosphate buffered (Oxoid, Chemical, England) was dissolved in water and made up to 1l to give a solution with pH 6.8.

2.3.4. Parasite Levels: In each blood film, parasite levels were determined by examination under light microscope (Leitz Wetzler, Germany) at 40 fold. Parasitaemia was expressed as percentage of the number of parasitized red blood cells including multiply parasitized cells, seen per 1000 red cells.

2.4. MALARIA TREATMENT

After the desired parasitaemia level had been established, usually in about the 3rd or 4th post-infection day, chloroquine sulphate (Quinogal, Galenika, Germany) 0.4mg/20gm body weight (Obih, 1982) was administered intraperitoneally daily for 4 days in one group and 7 days in another group. Only the mice were treated.

2.5. ANTICOAGULATED BLOOD SAMPLES

2.5.1. Anticoagulants: The anticoagulants used in these studies were trisodium citrate (Sigma), heparin (Pularin Heparin Injection B.P, Evans Medical Ltd., Speke, Liverpool, U.K.), Ethylenediamine tetracetic acid (EDTA, BDH, Poole, England) or acid citrate dextrose (ACD) where appropriate. These anticoagulants were prepared as described below.

2.5.2 Trisodium Citrate: A 3.8% solution of trisodium citrate dihydrate (Sigma Chemicals Co. St. Louis, U.S.A.) was prepared in distilled water. For blood collection, 1 part citrate was required for 9 parts of blood.

2.5.3. Heparin: Blood samples for mouse platelet aggregation were collected into heparin (Pularin, Heparin Injection BP, Evans Medical Ltd., Speke Liverpool, U.K.). From a 5000u/ml stock, a 1:10 dilution was made with sterilized normal saline to get a 500u/ml solution.

2.5.4. ACD: This was used to collect rat blood for platelet survival studies and human blood for platelet-plasmodium interaction studies. It was prepared by the Aster and Jandl method (1964) and contained

Trisodium citrate 2.H20	12.5g
Citric acid	7.5g
Dextrose	10.0g

dissolved in 500mls of distilled sterilized water. The pH was adjusted to 4.5 and 1 part ACD was used for 6 parts of blood.

2.6. BLOOD COLLECTION:

2.6.1. Human: All human blood samples were aseptically collected at U.C.H. Blood Bank through antecubital venous puncture. Samples for washed

human platelets were collected into ACD from healthy volunteers who had not ingested aspirin or other drugs known to interfere with platelet function for at least 7 days. Disposable polyethylene tubes were used for blood collection. Platelets were pooled for platelet washing.

2.6.2 Mice: After lightly anaesthetizing each animal with diethylether, blood was collected by clean cardiac puncture through exposed heart and was dispensed into either trisodium citrate (9:1, v/v, blood : citrate) for coagulation studies or into heparin (15-20u/ml of blood, Rosenblum et al 1983) for aggregation and release studies.

2.6.3 Rats: Blood was obtained from adult Wistar rats by the same method as is described above for mice (Winocour et al 1983). The samples were mixed with ACD in a ratio of 1:6 (ACD : blood).

2.7. BLOOD CELL COUNTS

These were performed by standard visual methods using the improved Neubauer counting chamber and counted under a light- or phase contrast-microscope. All dilution fluids were stored at $4^{o}C$.

2.7.1. White Blood Cell (WBC) Count: 20ul of blood was mixed with 380ul of diluting fluid (2% oxalic acid solution coloured pale violet with gentian violet) to give a 1:20 dilution in a glass tube. After mixing, the counting chamber with a cover glass was charged with the dilute WBC, using a clean Pasteur pipette. This was left in a moist chamber for 3 minutes for the cells to settle. The areas designated for WBC were counted and expressed to 10^{9}/litre after taking the dilution factor into account.

2.7.2 Red Blood Cells (RBC) Counts: These were counted by making a 1:200 dilution of blood (20ul of blood into 4mls of counting fluid) in a glass or polyethylene tube. The dilution fluid was a solution of 10% formalin in a 31.3g/litre solution of trisodium citrate. The diluted blood was mixed thoroughly before it was charged into the counting chamber and left for 3 minutes in a moist chamber for the cells to settle. RBC's were counted and the results expressed to 10^{12}/litre.

2.7.3 Platelet Counts: Platelet dilution fluid was prepared as a 1% ammonium oxalate solution in distilled water, coloured blue with a few cystals of copper sulphate. This was filtered, sterilized and stored at $4^{o}C$. 20ul of blood was diluted in 380ul of diluting fluid to give a 1:20 dilution. Platelet counts were done by the method of Bretcher and Cronkite (1950) using a phase contrast microscope.

2.8. BLOOD CELL CHANGES AND COAGULATION STUDIES IN ACUTE P. BERGHEI INFECTION IN MICE

2.8.1. Platelet and White Cell (WBC) Counts: A total of 46 animals were studied. They were divided into 4 groups for this study. Group A comprised of 16 animals infected with P. berghei berghei as described above and was treated with chloroquine for 7 days from the 4th post-infection day to the 10th. Group B comprised also of 16 animals infected as those in group A but received chloroquine treatment for 4 days only from the 4th to the 7th post-infection days. The remaining two groups served as controls. Group C, with 7 animals, received blood similarly treated as for infected mice from uninfected mice and served as the placebo control group. Group D, also with 7 animals, was plain control mice which were not treated in any way.

Both control groups C and D were not treated with chloroquine.

2.8.2. Sample Preparation for Coagulation Studies: Blood was usually collected from 4-5 mice and pooled before platelet rich-plasma (PRP) and platelet poor plasma (PPP) samples were prepared as described below. Blood samples were obtained from animals 3-4 days (for fibrinogen determination some of the animals were bled 48 hours after infection) after infection, when parasitaemia had usually attained levels of 20-30%. The blood from each mouse was collected and mixed in a polypropylene tube containing trisodium citrate (3.8%) in a ratio of 9:1 (v/v, blood : citrate, Dacie and Lewis 1975). The pooled blood sample from the animals was centrifuged at 2600g in a Sorvall RC3B centrifuge for 20 minutes at room temperature. The PPP thus prepared was used for coagulation studies usually within 2 hours of collection, the samples having been kept at room temperature meanwhile.

The coagulation tests were carried out in duplicate and included prothrombin time (PT), activated partial thromboplastin time (APTT) and clottable fibrinogen determined by the clot weight method of Hardisty and Ingram (1975).

2.8.3 Prothrombin Time: 0.1ml of mouse PPP was dispensed into washed glass tubes 75x2.5mm and mixed with 0.1ml of human brain thromboplastin (Homemade after Ingram et al 1975) and placed in a 37°C water bath and left for 1 minute. 0.1ml of 0.025M calcium chloride maintained at the same temperature was then added and simultaneously a stopwatch was started. The tube was gently shaken periodically and immediately the first sign of clot formation was noticed, the stopwatch was stopped and the time recorded in seconds. Duplicate tests were performed for each test and the mean time taken.

2.8.4 Activated Partial Prothrombin Time (APTT): This was carried out as described by the manufacturer of the reagent. To 0.1ml of mouse PPP in an acid washed glass tube (75x2.5mm), was added 0.1ml of prewarmed (37°C) Ortho activated PTT reagent (Activated thromboplax Reagent Optimized, Ortho Diagnostic System Inc. Raritan, N.J., USA) and left standing in a 37°C waterbath for 4 minutes. 0.02M Calcium chloride was then added and the stopwatch was started. The time at end point when clot first appeared gave the APTT in seconds.

2.8.5 Fibrinogen Concentration by Clot Weight Method: To 0.1ml of PPP placed in a 10ml acid cleaned glass tube was added an equal volume of 25mM calcium chloride solution and mixed by inversion. An applicator stick was placed in the tube and left in a 37°C waterbath for 10 minutes for clot to form. At the end of this interval, the clot formed was harvested by gently winding it unto the stick and pressing out the fluid against the side of the tube. The clot covered stick was brought out of the tube, rinsed in distilled water once and the clot was then peeled off the stick and then placed in acetone for 5 minutes before drying in an oven maintained at 100°C. The clot was then placed in a dessicator to cool. The fibrinogen obtained was weighed in a sensitive chemical balance (Ultramatic Model 3, Stanton Instruments, Ltd. England). The fibrinogen formed was expressed in mg/dl:

$$\text{Fibrinogen} = \frac{\text{Clot Weight (mg)}}{\text{vol of plasma (ml)}} \times \frac{100}{1} \text{ mg/dl.}$$

2.9. MOUSE PLATELET FUNCTION STUDIES

2.9.1. Preparation of PRP and PPP: Blood samples were collected as described previously. Normally, samples from 5-7 animals (infected or normal) were pooled together in order to obtain 1-2mls of platelet rich plasma (PRP). The pooled blood was centrifuged at 100g (Rosenblum et al, 1983) at 23-25°C for 30 minutes. The PRP was separated by non-contact process into another polypropylene tube and kept at room temperature for use immediately. The remaining blood was centrifuged at 2600g for another 20 minutes to obtain platelet-poor plasma (PPP), an aliquot of which was used to adjust the PRP to a platelet count in the $400-600 \times 10^9/l$ range.

2.9.2. PREPARATION OF REAGENTS:

i) Adenosine diphosphate (ADP, Sigma Grade I): A stock solution of 10^{-2} M was prepared in deionized, double distilled water and dispensed in 100ul aliquots into tissue culture tubes. These were stored at -20°C until used by reconstituting with Tyrode solution, to the required working concentration.

ii) Collagen: Acid soluble collagen (ASC; Sigma Chemical Co., St. Louis, U.S.A.) was prepared by the method of Cazenave, Packham and Mustard (1973). Bovine tendon collagen (1gm) was soaked in 200ml of 6% glacial acetic acid for 20 minutes at room temperature until the collagen was well hydrated. This mixture was then homogenized for 10 minutes in sets using a 100ml bucket of the homogenizer (Silverson Machines Ltd., Waterside, Chesham,

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Bucks, England). Both the homogenate and the foam formed were then centrifuged at 2500g for 15 minutes at room temperature. After discarding the foamy film on top the centrifuged collagen, both supernatant fluid and gelatinocis button were pooled, diluted with 200ml of distilled water and mixed gently to avoid foaming. This was then centrifuged at 1000g for 10 minutes at room temperature, and the supernatant material stored at $4^{o}C$. This solution was taken as 100% ASC concentration (2500ug/ml). The concentration of collagen used in the studies were expressed as $^{1}/1000$ or $^{1}/500$ and were equivalent to 2.5ug/ml or 5ug/ml collagen respectively. Dilutions of collagen solution were made with isotonic saline solution.

2.9.3 PLATELET AGGREGATION STUDIES:

Platelet aggregation was tested in PRP at $37^{o}C$ using a Payton aggregometer module (8COB) and recorder (Payton Associates, Scarborough Canada) running at one inch per minute. Each PRP aliquot was stirred at 1000 rpm and stimulated with Adenosine diphosphate (ADP, 0.5uM, 1.0uM) or Collagen (2.5, 5.0ug/ml). The points of minimum and maximum light transmission for each sample were preset using a PRP and the corresponding PPP respectively.

Aggregation was allowed to run for 3 minutes in each sample. All aggregating agents added were in volumes of not more than 20ul to give the desired concentration and the total volume in the cuvette was 500ul (agent + PRP). All concentrations of stimulating agents were given as final concentrations after all additions. Aggregation was expressed as a percentage of preset range. Where [illegible] was followed by rapid

rapid deaggregation, the point of maximum light transmission was used to determine the maximum % aggregation.

2.10. UPTAKE AND RELEASE OF ^{14}C-5HT-LABELLED MOUSE PLATELETS:

The uptake and release of ^{14}C- serotonin (5-hydroxy (side chain 2-^{14}C) tryptamine creatinine sulphate) with specific activity of 55mCi/m mole, Amersham Radiochemicals, England) were examined in normal and P. berghei infected mouse PRP. The pooled platelets were labelled by incubating PRP for 20-40 minutes at room temperature with 0.05uCi ^{14}C-5HT/ml of PRP after mixing thoroughly by inversion (Munn 1981). Samples were processed for measurement of uptake and release of the labelled serotonin as described by Greenberg and associate (1975).

50ul aliquot of the labelled PRP was placed in a Packard counting vial containing scintillant immediately after mixing the label with PRP. let the count be x. After the incubation, a 0.1ml aliquot of the labelled PRP was removed into an eppendorf vial, centrifuged at 12000g for 2 minute in an Eppendorf centrifuge (Brinkman, Rexdale, Ontario, Canada) from which another 50ul of supernatant was taken into a counting vial. let this value less background be y. This count represents the radioactivity that was not taken up the platelets after incubation.

Prior to any secretion studies, Imipramine (2uM, Ciba-Geigy, U.K.) was added to the labelled PRP before the aggregation test to prevent any reuptake of released ^{14}C-5HT (Dangelmeir and Holmsen 1983). To induce platelet secretion, 490ul aliquot of labelled platelets was stimulated with 5ug collagen or 1uM ADP and their release determined (The final volume in the cuvette was 500ul). For release determination, the postaggregation

supernatant of each sample was decanted into an Eppendorf tube and centrifuged for 2 minutes at 12000g in an Eppendorf centrifuge. Then 50ul aliquot of each of the centrifuged postaggregation supernatant was removed and placed in a scintillation vial, and processed for counting as described below. Le this count be z.

To each sample, including the blank which was made up of the diluent and scintillant only, was added 200ul of Hyamine hydroxide solubilizer (NEN, Pilot Chemicals Div., Boston, Mass. U.S.A.) vortex mixed and left at room temperature for 20 minutes, before a further thorough mixing with 2mls of absolute ethanol, 10mls of scintillant (made up of 5gm of 2,5, diphenyloxazole (PPO, New England Nuclear), 0.3gm of 2,2-P-phenylene bis (5-phenyloxazole) (POPOP, New England Nuclear) dissolved in 1 litre of scintillation grade Toluene. The votex mixed samples were then each counted for 5 minutes in a Packard Tri Carb-Liquid Scintillation Spectrophotometer model 3390 (Packard Instruments Co. Inc. Illinois U.S.A.). The background counts were subtracted from the counts after appropriate setting of the equipment.

CALCULATIONS: Uptake and secretion (release) were expressed as a percentage of the total radioactivity introduced and taken up into the platelets respectively:

Thus:

Let amount of radioactivity

added to PRP = x cpm

Let the amount of radioactivity in supernatant after incubation and centrifugation of PRP = y cpm

∴ % Uptake of radioactivity by platelets = $\left(\frac{x-y}{x}\right) \times \frac{100}{1}$ %

And for secretion release:

Total radioactivity within platelets = (x-y) cpm

Radioactivity in postaggregation supernatant of a particular sample = z cpm

∴ % Secretion = $(\frac{z-y}{x-y}) \times \frac{100}{1}$ %

2.11. REACTIONS OF IN VITRO PLASMODIUM FALCIPARUM INFECTED ERYTHROCYTES WITH WASHED HUMAN PLATELET SUSPENSIONS (WPS):

2.11.1. Preparation of Silicone-coated glass wares: In all studies, when washed platelets were used, polycarbonate or siliconized glasswares were used in order to avoid platelet adhesion to glassware and its subsequent activation. The siliconized glass-wares were prepared by immersing the acid washed, well rinsed, heat dried glassware in a 10% silicone (Silicone SC-87, Canadian General Electric, Dri-Film, Canada) - solution in carbontetra-chloride, rinsing them again several times in distilled water and drying them in an oven set at 100°C.

2.11.2. Erythrocyte Washing: Unsynchronized Plasmodium falciparum infected erythrocytes maintained in the culture medium RPMI-1640 using the candle jar method (Trager and Jensen 1976) were supplied by Dr. O. Sodeinde, Paediatrics Department, College of Medicine, University of Ibadan, Ibadan. The parasitized erythrocytes were washed thrice in isotonic saline and the counts adjusted to 18-20 x 10^{12}/l. Uninfected red blood cells from the same stock as that used in the parasite cultures were similarly treated in the culture medium (i.e. were sham-cultured) washed and used as controls. The final red cell suspension medium contained 5U/ml Apyrase.

2.11.3. Preparation of Reagents for platelet washing (Mustard et al 1972):

100ml of Hepes-buffered Tyrode albumin solution contained

5mls of stock solution I

1ml of stock solution II

2mls of stock solution III

0.35g Pentax fraction V, Bovine serum albumin (Sigma)

0.10g D-glucose, and the pH was adjusted to 7.35 with hydrochloric acid. This was prepared daily just before use.

STOCK SOLUTION I contained

160g of NaCl

4g of KCl

20g of $NaHCO_3$

1g of NaH_2PO_4

This was dissolved in 1 litre of distilled water and stored at $4^{o}C$.

STOCK SOLUTION II contained

20.3g of $MgCl2.6H_2O$ made up to 1 litre with distilled water and stored at $4^{o}C$.

STOCK SOLUTION III was made up of

21.9l of $CaCl_2\ ^{6}H_2O$ dissolved in a litre of distilled water and stored at $4^{o}C$.

HEPES BUFFER: A 1M Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid; Sigma Chemicals Co., St. Louis MO. USA) solution was prepared by dissolving the accurately weighed powder in half the required volume of distilled water and the final volume

was then made up by dropwise addition of 10N NaOH to adjust the pH to 7.4.

Fibrinogen: A 1% solution of Fibrinogen (Human Fibrinogen, Sigma) was prepared by dissolving 4gms of fibrinogen in 60mls of 0.9% NaCl solution. This solution was adsorbed with 6mls of aluminium hydroxide (BDH moist gel, prepared by sonicating 10gms of $Al(OH)_3$ in 40mls of 0.9% NaCl for 3 short bursts of 30 seconds each using a Sonifier Cell Disruptor [model W185, Heat Systems Ultrasonics Inc. Plainview, L.I. New York]. The mixture was then slowly shaken periodically and kept for 10 minutes at room temperature. It was then centrifuged at 2000g for 10 minutes at room temperature and the supernatant readsorbed with 6mls of aluminium hydroxide. The final supernatant obtained after recentrifugation for the same speed and duration, was dialyzed overnight at room temperature against 0.9% NaCl solution. The dialyzed fibrinogen solution was centrifuged at 37000g (Sorvall Superspeed ultra-centrifuge) for 20 minutes at room temperature. The supernatant was then stored in 0.4ml aliquots at -20°C and thawed once just before use.

Apyrase (Grade I Sigma): A 5mg/ml solution of Apyrase was prepared by dissolving this weight in double-distilled, deionized water at room temperature. It was stored in 0.1ml aliquots at 4°C. This concentration was arrived at after a trial run to determine the optimal concentration required for platelet washing.

2.11.4. Preparation of Washed human platelet suspension (WPS):

This was prepared as described by Mustard and associates (1972). Blood samples were collected as previously described into

polycarbonate tubes containing ACD anticoagulant in proportions of 6:1 (v/v, blood: ACD). For each washing, 200mls of ABO group compatible blood was pooled, 50ml per donor.

To obtain PRP, the blood was centrifuged at 190g for 15 minutes at 37°C using a Sorvall Superspeed RC2B Centrifuge (Automatic refrigerated centrifuge, Sorvall Inc. Newton, Connecticut, USA). The PRP was aspirated into clean 20ml polycarbonate universal tubes avoiding red cell contamination as much as possible. The PRP was then recentrifuged at 2500g for 15 minutes at 37°C to obtain platelet button. The supernatant PPP was discarded and the platelets were resuspended gently in the first washing fluid already dispensed in a siliconized 15ml volume conical glass tube.

The first washing fluid was made up of 10ml HEPES-buffered Tyrode albumin solution (see 2.11.3 earlier) containing 0.55mg/ml apyrase, 5CU/ml heparin. The pH was adjusted to 7.35. The tube was kept in a 37°C waterbath to maintain that temperature. The platelets were incubated in the first washing fluid for 15 minutes before centrifugation for 10 minutes at 1200g at room temperature. The supernatant was discarded and the platelets were resuspended for 10 minutes at 37°C in a second washing fluid kept at the same temperature. It contained Hepes-buffered Tyrode albumin solution, appropriate volume of the apyrase solution but no heparin. The platelet suspension was again centrifuged at 1200g for 10 minutes, the supernatant discarded and the platelets gently resuspended in the final Hepes-buffered-Tyrode albumin solution containing 0.1mg/ml of apyrase and 1mg/ml of glucose.

The washed platelet suspension (WPS) was kept in a siliconized centrifuge tube in a 37°C waterbath. The tube was covered with parafilm throughout the experiment to minimize pH fluctuation caused by contact with free air current (Joist JH, personal communications). The platelet count was adjusted with the Tyrode albumin solution so as to give a final count of 450-650x10^9 platelets/litre suspension in the experiment. Platelets prepared and stored in this way remained viable as assessed by their response to ADP stimulation for periods of 3-5 hours.

2.11.5. PLATELET-ERYTHROCYTE INTERACTIONS:

Description of Experimental model: 1.00ml of WPS was mixed with 0.5ml of washed normal or parasitized erythrocytes in a sterile tissue culture tube. The mixture was agitated in a 37°C waterbath with a shaker attachment, for periods of 1,2 or 5 minutes. The erythrocytes were then sedimented by rapid differential centrifugation in an Eppendorf microcentrifuge at 12000g for about 1-2 seconds. This duration determined experimentally, was found to separate out both cells, leaving a platelet-rich supernatant (PRS) and red cell sediment. The PRS was then gently aspirated, avoiding red cell contamination and was immediately (within 1-2 minutes) used in the aggregation tests. The volumes of platelet and red cell suspensions used in the interaction were adjusted to give approximately a 1:20 ratio of platelets to red cells which approximates to that in vivo. Platelet count in PRS was 300-400x10^9/l.

Two platelet rich supernatant (PRS) volumes were obtained during each experiment. One sample, PRS-i, was the supernatant

obtained following platelet interaction with P. falciparum infected rbcs. The other, PRS-c was obtained after platelet interaction with sham-cultured control erythrocytes.

2.11.6. EXPERIMENTS WITH PLATELET-RICH SUPERNATANTS:

Platelet aggregation: Platelet aggregation with PRS-i or PRS-c was tested by a modification of Born's method (1962) described previously (Section 2.9.3). In order to preset the limits of light transmission in the aggregometer, WPS and platelet washing fluid were used to adjust the minimum and maximum light transmission points respectively. Total volume of WPS including added reagents was kept at 500µl and duration of aggregation was 4 minutes for each run. A 0.25µM ADP in the presence of 0.04% fibrinogen (Greenberg et al 1975) and collagen (2.5 or 5ug) prepared as previously described were used as aggregating agent. All concentrations of reagents refer to the final concentration in the cuvette.

2.11.7. ^{14}C-5HT Uptake and Release Studies with PRS-i and PRS-c:

Thrice washed human platelets used in these studies were prelabelled (before interaction with erythrocytes) in the first washing fluid by incubation for 15 minutes at 37°C with 0.06uCi/ml of ^{14}C - Serotonin (Amersham Radiochemicals England, Specific activity 55mCi/mole; (Greenberg et al 1975). (Preliminary experiments have revealed that interaction of labelled-platelets with erythrocytes did not affect uptake as measured by platelet total radioactivity before and after interaction).

Imipramine was added 15 seconds before any other addition to the platelet suspensions (PRS-i or PRS-c) to inhibit reuptake of released serotonin (Packham et al 1977). ^{14}C-5HT release was induced with 5ug Collagen, or 1.0uM ADP. Samples were prepared for counting and data analysis was carried out as previously described (see Section 2.10).

2.11.8. Effect of ADP inactivation on PRS-c and PRS-i reactions:

Creatine phosphate (CP, 5.0mM, Sigma) in the presence of creatine phosphokinase (CPK, 4.0U/ml, Sigma) (Mann et al 1983) and Pyruvate Kinase (PK, 20U/ml; Sigma) in the presence of phosphoenulpyruvate (PEP, 50uM, Sigma) (Saniabali et al 1984) were used to check the effect of ADP inactivation on ADP-induced aggregation of PRS-c and PRS-i platelets. These enzyme systems were freshly prepared by dissolving them in physiological saline. The solutions were preserved on ice prior to and during use. CP/CPK and PK/PEP enzyme systems were incubated for 20 and 30 seconds respectively with the post-interaction platelet-rich suspensions before stimulation with ADP.

2.11.9. Effect of Thromboxane Synthetase inhibitor on ADP- or Collagen-induced aggregation of PRS-c and PRS-i platelets:

Dazoxiben (UK-37,248-01) (4-[2-(1H-imidazol-1-yl) ethoxy] acid hydrochloride) a gift of Pfizer Research, Sandwich, U.K.) is an imidazole derived selective inhibitor of thromboxane synthetase in human platelets (Tyler, Saxton and Parry, 1981). The in vitro effect of dazoxiben inhibition on human platelet thromboxane TxA_2 generation induced by arachidonic acid is followed by the prevention of aggregation in some individuals,

known as "responders" and not in others, "non-responders" (Bertele et al 1981). Bertele and his group (1984a,b) have shown that 20uM-80uM dazoxiben completely prevented platelet thromboxane B_2 (a breakdown product of TxA_2) generation.

In this study, dazoxiben was used to inhibit thromboxane synthetase activity in PRS-c or PRS-i platelets before aggregation was induced with ADP (0.25uM) or collagen (2.5ug). For this test, dazoxiben was dissolved in normal saline and the solution was made up daily as required. 20uM Dazoxiben was incubated for 1 minute at 37°C with continuous stirring at 1100rpm with the erythrocyte interacted platelets before inducing platelet aggregation. This concentration gave a complete inhibition of thromboxane A_2 generation (Bertele et al 1984).

2.11.10. Experiments with Erythrocyte derived supernatants (EDS):

0.2ml of the washed erythrocyte suspension (prasitized or control) was centrifuged at 12000g for about 5 seconds in the Eppendorf centrifuge and the supernatant collected (i.e. EDS). Thus, we had parasitized erythrocyte derived supernatant (EDS-p) or control erythrocyte derived supernatant (EDS-c).

2.11.10.1. Effect of EDS-p or EDS-c interaction on washed platelet suspension: Increasing volumes (25ul-150ul) of EDS (control (c) or parasitized (p) were mixed with washed human platelet suspensions, stirred at 37°C for 1 minute before inducing aggregation with 0.25uM ADP in the presence of 0.04% fibrinogen. Preliminary experiments to investigate the effect of adding the supernatants to washed platelet suspensions and stirring for up to 4 minutes in the aggregometer had revealed no aggregation inducing effect.

2.11.10.2. Effect of Thrombin Inactivation on addition of EDS-p or EDS-c to WPS before aggregation: EDS-p and EDS-c were incubated overnight in 37°C waterbath to inactivate thrombin (Kwaan et al 1972). 100ul of these erythrocyte derived supernatants was interacted for 1 minute with freshly prepared human WPS at 37°C before the aggregation test was completed with addition of 0.25uM ADP.

2.11.10.3. Effect of heating on EDS-c or EDS-p on platelet aggregation:

The EDSs were heated for 1 hour at 56°C, allowed to cool before interacting with freshly prepared washed human platelet suspension for 1 minute as already described. The platelets were separated by rapid centrifugation and resuspended in platelet suspending fluid before aggregation response to 0.25uM ADP was tested.

2.11.11. Phospholipd Analysis: The following samples were analysed for their total phospholipid contents

(a) Cell free supernatants of PRS-i or PRS-c. This was obtained by centrifuging the PRS(i- or -c) at 12000g for 1 minute and aspirating the supernatant for this test.

(b) PRS-i or PRS-c post aggregation supernatant. After aggregation, the post-aggregation PRS-i or PRS-c was spun at 12000G for 1 minute and the cell-free supernatant of each tested for its total phospholipid content.

The original phosphorous assay method of Fiske and Subbarow (1925) as modified by Varley (1978) was used. The samples were tested for phospholipid in the "Lipid Research laboratory in Chemical Pathology Department, U.C.H. (see Appendix).

2.11.12. SDS- PAG Electrophoretic analysis of Supernatants from PRS-i/PRS-c and post PRS-i/PRS-c aggregation supernatants:

2.11.12.1. Principles: Sodium dodecyl sulphate (SDS)- polyacrylamide gel electrophoresis (PAGE) is used to resolve and characterise the number and size of protein or protein fragments in a protein preparation. The protein is treated by heating at 100°C with an excess of soluble thiols (R-SH e.g. B-mecaptoethanol or dithiotreitol) and SDS to disrupt completely all polypeptide chains by breaking the disulphide (-S-S-) bonds from one another. The polypeptide chains then bind to a large quantity of SDS, thereby gaining a strongly negative charge.

The anionic denatured protein chains are resolved electrophoretically in a buffered environment that contains thiols and SDS plus a high polyacrylamide gel concentration. The thiol and the SDS maintain the denatured state of the disrupted proteins or protein subunits, while the SDS generates a constant charge to mass ratio. The high concentration of polyacrylamide gel generates molecular sieving in which viscosity and pore size of gel define mobility of the protein subunits. The relative mobility of each of the anionic, denatured proteins (as polypeptide chains) is a logarithmic function of the molecular weight of tha polypeptide chain (Clark and Switzer 1977).

The polyacrylamide gels are formed as a result of polymerization of acrylamide and N-N1- methylene-bis-acrylamide. The reaction is catalysed by N,N, N1,N1, - tetramethylene diamine (TEMED) in the presence of ammonium per sulphate. Bromophenol blue was used as the protein tracking dye while the gel was stained in Coomasie brilliant blue. The SDS-PAG Electrophoresis

experiments were performed in the Biomembrane Laboratory of the Biochemistry Department, University of Ibadan, Ibadan.

2.11.12.2. Preparation of Reagents: All reagents were prepared as described by Bewaji (1983)

(a) Acrylamide-bisacrylamide solution: A 100ml solution containing 30% (w/v) acrylamide and 0.8% of bisacrylamide solution in distilled water was made, filtered through Whatman No. 1. paper to remove impurities. This was stored at $4^{o}C$. (This solution was prepared under a hood as acrylamide is toxic). Both reagents were made by BDH Chemicals Ltd. (England). The ratio (g:g) of bisacrylamide to acrylamide of 0.027 which was maintained throughout the gradient is recommended for polypeptide analysis (Nurden et al 1981).

(b) Polyacrylamide (BDH): A 3% solution was prepared in distilled water while being stirred vigorously to obtain a homogenous mixture.

(c) Stacking gel buffer: This solution contained

Tris - Chloride (Calbiochem-Boerhinger USA)	0.5M
SDS (Fluka-Garantie, Switzerland)	0.1%
EDTA (Sigma)	8.0mM
pH was adjusted with NaOH to	6.8.

The solution was made up in distilled water to an initial volume of 500mls. The pH was then adjusted and the volume was then made up to 1 litre.

(d) Running (Seperating Gel) buffer: This buffer was made up initially to 500ml as follows

Tris	1.5mM
EDTA	8mM
SDS	0.1%

The pH was adjusted to 8.8 and the volume then made up to 1 litre. Both the running and the stacking gels were stored at $4^{o}C$.

(e) Electrode buffer: Contains

24g of Tris-base

115.2g of glycine (Hopkins and Williams, England)

4.0g of SDS

2.7g of EDTA,

dissolved in 4 litres of distilled water. The solution was prepared with constant stirring.

(f) Sodium phosphate buffer: Contained

30.5mls of 0.2M $Na_2HPO_4.12H_2O$ solution

and 19.5mls of 0.2M $NaH_2PO_4.2H_2O$ solutions

with a pH of 7.0. Both solutions were prepared in distilled water.

(g) Sample buffer: (3 times concentrated): This was prepared and dispensed into test tubes (5ml tubes) in aliquots of 1ml and stored at $-20^{o}C$ until use. The sample buffer contained

1.2ml	of	sodium phosphate buffer
6.0 ml	of	glycerol
1.5g	of	SDS
30mg	of	Dithiothreitol (DTT, Sigma)
10mg	of	bromophenol blue
20ml	of	distilled water.

(h) Preparation of Protein samples: The protein concentration of supernatants from PRS-i, PRS-c, or their post aggregation cell free supernatants (both samples were cell-free obtained after centrifuging for 3 minutes at 12000g and collecting the supernatant for the test) which had been stored at -20°C, were determined by Lowry's method (1951). The protein contents of each sample was then adjusted to 25-30ug, which was considered to be optimal for the SDS-PAGE. Two volumes of each adjusted sample was then mixed with 1 volume of thrice concentrated sample-buffer and the mixture was then heated at 100°C for 3 minutes.

Protein Estimation: Protein concentrations in samples for SDS-PAGE and sialic acid were determined by the Lowry's method (1951) using bovine serum albumin as standard.

Principle: The colour reagent used in this method is the phosphomolybdate-phosphotungstate complex. The colour formed by the Folin-Ciocalteu reagent is caused by the reaction of proteins with alkaline copper in the reagents and the reduction of the phosphomolybdate-phosphotungstate salts in the reagents by the tyrosine and tryptophan of proteins. This reaction is caused by the reduction of the phenol groups in the protein aminoacids giving a blue colour at alkaline pH.

Since the complex interacts at alkaline pH, an excess of the reagent must be added to take the reaction to completion. Lowry et al (1951) demonstrated pretreatment of protein samples with alkaline copper markedly increased colour development in the reduction reaction with the

reagent. In their assay system, they also added a mixture of Na_2Co_3-NaOH to buffer the pH around 10 and to neutralize the phosphoric acid produced by the degradation of the phosphomolybdictungstic complex at alkaline pH.

Reagents:

Reagent A: This was a solution of 2% Na_2CO_3 (anhydrous) in 0.1N NaOH made up to a volume of 500mls.

Reagent B: Was a 100ml solution of 0.5% $CuSO_4.5H_2O$ made up in 1% sodium citrate.

Reagent C: This was prepared fresh just before use by mixing 50mls of Reagent A and 1ml of reagent B.

Reagent D: This was the Folin-Ciocalteu reagent (BDH Chemicals Ltd., England) which contained the phosphomolybdictungstic complex, lithium sulphate and bromine water. The working solution was a 1:3 dilution using distilled water as diluent.

Protein standards: 10-100ug/ml solutions of bovine serum albumin (BSA, Sigma Chem. Co. St. Louis USA) from a stock of 1mg/ml BSA was used. The protein standards were made up in distilled water to a volume of 0.6ml each. 6 standard tubes contained 10 to 100ul of standard (x) were prepared.

Blank: This was 0.6ml of distilled water.

Solutions:	Test	Blank	Standards
Protein standard	-	-	x mls
Test sample	10ul	-	-
Distilled water	590ul	600	0.6-x mls
Reagent C	3.0ml	3.0ml	3.0ml

The mixtures were vortex mixed rapidly and left for 10 minutes, after which 0.3ml of Reagent D was added to each tube and each sample at 750nm was read after leaving it to stand at 30 minutes and room temperature. A Bausch and Lomb Spectrophotometer (Spectronic 21, Bausch and Lomb, USA) was used to determine the optical density.

Various protein standard concentrations were treated as the test sample. The O.D. values were plotted against protein concentrations on a plain graph paper and the value of the test sample obtained from reading its O.D. off the corresponding protein concentration on the graph.

(i) Preparation of Stacking and Running gels: The composition is given below

Solutions	5% Stacking(mls)	12% Running gels(mls)
30% Acrylamide -0.8% bisacrylamide	1.3	8.0
Stacking gel buffer	2.0	-
Running gel buffer	-	5.0
TEMED (Sigma)	0.005	0.01
3% Polyactylamide ($MW.5x10^6$)	1.3	3.3
Distilled water	3.3	3.6
10% Ammonium per sulphate	0.16	0.10
Total	8.0ml	20ml

Solutions:	Test	Blank	Standards
Protein standard	-	-	x mls
Test sample	10ul	-	-
Distilled water	590ul	600	0.6-x mls
Reagent C	3.0ml	3.0ml	3.0ml

The mixtures were vortex mixed rapidly and left for 10 minutes, after which 0.3ml of Reagent D was added to each tube and each sample at 750nm was read after leaving it to stand at 30 minutes and room temperature. A Bausch and Lomb Spectrophotometer (Spectronic 21, Bausch and Lomb, USA) was used to determine the optical density.

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Stacking gel buffer	2.0	-
Running gel buffer	-	5.0
TEMED (Sigma)	0.005	0.01
3% Polyactylamide (M_w.5x10^6)	1.3	3.3
Distilled water	3.3	3.6
10% Ammonium per sulphate	0.16	0.10
Total	8.0ml	20ml

The ammonium per sulphate was added immediately before pouring the gel while the 3% polyacrylamide solution had to be added from a large-bored pipette and stirred vigorously to give a homogenous mixture.

2.11.12.3. Procedure: The apparatus described by Reid and Bieleski (1968) was used. The discontinous pH, 5-12% polyacrylamide gradient slab gel system of Laemmli and Favre (1973) was used. A sandwich was made with 2 glassplates separated by plastic spacer strips and was clamped with 2 large binder clips at each side of the electrophoresis apparatus. The space was filled with the running gel to 3cm level from the notched plate, using a 50ml syringe with a 16 or 17G needle. The upper surface was gently overlayered with 1/4 concentrated running gel buffer using a Pasteur pipette. The gel was allowed to set for 2-3 hours.

The overlayered solution was then poured and the remnants blotted with filter paper. The stacking was then poured over the running gel leaving a 5mm space from the top. A Teflon comb which form the sample wells was carefully inserted to eliminate air bubbles. The comb was slowly removed after an hour avoiding distortion of the well.

Samples prepared as described above in equal volumes of 60ul each, were underlayered using a Hamilton microsyringe. The two outerwells were filled with only sample buffer while the other wells were filled with samples. The protein content of each sample was adjusted by dilution with normal saline where necessary to 25ug/ml.

The positive lead was connected to the lower electrode while the negative lead was connected to the upper electrode. The gel was run overnight at 35mA and a constant voltage of 30V at room temperature (In some experiments the gel was run at 35mA at a constant voltage of 50V at room temperature) until the tracking dye was within 1.0cm from the bottom.

After separating the gel from the plate and cutting off the lower left corner of the gel to mark sample 1, the proteins were fixed in the gel for 30-60 minutes with 10% trichloroacetic acid in 50% methanol. This was then rinsed in distilled water and stained for 2 hours with 0.25% Coomassie Brilliant blue in 50% methanol/7.5% acetic acid (Reid and Bieleski 1968). The gel was rinsed twice with distilled water before they were destained by repeated washing in a solution containing 7.5% acetic acid in 20% methanol. Destaining was facilitated by adding a little cotton wool until the bands on the gels became clear.

2.12. PLATELET SURVIVAL STUDIES IN RATS INFECTED WITH P. BERGHEI BERGHEI:

2.12.1. Suckling and adult Wistar rats were used in these experiments. The adult rats provided the homologous platelets used for labelling. Infection of suckling rats and blood collection in adult rats have previously been described (Section 2.2 and 2.6 respectively). About 6-8mls of blood was obtained from each adult rat.

2.12.2. Reagents for rat platelet washing (Arlie et al 1970; Kinlough-Rathbone et al 1983).

100mls of calcium free Tyrode albumin solution contained

5mls of stock I

2mls of stock II

0.35g of Pentax Fraction V BSA

0.10g of D - Glucose

2mls of 1M HEPES buffer hydrochloric acid.

The composition of stock solution I, II, and III were as previously described (Section 2.11.3.).

EGTA stock: 3.8g of Ethyleneglycolbis (aminoethyl) tetraacetic acid (EGTA, Sigma) was dissolved in 80mls of distilled water and the remaining volume made up by dropwise addition of 10M NaOH solution to make a 3.8% solution at pH7.0. A 20% solution of EGTA was prepared from the stock by adding 9mls of isotonic saline to 10mls of Stock EGTA solution.

2.12.3. Preparation of washed rat platelet suspension: This was prepared with adequate sterile precautions as described by Arlie et al (1970). All containers used were autoclaved for 20 minutes at 15 bar pressure. Polycarbonate tubes or siliconized heavy duty conical centrifuge tubes were used. PRP was prepared by centrifugation of adult Wistar rat blood for 15 minutes at 190g and the platelet rich supernatant aspirated. Unlike in the preparation of washed human platelet, the washing procedure was carried out at room temperature. Other details for obtaining platelet button were as used with human platelet. The platelet poor plasma (PPP) thus obtained was preserved for further use.

The platelet button obtained was immediately gently resuspended in 10ml of sterilized calcium-free Tyrode albumin solution in a siliconized glass tube to which was also added 50ul of 2% EGTA. The platelets were labelled in this first washing fluid with 50uCi of sodium ^{51}Cr - Chromate ($Na_2\ ^{51}Cr\ O_4$, 1m Ci/ml, 2.3ug Cr/ml specific activity; Amersham International plc, Amersham U.K) per rat (Winocour et al 1983). This mixture was left for 15 minutes at room temperature. The labelled platelet suspension was centrifuged for 10 minutes at 1200g, the supernatant discarded and the platelet button obtained, again resuspended for another 10 minutes in calcium-free Tyrode albumin solution without EGTA. The centrifugation and resuspension of platelet button were repeated. The final platelet suspension medium was in the PPP at a concentration of $1.50\text{-}2.50 \times 10^9$ platelets/ml (Winocour et al 1982.

2.12.4. Platelet Survival: A 0.5-1.0ml volume of the labelled platelets was injected using a 25G needle into the tail vein (near the tail base) of both the already infected and normal suckling rats which were previously anaesthetized lightly with diethylether. The veins were dilated by cleaning with xylene, before cleaning with methylated spirit. Backflow of injected labelled-platelet suspension was checked by pressing hard at the point of injection for 1 minute before releasing the animal. The control group consisted of 4 rats while 8 rats made up the infected group. The % parasitemia in the injected group was determined as previously described (Section 2.3.4.).

2.12.5. Sampling and determination of radioactivity: This was carried out as described by Winocour and associates (1982, 1983). The radioactivity in 100ul of labelled rat platelets was determined in a Packard Auto Gamma Scintillation Spectrophometer (Model 5360, Packard Inst. Co. Inc. Illinois, U.S.A.). The count was later used to estimate the total radioactivity injected into each animal.

Four hours after the injection of ^{51}Cr-labelled platelets, blood samples were collected from freshly cut tail ends of each rat into heparinized hematocrit tube (Celman-Hawksley Ltd., Sussex England). (The 4 hour interval was allowed for total recirculation and even distribution of the injected labelled platelets). The hematocrit tubes were sealed at one end with plasticine and the length of the blood columns recorded immediately. From the weight of known length of water in a number of hematocrit tubes (12), a standard diameter of the tube was calculated (0.0449 $\pm$ 0.00095cm; see Appendix table 4) from which the volume of each blood sample was estimated.

From this volume the radioactivity per ul of blood was calculated. The blood sample taken at the 4th hour period after labelling formed the 100% sample. Subsequent samples were taken by the same procedure at 17.0, 42.5 and 66 hours after labelling. Radioactivity of these samples were determined at the end of the experiment (after 66 hour sample collection) thus evening out decay. The relatively short period of sample collection (17-66 hours) minimized the decay of radioactivity (Winocour et al 1983).

CALCULATIONS:

Radius of hematocrit tubes:

Let weight of column of water in hematocrit tube = W gm

Height of water column in hematocrit tube = H cm

Density of water at room temperature = Dg/cm^2

But Density $D = \frac{\text{Mass}}{\text{Volume}} \triangleq \frac{\text{Weight}}{\text{Volume}} = \frac{W}{V}$ ... (1)

Where V = volume of water

The volume of a cylinder is given by the equation $V = \pi r^2 H$

(where r = radius of tube)

By substituting for V in (1)

$$\therefore \quad r = \left(\frac{W}{D\pi H}\right)^{\frac{1}{2}} \quad \ldots\ldots\ldots \quad (2)$$

Since W, H and D are known the radius was easily calculated.

Calculation of total radioactivity administered into animals :-

Let volume of label injected into each animal = V_1

Let the count from 100ul of labelled-washed platelets = N_1

$\therefore$ radioactive count/ul of labelled platelet suspension = $\frac{N_1}{100ul} = N_2$

$\therefore$ Total radioactivity injected into animal = $\frac{N_1 V_1}{100}$

$= N_2 V_1 = R$ ..(2)

% Recovery according to Barret and Butter (1983) is given by

$$\% \text{ Recovery} = \frac{\text{Blood volume} \times \text{cpm/ml of blood}}{\text{Total count injected into animal}}$$

where cpm = count per minute.

From the value of R obtained in (3) the volume of labelled blood sample in the hematocrit tube was calculated since the height of blood was known.

Let this volume be V_2 (ul)

Let the total count be P

:. cpm per ul of blood = $\frac{P}{V_2}$

:. cpm per ml of blood = $\frac{10^3 P}{V_2}$ = T

:. % Recovery = $\frac{BT}{R}$ where B = blood volume of animal

R = Total radioactivity injected into animals

T = cpm/ml of blood.

The blood volume of the suckling rat used in the calculation is given as 7.59% of the body weight (Belcher and Harris 1957) i.e. 2.31ml where the mean weight of rats used was 30.47 ± 1.75g as determined from 12 rats (4 controls and 8 infected).

2.12.6 Platelet Survival Parameters: Platelet survival curves were obtained for both the control and infected groups of animals by plotting recovery values as a percentage of initial recovery (i.e. recovery at time zero, taken as the count 4 hours after injection) against the time of sampling (Aster 1967). Recovery was expressed as the value of the circulating platelet specific activity expressed as a percentage of recovery which would have been obtained if all the infused radioactive platelets had appeared in the recipient rat circulation (Ebbe et al 1965).

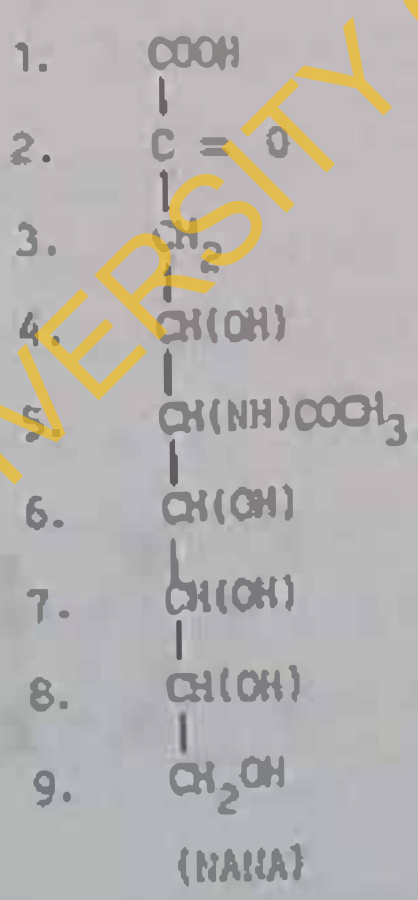
From the survival curves, the mean survival time and half-disappearance time ($\frac{t}{2}$) for each rat group were determined. The mean survival time was given by extrapolating the platelet survival curve to the abscissa and the value at this point read

off the survival curve and was the time (in hours) which it took half the platelet radioactivity to disappear from circulation (Aster 1969).

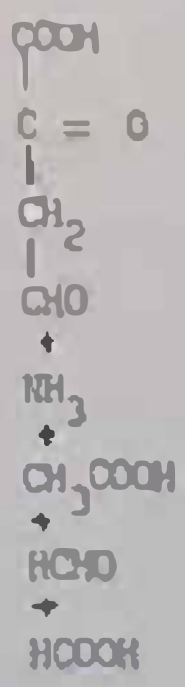
2.13. DETERMINATION OF TOTAL SIALIC ACID CONTENT IN WASHED PLATELET SUSPNSION FROM CONTROL AND P. BERGHEI INFECTED RATS:

2.13.1. Principle: Platelet free sialic acid (SA) in the presence of periodate, undergoes oxidation to form B-formylpyruvate, the excess of the periodate oxidizing agent being neutralized with either sodium arsenite (Warren 1959, Aminoff 1961) or sodium thiosulphate (Denny et al 1983). The free chromogen thus formed (presumed to be similar to that formed by 2-oxo-2-deoxysugar acids) was coupled to thiobarbituric acid and the final colour extracted into butan-1-ol (Aminoff 1961) or cyclohexanone (Warren 1959) for colorimetry.

The reaction is summarized as follows:



(NANA)



Oxidative cleavage of N-acetylneuraminic acid (NANA) by periodate occurs between C4 and C5 (Aminoff 1961).

2.13.2. Infection of Animals: Blood collection and determination of parasitaemia in suckling rats were as earlier described.

2.13.3 Washed rat platelet suspension: Washed rat platelet suspensions from normal and P. berghei infected animals were prepared as described by Arlie et al 1970 (Section 2.12.3) with some modifications. The modification was that the platelets were finally resuspended gently in citrated Tris-buffered saline (TBS; containing 0.15M NaCl, 20mM Tris, 10mM sodium citrate, 5.6mM citric acid, pH 7.4) with the least possibledisturbance of the erythrocyte button (Soslau and Giles, 1982). Platelets from 4-5 animals were pooled and the final volume of the WPS for each set was 1.1ml with a count of $200x10^9$ per ml (Greenberg et al 1975). The platelets were suspended in TBS to avoid a possible effect of glucose on the platelet SA content to be estimated. Platelets were counted by the method of Bretcher and Cronkite (1950) using phase contrast microscopy.

2.13.4. Protein estimation: Platelet protein was determined by Lowry's method (Lowry et al 1951) previously described in section 2.11.12.2 with bovine serum albumin as standard. Protein contents were expressed as mg of protein per 0.5ml of WPS. A standard curve was determined for each assay. A 0.5ml volume of WPS was lysed with 125ul of 20% Triton-X100 (BioRad Laboratories, California, USA) made up in normal saline. The lysate was incubated for 5 minutes at 37°C before protein estimation (Bunting et al 1978).

2.13.5. Total Sialic acid determination: Total platelet sialic acid was measured in both control and infected samples by the thiobarbituric acid method of Aminoff (1961) described below. A 0.5ml volume of

the WPS was placed in a heavy walled glass tube containing an equal volume of 0.2N H_2SO_4. After mixing the solution was incubated in a 50°C water bath for 1 hour to release, by mild hydrolysis, membrane bound sialic acid (N-acetylneuraminic acid, NANA) without degradation (Warren 1959). The hydrolysed platelet suspension was centrifuged at 1500g for 5 minutes at room temperature, the supernatant decanted into a clean tissue culture tubes and stored at -20 °C until analysis, usually within 4 days of preparation. For total platelet sialic acid determination in both control and infected samples.

0.25ml of 25mM periodic acid (prepared in 0.125M H_2SO_4, pH 1.2) was added to 0.5ml of each sample. A blank (butan-1-ol) or standard (1-40ug of NANA, Sigma Chem. Co. Ltd. USA) dispensed in a clean glass tube was included with each run. The tubes were incubated for 30 minutes in a 37°C waterbath and the excess periodate was reduced by mixing without delay, 0.2ml of sodium arsenite (2% solution of sodium arsenite in 0.5M HCl) with each sample. A yellow colour was observed. Immediately the yellow colour disappeared, 2mls of thiobarbituric acid (TBA, 0.1M solution of TBA in distilled water, pH adjusted to 9.0 with NaOH, and stored in a dark bottle at 4°C) was added and the mixture heated to 100°C for 15 minutes (Denny et al 1983) to achieve optimum colour production. The tubes whose contents showed pink colour were cooled in crushed ice-pack. A 5ml volume of acid butan-1-ol (butan-1-ol containing 5% v/v, of 10M HCl) was added to each tube, capped and shaken vigourously. Complete phase seperation was affected by centrifugation of each tube at 400g for 5 minutes.

The top butanol phase was carefully removed and the intensities of the colour in this butanol phase read off against the blank using a Bausch and Lomb Digital Spectrophotometer (Spectronic model 21, USA) at 549nm.

CALCULATIONS:

The absorption spectrum of the colour obtained with N-acetyl neuraminic acid (NANA) was identical with that obtained by Warren (1959). The molar extinctions ratio at 549nm was 70.7×10^3 and this was directly proportional to the concentration of NANA (Aminoff 1961). The sialic acid contents of the samples were calculated from Warrens equation (see below):

$$\text{nMoles of } \overline{/NANA/} \text{ in samples} = \frac{V \times O.D_{549nm}}{E}$$

where V = final volume in test solution (2.95 ml)

E = molar extinction coefficient

$= 7.07 \times 10^4$ (Aminoff 1961)

O.D = optical density at 549nm

Values were obtained by calculation and these were very close to those obtained by reading off the standard curve.

CHAPTER THREE

RESULTS

3.1. CHANGES IN PLATELET COUNT DURING P. BERGHEI INFECTION IN MICE:

In all the 4 groups of mice studied (i.e. groups A, B, C and (D) the baseline platelet counts were within the normal range of 716-1314x10^9/l. There was no significant difference between the two control groups (C and D) as shown in Table 2.

There was a rapid drop in platelet count with increasing parasitaemia leading to death in 6-7 days if the animals were untreated (unpublished data). Plate 1 shows a typical blood film from a P. berghei infected mouse. Infected mice on treatment were in two groups. Group A animals received 0.02mg of Chloroquine per gm body weight for 7 days (i.e. post-infection days 4-10) while group B animals were also treated with the same dose of chloroquine but only for 4 days (days 4-7). A significant fall in platelet count was recorded by the 4th post-infection day for animals in group A ($p < 0.005$; $t=9.576$) as shown in Table 3. The platelet count continued to fall up to a day after chloroquine treatment was stopped on day 11. It was also observed that for animals in group A, the rate of fall in platelet count from day 0 to 11 (844.2±46.8 on day 0, to 317.5±62.8 on day 11) was not as steep as that observed in group B animals during the same interval (894.2±146.8 on day 0 to 81.5±13.5 on day 11; Table 4).

In both groups of animals (A and B), it was observed that the platelet count continued to drop even though the parasitaemia was gradually falling. In group A animals that received treatment for 7 days, parasitaemia was cleared by the last day of treatment, while in group B animals on the 4 days of treatment, a low level of parasitaemia persisted to the 27th post-infection day.

TABLE 2

Summary of WBC and Platelet Counts in normal Swiss albino mice.

DAYS	GROUP C		GROUP D	
	PLATELET COUNT ($x10^9/l$)	WBC COUNT ($x10^9/l$)	PLATELET COUNT ($x10^9/l$)	WBC COUNT ($x10^9/l$)
0	931.3±41.0	10.46±1.27	906.6±32.2	8.26±1.34
2	880.6±28.9	10.23±0.71	926.0±26.6	8.43±1.28
4	924.6±80.1	10.56±1.32	919.8±53.7	8.04±0.6
7	888.6±36.2	10.4±1.14	936.4±29.9	8.38±1.57
11	900.4±19.3	11.67±0.68	950.0±30.5	8.74±1.89
27	850.3±20.2	10.02±84	901.9±42.5	7.95±1.80

Group C animals were innoculated intraperitoneally with normal mouse red cells while Group D animals served the plain control group (see text).

n = (no. of animals studied) = 7 for each group.

It is seen that there was no difference in platelet counts between groups C and D throughout the duration of the experiments. The presence of red blood cells in the peritoneum however caused a slight but not statistically significant increase in white cell count (see Group C of D). Values represent Mean ± S.D.

Plate 1: A blood film of P. berghei infected mouse erythrocytes. (x 1000)

TABLE 3: Summary of Platelet and WBC counts in Swiss albino mice infected with P. berghei (Group A) and treated with chloroquine sulphate for 7 days (4th - 10th post-infection days).

DAYS	PLATELET COUNT ($x10^9/l$)	W.B.C. COUNT ($x10^9/l$)	% PARASITAEMIA
0	844.2±46.8	8.21±1.28	0
2	794.6±53.4	10.03±1.31	2.92±2.27
4	514.0±78.2	14.38±1.04	19.6±6.1
6	ND	ND	8.3±2.4
7	348.1±98.1	12.56±1.57	1.81±0.91
11	317.5±62.8	11.16±1.22	0
14	521.9±63.9	8.75±1.24	ND
27	812.0±82.8	8.91±0.47	0

n (no. of animals studied) = 16

ND = not determined.

Values represent Mean ± S.D.

TABLE 4: Summary of WBC and Platelet counts in Swiss albino mice infected with P. berghei and treated with chloroquine sulphate for 4 days (4th-7th post-infection days) /Group B7.

DAYS	PLATELET COUNT ($x10^9/l$)	W.B.C. COUNT ($x10^9/l$)	% PARASITAEMIA
0	894.2±146.8	7.40±1.06	0
2	665.7±60.1	10.63±1.26	4.02±1.1
4	326.0±110.3	12.38±3.31	24.23±4.5
7	208.5±47.7	15.50±4.21	1.70±0.41
11	81.5±13.5	11.76±2.92	2.39±1.3
14	ND	10.12±5.34	ND
27	347.3±130.0	7.27±0.91	2.1±0.9

n (no. of animals studied) = 16

ND = not determined.

Values represent Mean ± S.D.

The platelet count in group A animals had by this time returned to baseline values. There was no statistically significant difference between the mean counts on day 0 and day 27 in this group ($p > 0.05$; $t=0.895$). By contrast, the platelet count in group B animals which received treatment for only 4 days was still significantly lower than baseline values by the 27th day ($p < 0.005$; $t=10.4$). Fig. 9 show changes in platelet count in all the animal groups together with % parasitaemia in the infected groups; it is seen clearly that peak parasitaemia was first attained before the lowest platelet count was recorded in both infected groups A and B mice.

3.2 CHANGES IN WBC COUNT:

With respect to the two control groups C (placebo group i.e. animals aseptically injected with normal mouse blood) and D (i.e. plain control mice), the WBC levels remained within normal range throughout the experimental period, with the placebo group (C) showing a slight increase in WBC count on day 11 but this remained within normal range of 5.0 - $16.7 \times 10^9/l$ obtained for mice (Archer and Jeffcoat 1978). No significant differences were detectable between the mean daily WBC counts of the groups (C and D) throughout the duration of the experiment.

In the other two experimental groups of mice A and B, infected with P. berghei but given different treatment regimes, WBC rose from $8.21 \pm 1.28 \times 10^9/l$ on day 0 to $14.38 \pm 1.04 \times 10^9/l$ on day 4 ($p < 0.005$; $t=9.90$) while in group B, it rose from $7.40 \pm 1.06 \times 10^9/l$ on day 0, to $12.38 \pm 3.31 \times 10^9/l$ on day 4 ($p < 0.005$; $t=8.05$). Fig. 10 summarizes the changes in WBC in all the animal groups A, B, C and D, and also relates WBC to percentage parasitaemia in groups A (on 7 days treatment) and B (on 4 days treatment).

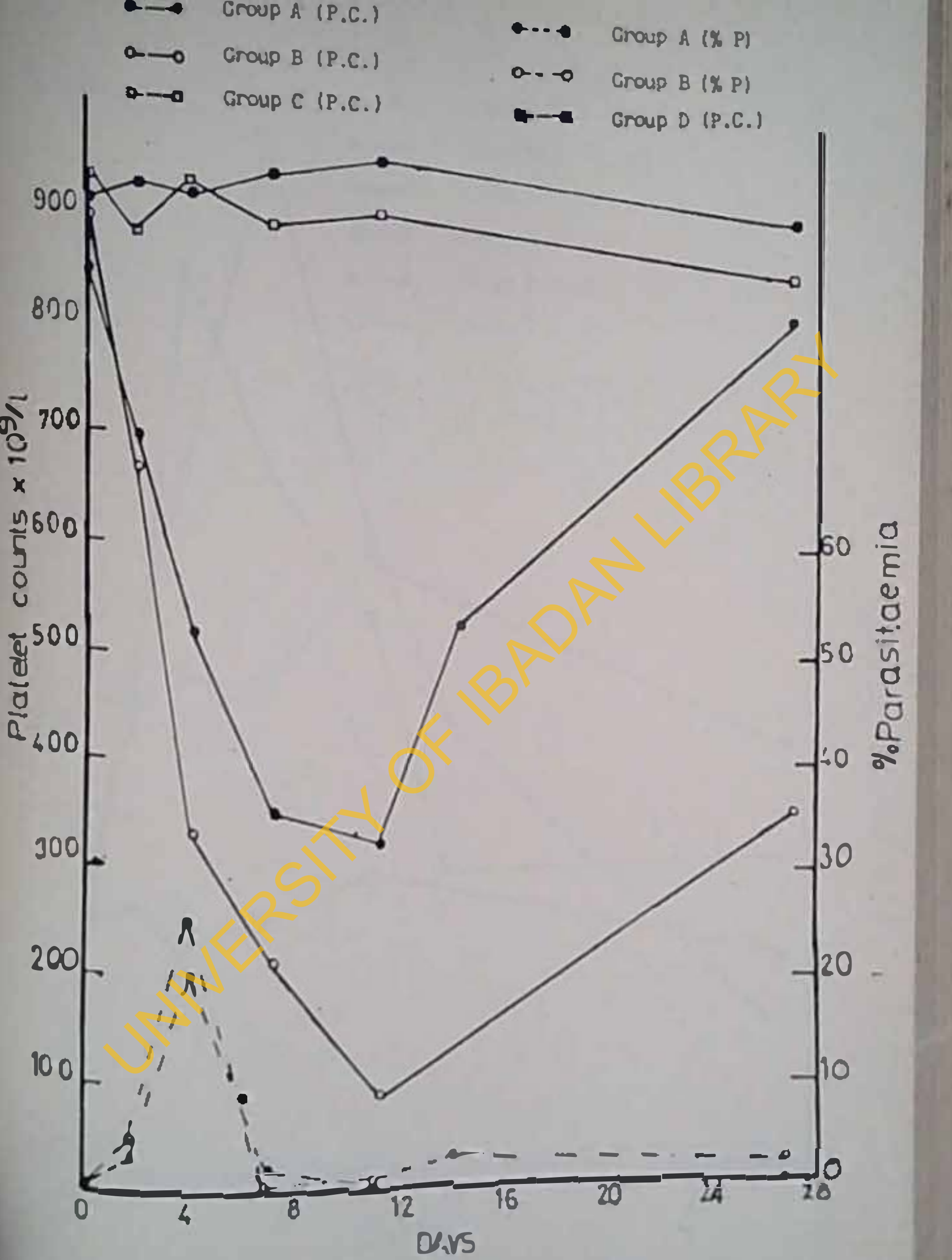


Fig. 9. Graph showing changes in platelet counts and % parasitaemia in infected mice (groups A and B) and platelet changes in the normal mice (groups C and D). (P.C. = platelet count; % P = percentage parasitaemia).

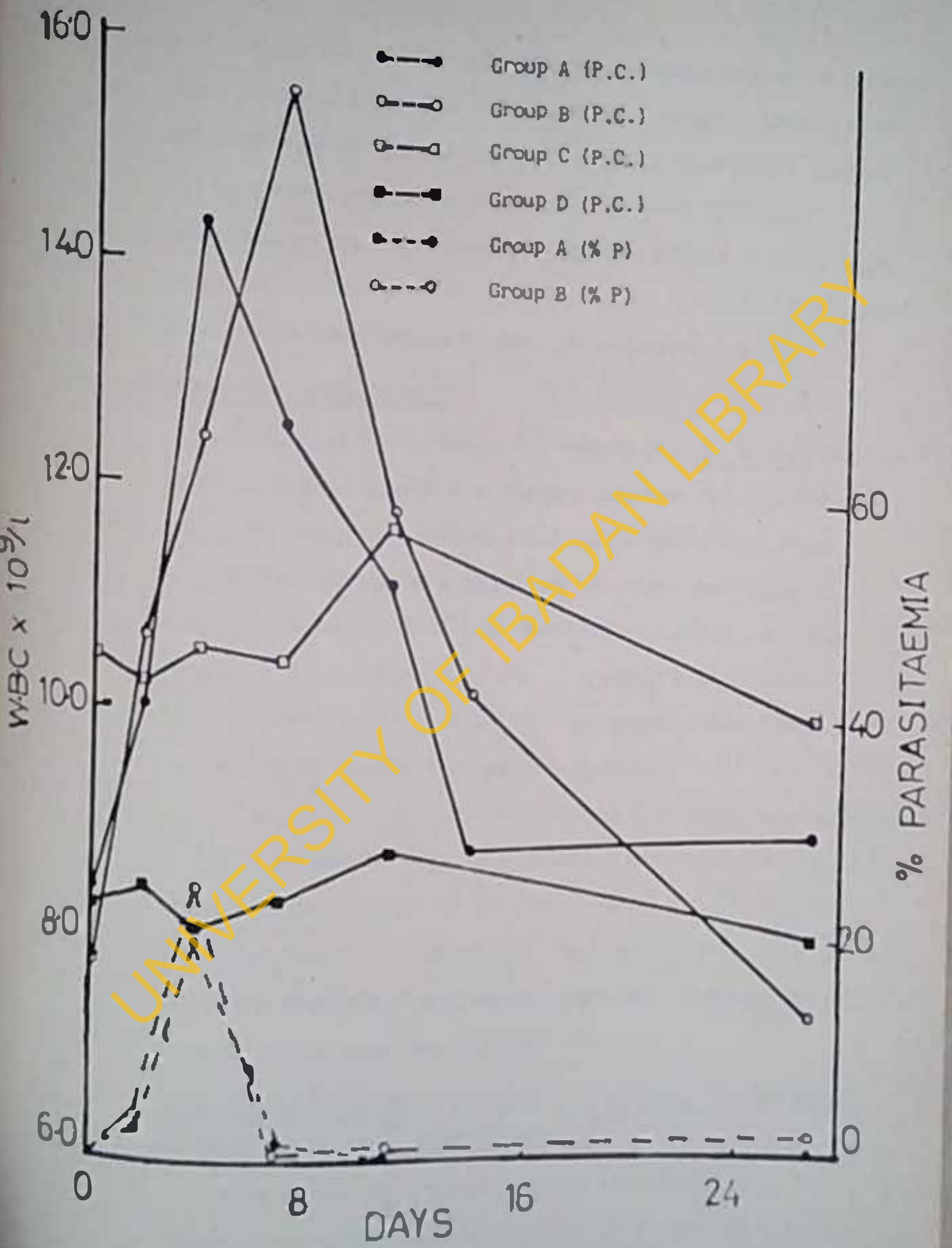


Fig. 10. Graph showing changes in WBC counts and % parasitaemia in infected mice (groups A and B) and platelet changes in normal mice (groups C and D).
(P.C. = platelet count; % P = percentage parasitaemia).

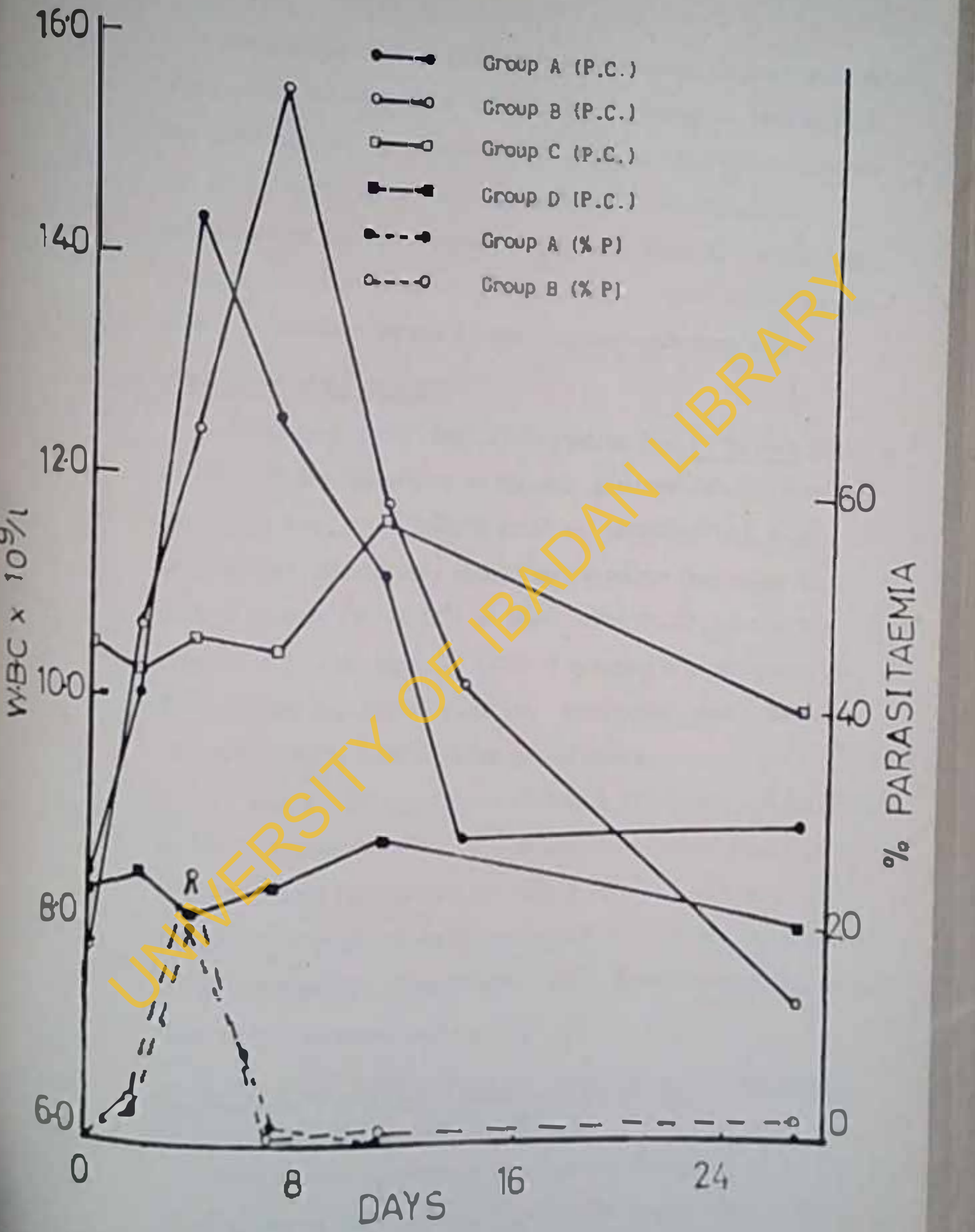


Fig. 10. Graph showing changes in WBC counts and % parasitaemia in infected mice (groups A and B) and platelet changes in normal mice (groups C and D).
(P.C. = platelet count; % P = percentage parasitaemia).

Unlike the fall in platelet count observed in group A animals which persisted long after initiation of chloroquine therapy, the WBC count started to drop with initiation of chloroquine treatment and by the 14th day had attained baseline level which was maintained to day 27. However, in group B animals, the WBC count continued to rise up to the 7th day ($15.5 \pm 4.21 \times 10^9/l$) but dropped slowly to baseline values by the 27th post-infection day.

3.3. BLOOD COAGULATION STUDIES:

Results of blood coagulation studies from *P. berghei* infected albino mice are summarized in Table 5 (also see appendix Tables 1-3). The mean PT(11.70 ± 2.55 secs) and APTT(45.5 ± 12.5 secs) in the infected animals were significantly longer than those in control animals (PT, 8.13 ± 1.94 secs; APTT, 29.0 ± 7.2 secs; Fig. 11). From Table 5 (also appendix Table 3) a negative correlation was found between parasitaemia and the prothrombin time. There was no correlation between APTT and parasitaemia.

A significant decrease in clottable fibrinogen concentration in the *P. berghei* infected mice plasma was observed when compared with the values from plasma of control animals ($p < 0.005$; $t = 3.77$). A negative correlation which was significant at both the 5 and 1% levels was observed between parasitaemia and fibrinogen concentrations in the infected animals (Fig. 12).

3.4. CHANGES IN MOUSE PLATELET FUNCTION (AGGREGATION), ^{14}C-5HT UPTAKE AND RELEASE) DURING *P. BERGHEI* INFECTION:

With respect to platelet aggregation stimulated by low concentration of ADP (0.5uM), infected PRP showed significantly higher % aggregation (38.45 ± 12.86 %) response than was observed

TABLE 5: Summary of some blood coagulation parameters in *P. berghei* infected and in normal mice plasma (platelet poor plasma: PPP)

TEST	CONTROL PPP	INFECTED PPP	p. Value
PROTHROMBIN TIME (Sec)	8.13±1.94 (n = 8)	11.70±2.55 (n = 10)	p < 0.005; t = 3,27
APIT (Secs)	29.0±7.2 (n = 11)	45.5±12.5 (n = 10)	p < 0.005; t=5.08
CLOTTABLE FIBRINOGEN (mg/dl)	253.9±41.5 (n = 10)	174.7±58.1 (n = 17)	p < 0.005; t=3.77

p = Student's t-test.

Values given for all tests are mean of duplicate determinations.

n = no of determinations.

Mean % *parasitaemia* in infected animals used was 28.5±17.6% (n=17)

Negative correlation existing between parasitaemia and blood coagulation parameters (PT and clottable fibrinogen) was significant at the 5% level (for PT, r = -0.560) and at 5 and 1% levels for clottable fibrinogen; (r = -0.675) (See Appendix tables 1 and 3).

Values represent Mean ± S.D.

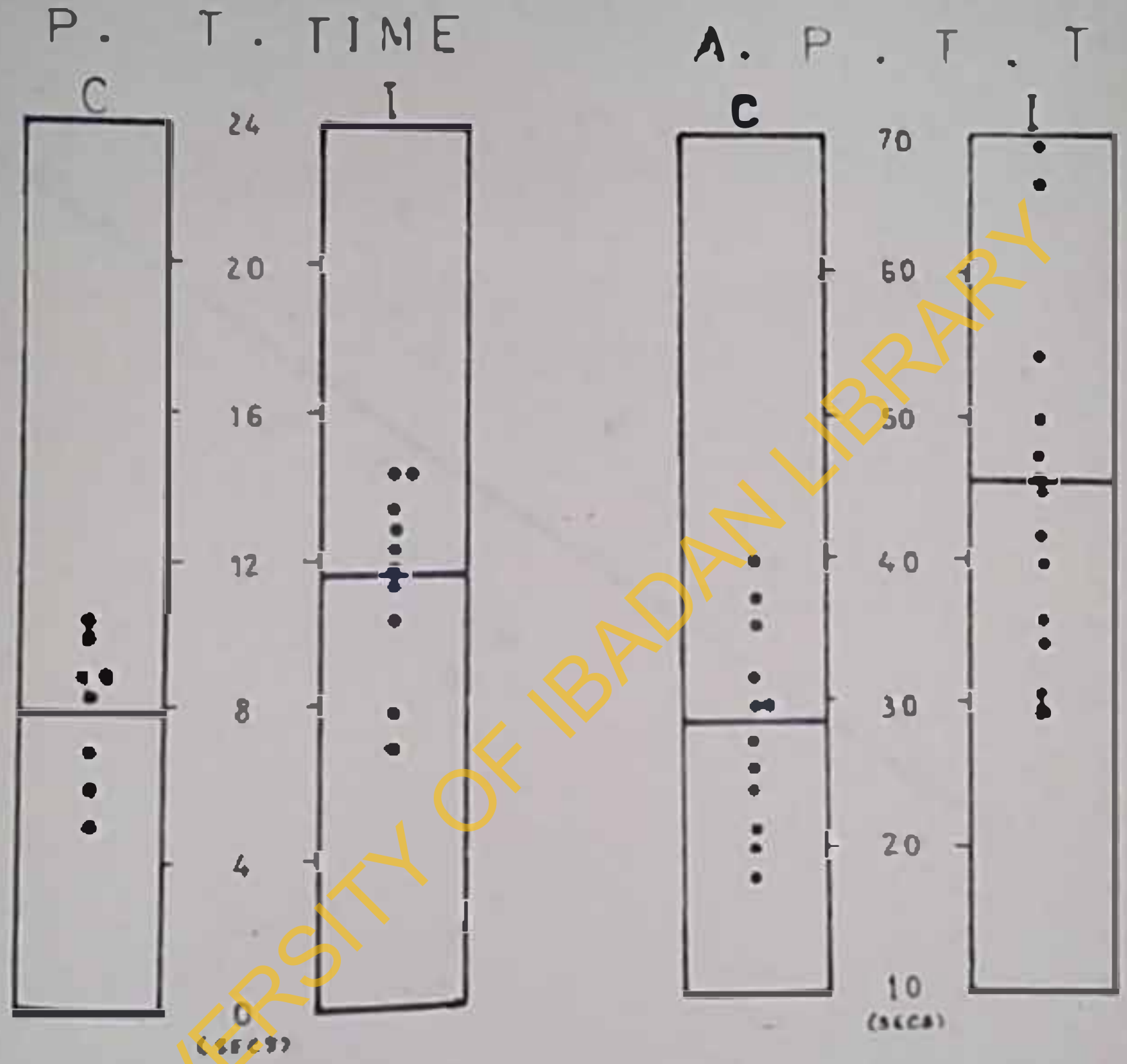


Fig. 11. Results of Prothrombin time and APTT in control and infected mice plasma. (C = control; I = infected).

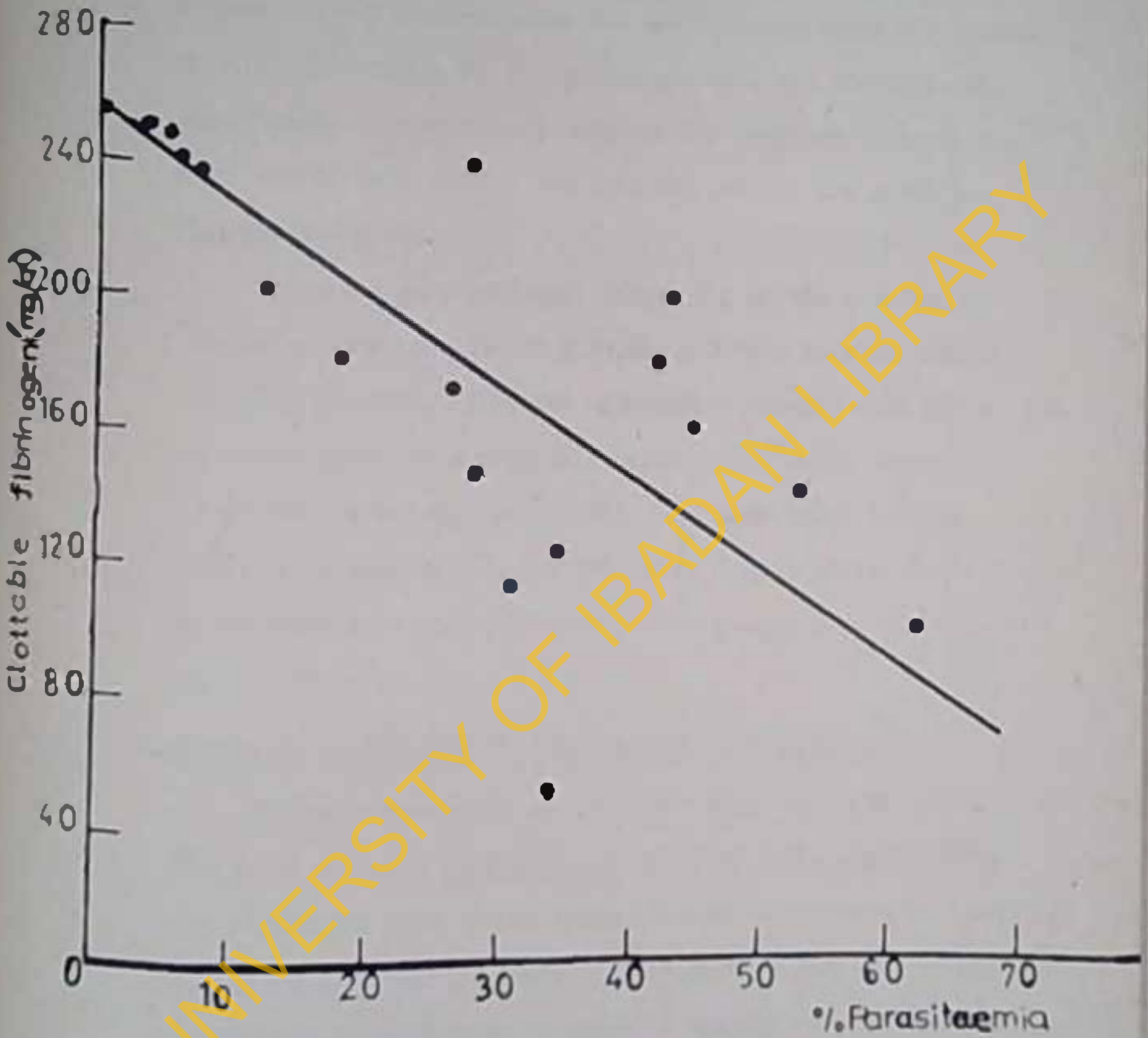


Fig. 12. A graph showing fall in clottable fibrinogen with rise in % parasitaemia.

in the control mice PRP(20.7±7.0%; $p < 0.01$; $t=2.83$; see Table 6). The differences observed in the mean percentage aggregation between that of control mouse PRP and infected mouse PRP induced by 1.0uM ADP, 2.5ug and 5.0ug collagen were not statistically significant. Representative aggregation tracings obtained for mouse PRP in both control and infected samples are shown in figures 13 and 14.

There was no significant difference in the % uptake of ^{14}C-5HT between control (77.93±3.89; n=3) and infected animals (75.75±8.03%, n=3). Platelet aggregation induced with ADP was not associated with any appreciable release of ^{14}C-5HT (control, 1.20±1.81%; infected, 1.41±1.03%). Collagen (5ug) induced 49.2±1.8% release of ^{14}C-5HT from control mice while 48.8±4.3% was released from infected mice. These values were essentially similar (Table 6).

3.5.1. RESULTS OF ERYTHROCYTE-PLATELET INTERACTION EXPERIMENTS:

Thrice-washed human erythrocytes (sham cultured control /PRSc/, or cultured *P. falciparum*-infected erythrocytes /PRSi/ were interacted with washed human platelet suspension as described in section 2.11.5 and a platelet rich supernatant (PRS) devoid of erythrocyte contamination or platelet aggregates was subsequently obtained after differential centrifugation. The platelet count in the platelet-rich supernatant (PRS) after interaction with control or infected erythrocytes ranged between 300 to 400x10^9/l. On stirring the PRS samples at 1100 rpm at 37^{o}C, a normal baseline testing was obtained indicating absence of significant platelet activation after the interaction. Fig. 15 shows aggregation curves

TABLE 6: Results of platelet aggregation and ^{14}C-5HT release studies in P. berghei infected and control mice PRP.

		% Aggreation induced with				% Release induced with	
		0.5uM ADP	1.0uM ADP	2.5ug Collagen	5.0ug Collagen	1.0uM ADP	5.0ug Collagen
CONTROL		20.7 ± 7.0	37.2 ± 15.98	43.5±6.2	88.55±16.2	1.20 ± 0.81	49.20±1.8
INFECTED		38.45 ± 12.86	47.14 ± 12.10	45.3±6.2	87.98±13.1	1.41± 1.03	48.8±4.3
p		<0.01; t=2.83	NS	NS	NS	NS	NS
n	c	5	4	10	5	5	10
	i	6	5	12	5	6	12

Parasitised mice PRP was prepared from animals with % parasitaemia in the range of 5.8-26.01%. p = students t-test.

n = no. of experiments using c (control PRP) and i (infected PRP).

NS = not statistically significant. Values represent Mean ± S.D.

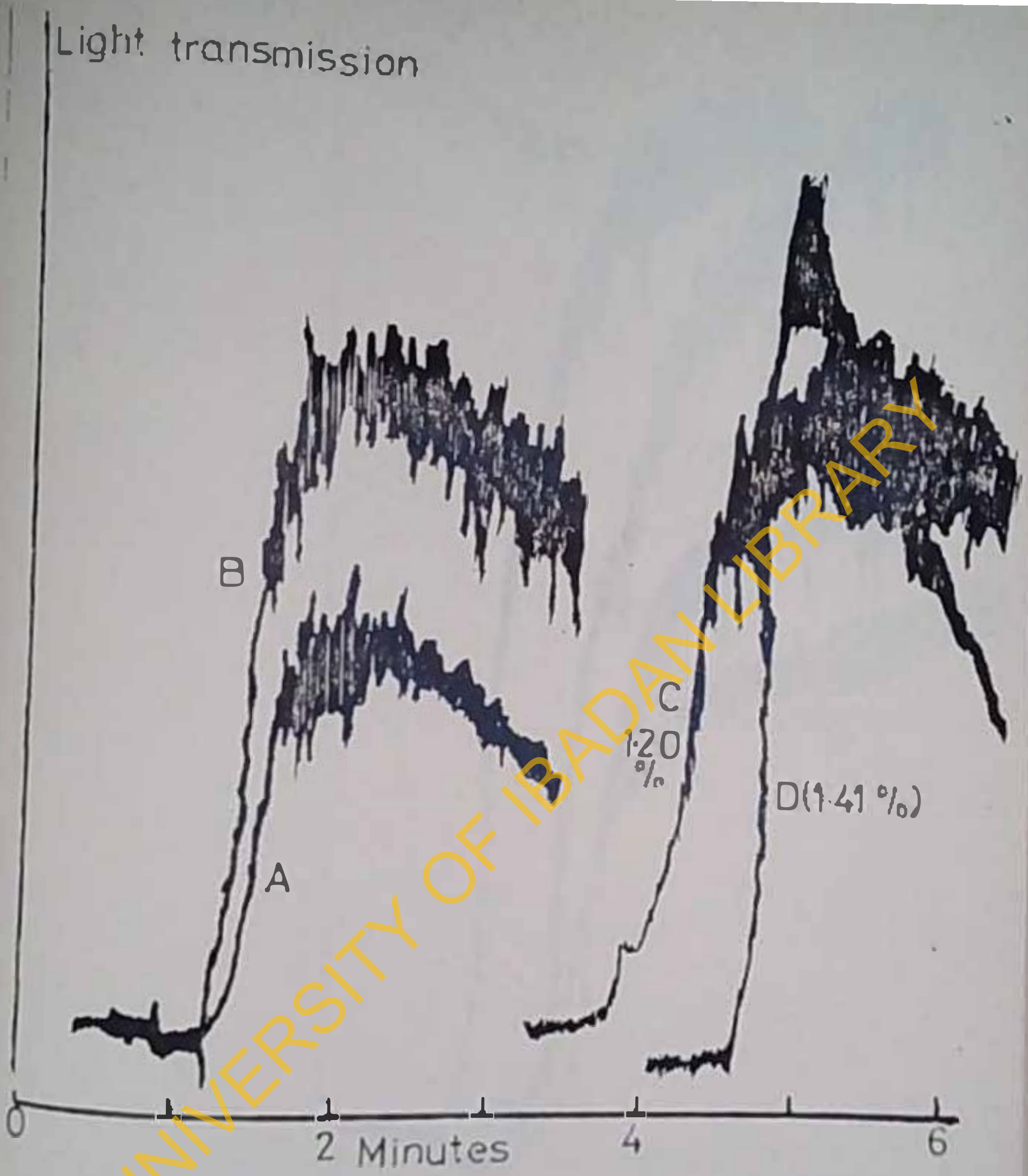


Fig. 13. Platelet aggregation following the addition of ADP to mouse PRP (control and infected).
Control mouse PRP = A, C
Infected mouse PRP = B, D
0.5uM ADP induced aggregation = A, B
1.0uM ADP induced aggregation = C, D.

% ^{14}C-5HT released from prelabelled platelets is shown beside the tracings.

Fig. 14. Platelet aggregation following the addition of collagen to mouse PRP (control and infected).
Control mouse PRP = A, C.
Infected mouse PRP = B, D.
2.5ug Collagen induced aggregation = A, B
5.0ug collagen induced aggregation = C, D.
% ^{14}C-5HT released from preloaded platelets is shown beside the tracings.

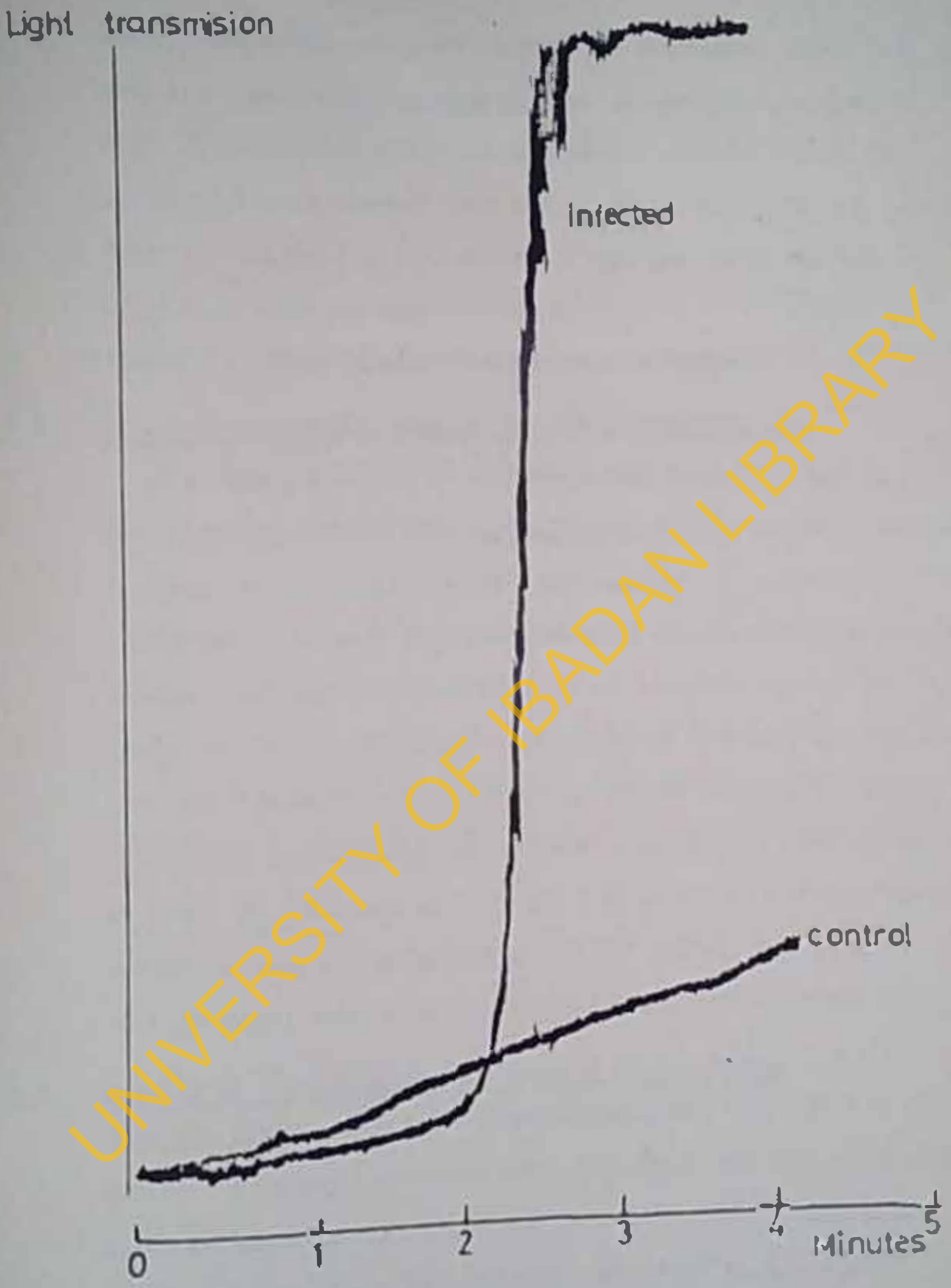


Fig. 15. PRSc and PRSi platelet aggregation induced with ADP (0.25u/l).

for PRS-c and PRSi using ADP (0.25uM) as stimulant. Blood film made from cultured P. falciparum human erythrocytes is shown in Plate 2. ADP-induced (0.25uM) aggregation in PRSi (90.3±7.5%) was significantly greater than that in PRSc (16.5±4.7%; $p < 0.005$, $t=41.7$). Collagen (2.5ug) induced % aggregation in PRSc was 62.2±36.0% while aggregation induced in PRSi was 67.1±28.8% (Table 7). These results were essentially similar.

3.5.2. Effect of interaction time on PRSc or PRSi aggregation:

Washed human platelet suspension was interacted for 1, 2, and 5 minutes with cultured P. Falciparum-parasitized erythrocytes or normal erythrocytes from the sham culture. The results summarized in Table 8 show that there was no significant difference between the % aggregation after 1, 2 or 5 minutes interaction either in PRSc or PRSi experiments. There were however, significant differences between % aggregation values of platelets after interaction with infected as compared with control erythrocytes at every incubation period. It was also seen that the duration of incubation time had no effect on ^{14}C-5HT uptake (PRSc uptake = 90.4.2% (n=2); PRSi uptake 93.1±2.9(n=2); unpublished results).

3.5.3. Results of ^{14}C-5HT Uptake and Release studies using PRSc and PRSi: The percentage aggregation induced with 0.25uM ADP in the presence of 0.04% human fibrinogen and that induced by 2.5ug collagen on PRSc are shown in Table 7. While aggregation induced in PRSi platelets differed significantly ($p < 0.005$; $t=41.7$) from that induced in PRSc by ADP (0.25uM), no significant difference was observed when 2.5ug collagen was used as stimulus (Fig. 16).

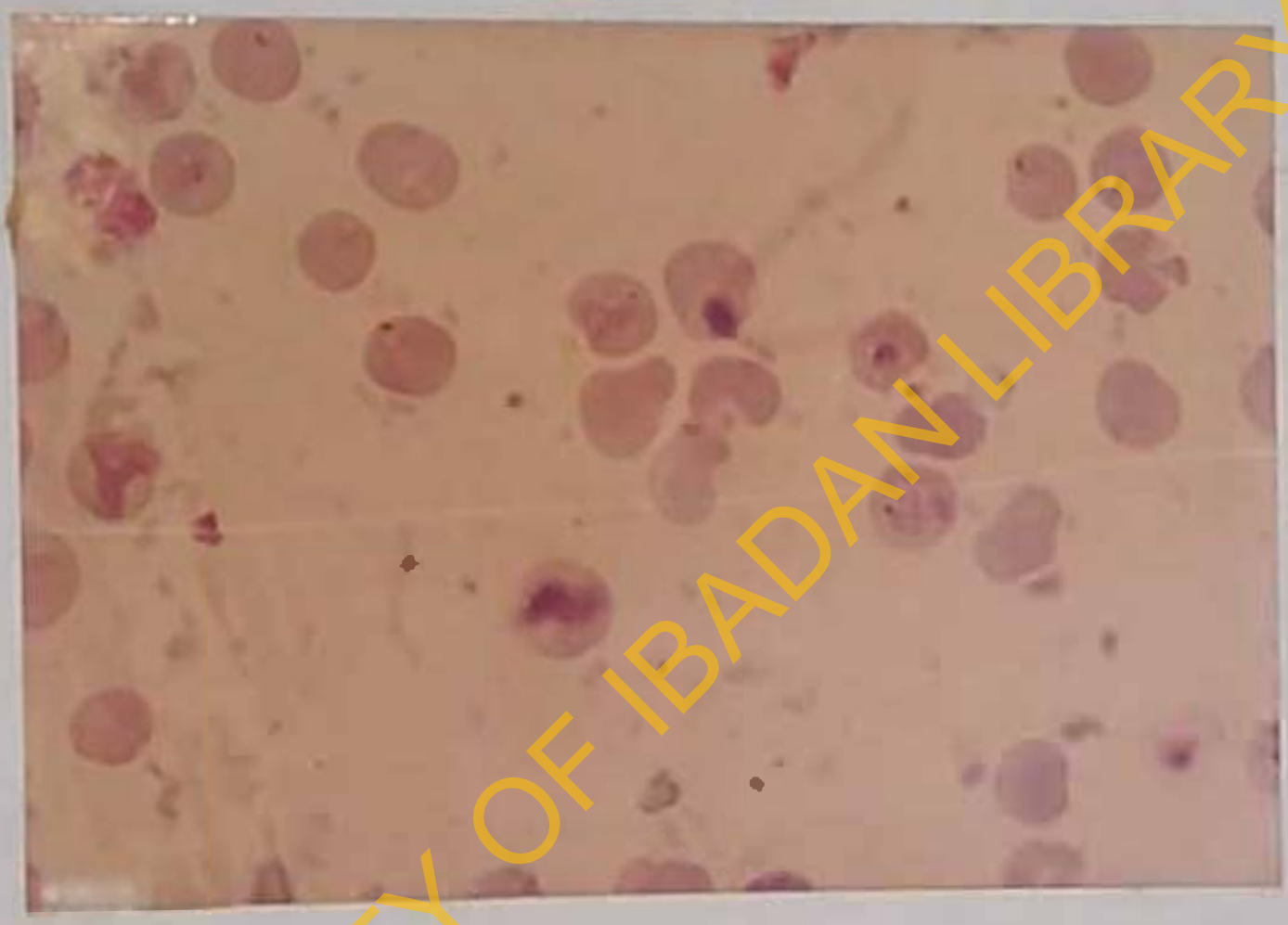


Plate 2: A blood film of cultured P. falciparum infected human erythrocytes. (x 1000)

TABLE 7: Platelet aggregation and ^{14}C-5HT Uptake and release following interaction with control (PRSc) and P. falciparum-infected (PRSi) erythrocytes.

	% Aggregation induced with		% ^{14}C-5HT Release induced with	
	ADP (0.25uM)	Collagen (2.5ug)	ADP (1.0uM)	Collagen (5.0ug)
PRS-c	16.5±4.7	62.2±36.0	8.1±7.8	22.3±7.7
PRS-i	90.3±7.5	67.1±28.8	17.2±10.1	40.1±8.7
p	<0.005;t=41.7	NS	<0.005;t=3.56	<0.005;t=6.1
n c	27	15	27	15
n i	27	16	27	16

9.1.44±8.03%(n=10) ^{14}C-5HT Uptake was recorded for the platelet suspension used in both PRSc and PRSi experiments.

n = no. of experiments using c (PRS-control) and i (PRS-infected)

p = student t-test. Range of % parasitaemia used for studies was 3.54-9.14. Values represent Mean ± S.D.

TABLE 8: Effect of incubation time on aggregation of PRSc and PRSi.

Duration of incubation (mins)	% Platelet Aggregation		p
	PRSc	PRSi	
1.0	22.2±18.3(n=11)	94.1±4.3(n=10)	< 0.005; t=12.13
2.0	18.4±14.2(n=8)	91.1-11.7(n=8)	< 0.005; t=11.17
5.0	17.5±8.7(n=9)	92.8±5.5 (n=8)	< 0.005; t=21.00

PRSc = Platelet rich suspensions after interaction with shaec-cultured erythrocytes.

PRSi = Platelet rich suspensions after interaction with cultured *P. falciparum* infected erythrocytes.

n = no. of experiments performed

% Parasitaemia was in the range of 4.7-6.6

p = student t-test.

Values represent Mean ± S.D.

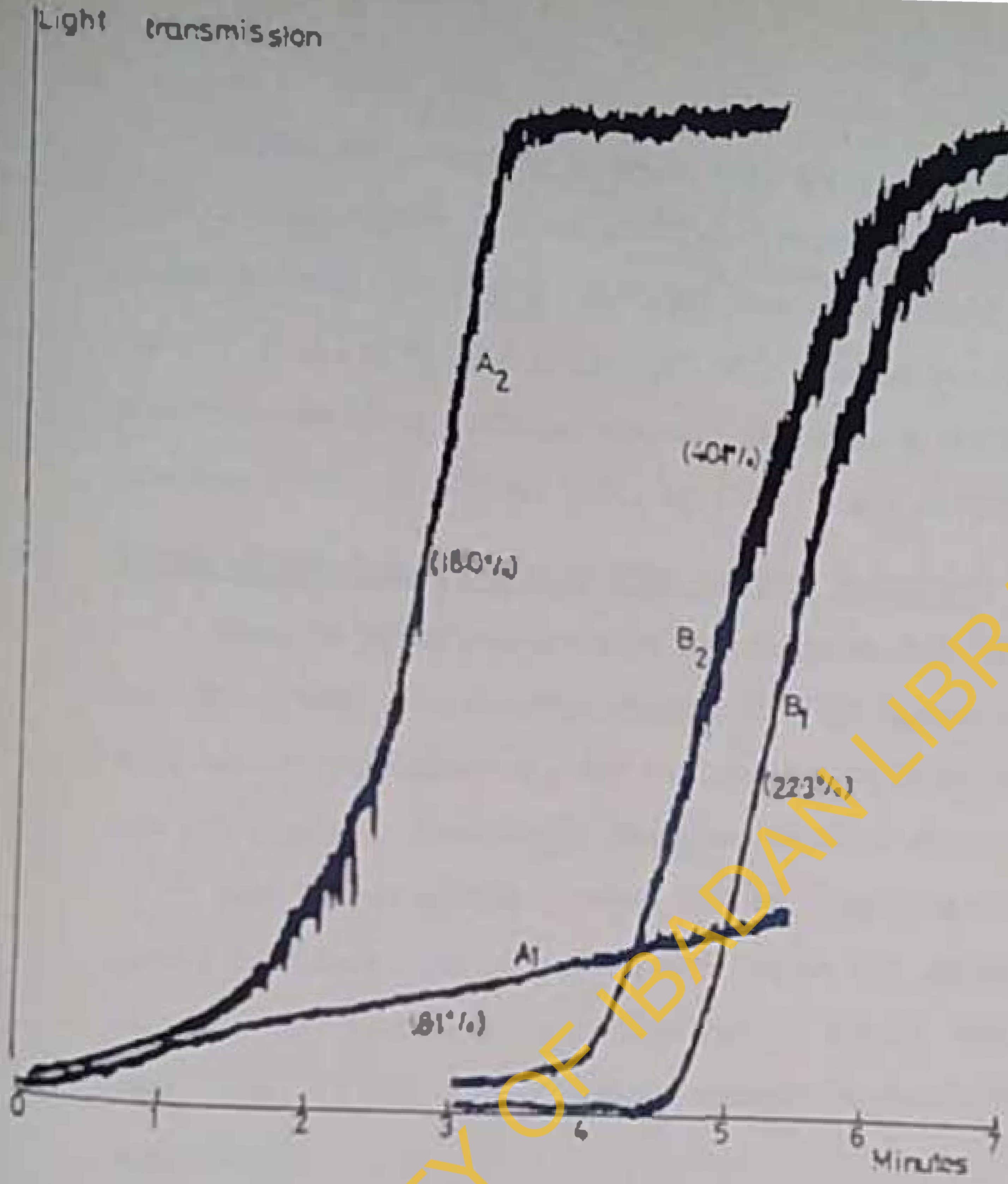


Fig. [illegible] Typical aggregation curves for [illegible] and [illegible] induced with ADP or collagen.

[illegible] = A1, B1 ; [illegible] = A2, B2

[illegible] ADP induced aggregation : A1 A2

2.5[illegible] collagen induced aggregation : B1, B2

% [illegible] released from [illegible] is shown beside each tracing

Unlike the situation in mouse PRP, ADP (1.0uM) induced a significantly higher release of ^{14}C-5HT from PRSi prelabelled platelets than from PRSc prelabelled platelets (see table 7) (PRSc=8.1±7.8% vs PRS-i = 17.2.±10.1%; $p < 0.005$, $t=3.56$). Similar results were obtained when 5.0ug collagen was used to induce platelet release reaction (PRSc, 22.3±7.7%; PRSi, 40.1±8.7; $p < 0.005$; $t=6.1$).

3.5.4. Effect of ADP-inactivation on PRSc and PRSi reactions:

Creatine phosphate-creatine phosphokinase (CP/CPK) and pyruvate kinase - phosphoenulpyruvate (PK/PEP) enzyme systems were used to inactivate any ADP that might have been present in the PRS samples. The results are shown in Table 9 and Fig. 17.

Both enzyme systems reduced the PRSi aggregation significantly to values similar to that observed in PRSc aggregation and indicate that there was significant ADP or ADP-like activity in PRSi. Fig. 18 and 19 show typical aggregation tracings for these experiments.

3.5.5. Effect of Dazoxiben, a thromboxane synthetase inhibitor on PRSc and PRSi aggregation:

In this study 20uM Dazoxiben was used to inhibit thromboxane synthetase in platelets interacted with cultured P. falciparum infected human erythrocytes (PRSi) and in platelets interacted with sham-cultured erythrocytes (PRSc). Aggregation was induced with either 0.25uM ADP or 2.5ug collagen. The results presented in Table 10 and Fig. 20 show that thromboxane mechanism contributed to the enhanced platelet aggregation in PRSi reactions with collagen. With ADP stimulated aggregation, dazoxiben failed to inhibit significantly PRSc and PRSi aggregation although a 18.5% inhibition was observed in PRSi aggregation.

TABLE 9: Effects of ADP-inactivating enzyme systems (CP/CPK and PK/PEP) on erythrocyte interacted platelet aggregation induced by 0.25uM ADP.

ADP-inactivating enzyme systems		% PRSc Aggregation	% PRSi Aggregation
PK-PEP	BEFORE	20.0±5.1 (n=5)	57.6±7.02 (n=5)
	AFTER	10.0±5.7 (n=5) (P = NS)	14.0±2.8 (n=5) (p< 0.005; t=12.9)
CP-CPK	BEFORE	19.4±13.8 (n=6)	62.7±7.2 (n=6)
	AFTER	10.6±4.0 (n=6) (P = NS)	13.2±8.7 (n=7) (p< 0.005; t=12.2)

% Parasitaemia of P. falciparum in erythrocyte culture was 8.72±2.6

Before = % aggregation before incubating PRS with enzyme system

After = % aggregation after incubating PRS with enzyme system

CP/CPK = creatine phosphate/creatine phosphokinase

PK/PEP = pyruvate kinase/phosphonulpyruvate

n = no. of experiments

NS = not statistically significant.

Values represent Mean ± S.D.

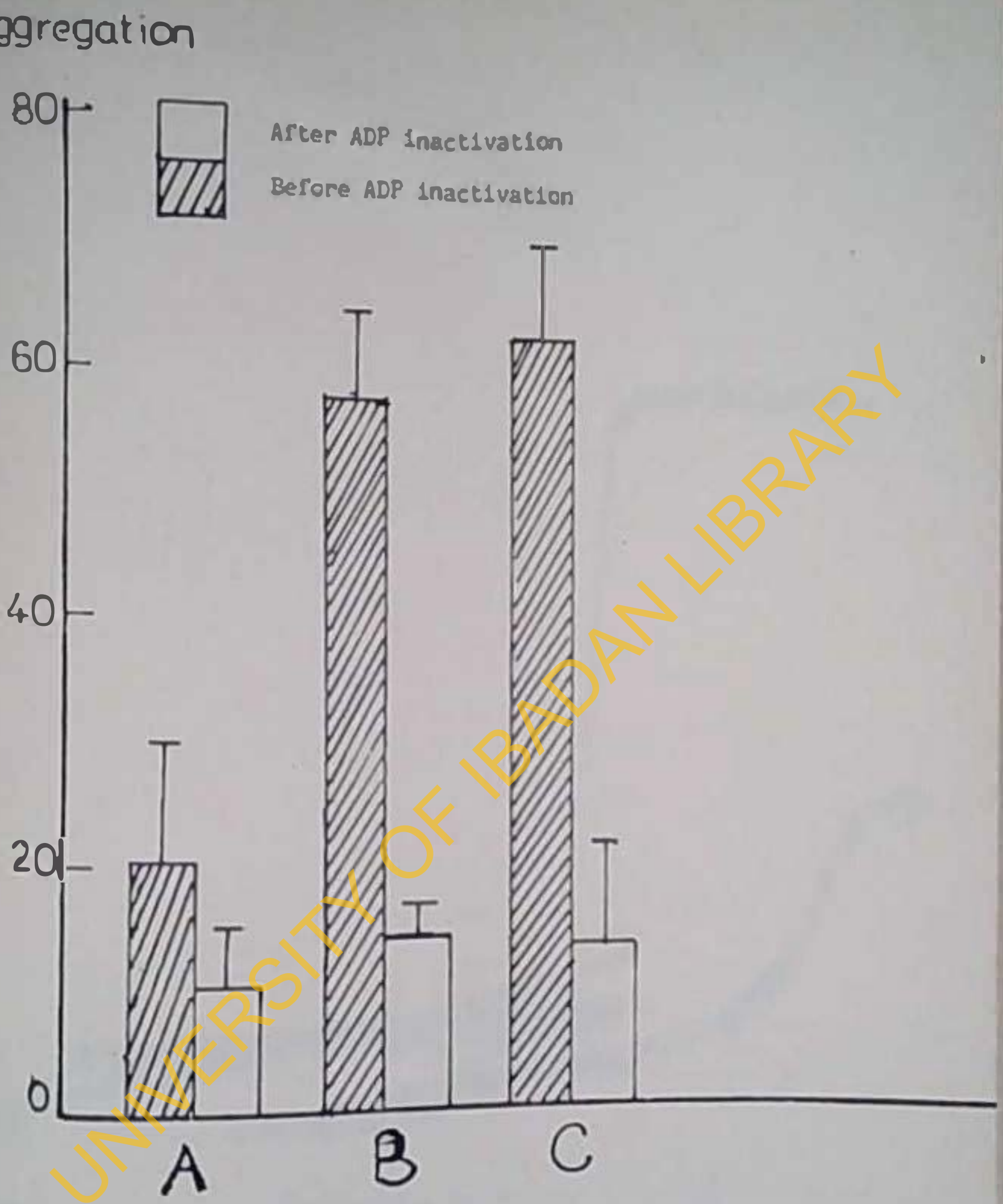


Fig. 17. Effect of ADP-inactivating enzyme systems on PRSc and PRSi aggregation.

A = PRSc aggregation

B = PK-PEP used as ADP inactivating enzyme system on PRSi aggregation

C = CP-CPK used as ADP inactivating enzyme system on PRSi aggregation.

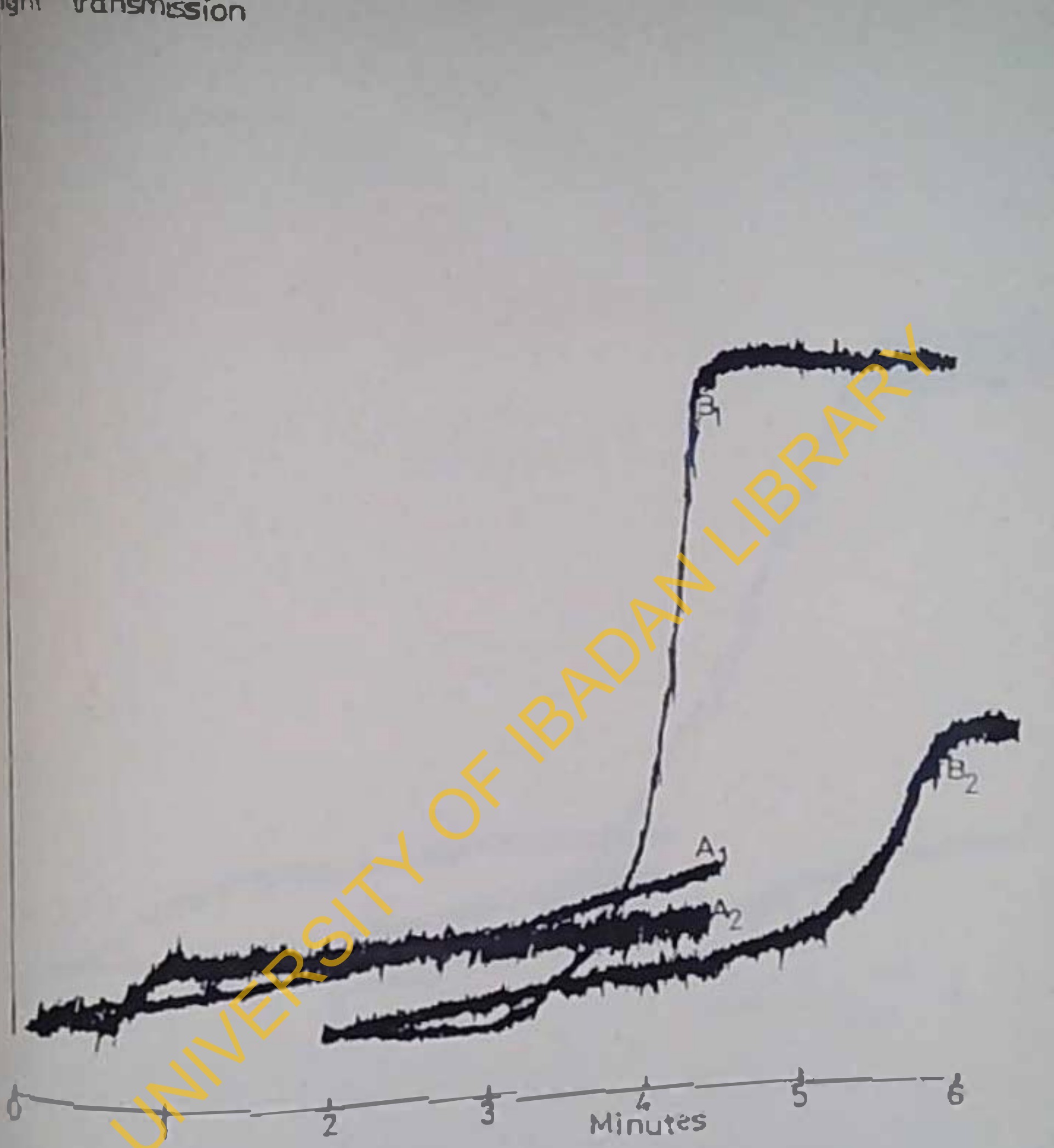


Fig. 18. Typical aggregation curves showing effect of CP/CPK on PRSc and PRSi aggregation induced with ADP.

PRSc aggregation = A_1 A_2

PRSi aggregation = B_1 B_2

Before ADP-inactivation = A_1, B_1

After ADP-inactivation = A_2, B_2

(CP = 5.0mM; CPK = 4.00/ml; ADP = 0.25uM).

Light transmission

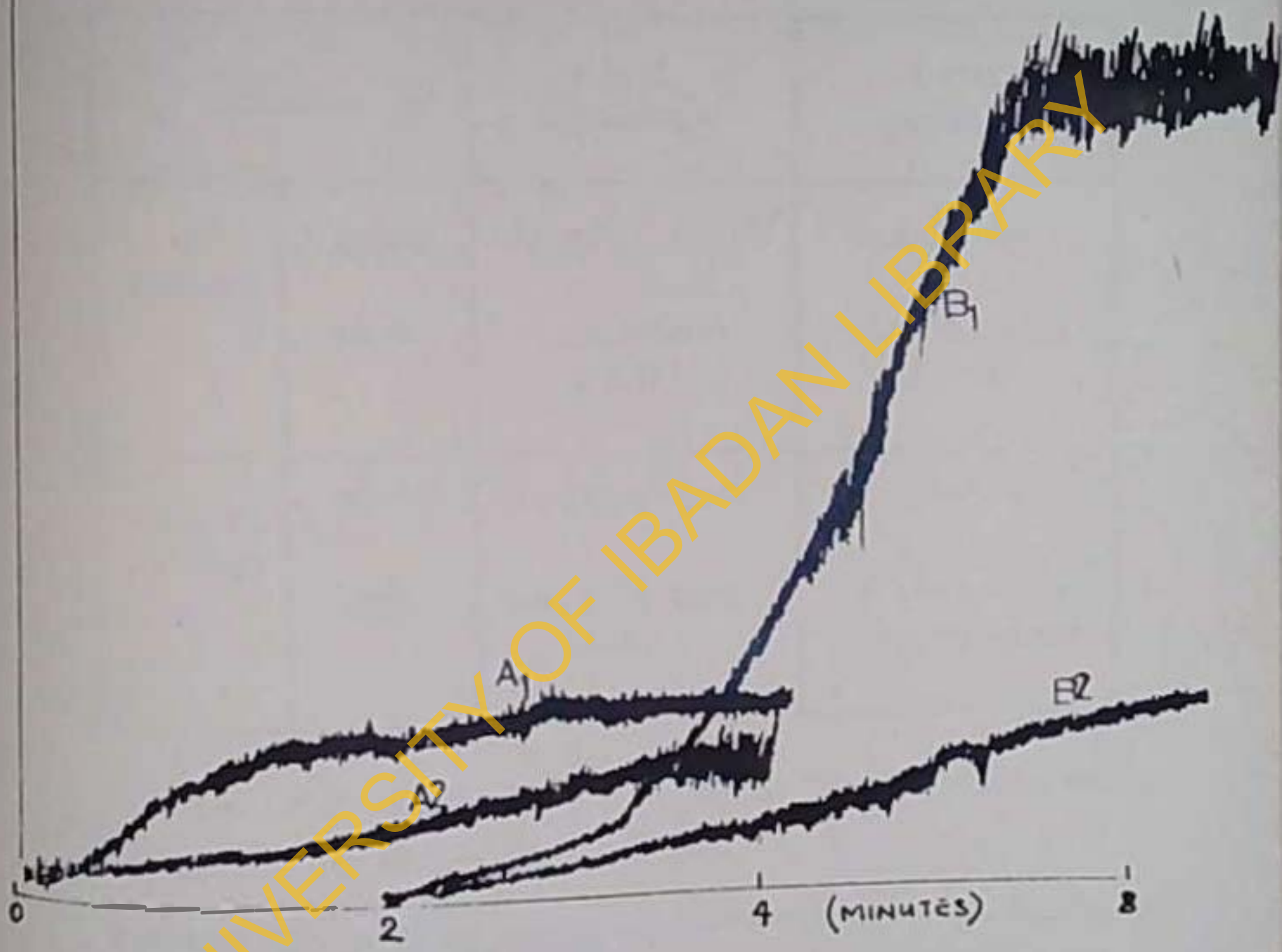


Fig. 19. Typical aggregation curves showing effect of PK/PEP on PRSc and PRSi aggregation induced with ADP.

PRSc aggregation = A_1, A_2

PRSi aggregation = B_1, B_2

Before ADP inactivation = A_1, B_1

After ADP inactivation = A_2, B_2

(PK = 20U/ml; PEP = 50U/ml; ADP = 0.25U/ml)

TABLE 10: Effect of Dazoxiben on PRSc and PRSi aggregation induced with ADP or Collagen.

DAZOXIBEN		% PRSc aggregation	% PRSi aggregation
ADP (0.25uM)	BEFORE	17.73±5.4 (n=4)	65.03±15.4(n=4)
	AFTER	14.4±4.2 (n=4) (P = NS)	53.0±12.8(n=4) (P = NS)
COLLAGEN (2.5ug)	BEFORE	48.6±13.1 (n=3)	48.1±6.75(n=3)
	AFTER	40.0±10.7 (n=3) (p=NS)	32.9±8.6 (n=3) (p < 0.05; t=2.54)

Dazoxiben (20uM) was incubated with PRS for 1 minute before inducing aggregation with either ADP or Collagen.

% Parasitaemia of P. falciparum infected erythrocytes was 5.25±1.83%

n = no. of experiments

p = student t-test; NS = not statistically significant.

Values represent Mean ± S.D.

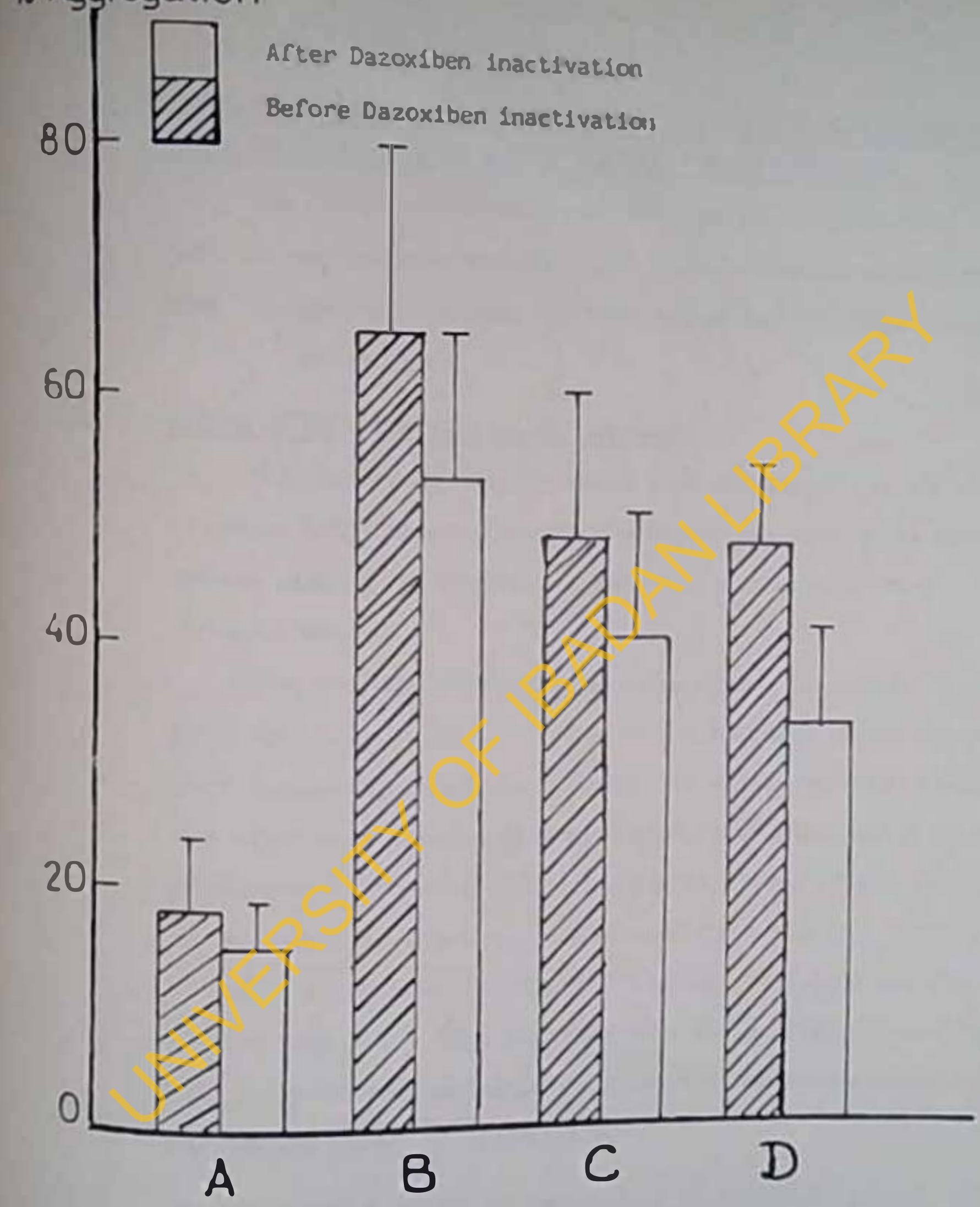


Fig. 20. Effect of Thromboxane synthetase inhibitor, Dazoxiben, on PRSc and PRSi aggregation.

PRSc platelet aggregation : A, C
PRSi platelet aggregation : B, D.

ADP induced aggregation : A, B
Collagen induced aggregation : C, D.

(Dazoxiben = 200μl; ADP = 1.25μM; collagen = 2.5ug).

3.6. RESULTS ON EXPERIMENTS USING ERYTHROCYTE DERIVED SUPERNATANTS (EDS) TO INTERACT WITH WASHED PLATELET SUSPENSIONS WPS:

The following abbreviations are used in this section:

EDSp = supernatants obtained from washed infected erythrocytes

EDSc = supernatants obtained from washed control (sham-cultured) erythrocytes.

3.6.1. Effect of EDSp and EDSc on WPS aggregation:

This experiment was conducted with the aim of finding out if the activity responsible for the higher aggregation in PRSi resided also in the supernatant fraction of these infected erythrocytes.

EDSp and EDSc were prepared as described in section 2.11.10. 100ul and 200ul of EDS were added to WPS to check if the former could induce aggregation on its own. This demonstration revealed that after 4 minutes incubation, 2 minutes of which was in the presence of 0.04% human fibrinogen EDSp and EDSc did not induce any aggregation. Rapid aggregation was induced by the addition of 0.25uM ADP only in the EDSp+WPS mixture and not in the EDSc+WSP mixture (Fig. 21). This suggested that the activity(ies) in the EDSp primed normal platelets and rendered them hypersensitive to low concentrations of exogenous ADP.

3.6.2. Volume-dependent effect of aggregation of washed platelets after incubation with EDSp and EDSc:

This experiment was performed to see if EDS had any dose (volume)-dependent effect on WPS aggregation. Volumes of 25, 50, 100 and 150uls of EDSc or EDSp were mixed with WPS such that the final volume of mixture did not exceed 1.0ml. The platelet count in the final mixture ranged from $350 \times 10^9/l$ to $450 \times 10^9/l$.

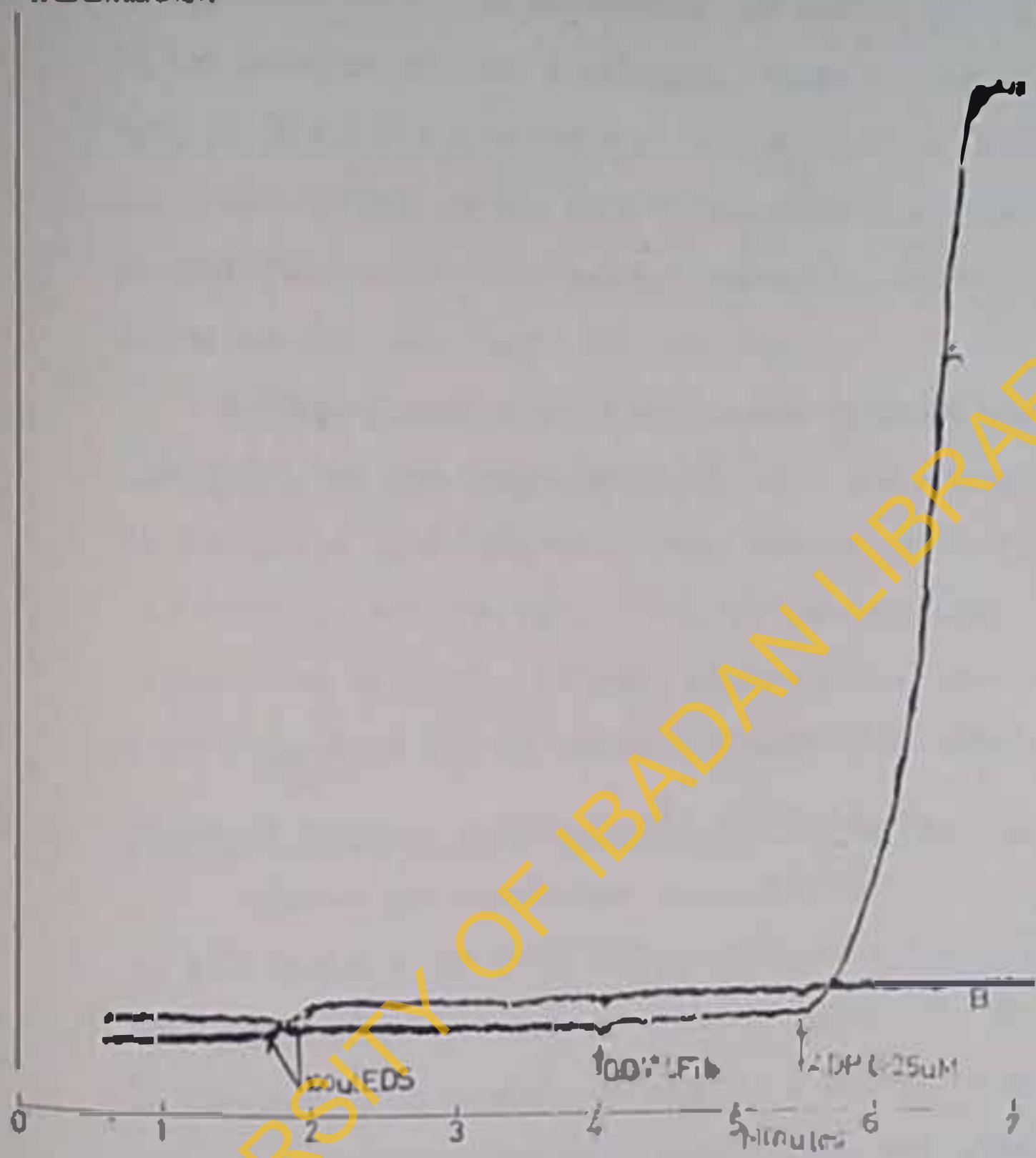


Fig. 21. Effect of EDS infected and control interaction with VPS treated on aggregation induced with ADP.

EDS1 aggregation : A

EDSc aggregation : B

0.25uM ADP used to induced aggregation.

Aggregation was induced in 500ul of the mixture with 0.25uM ADP in the presence of 0.04% fibrinogen. Table 11 show that no dose related effect was observed when EDSc was used at these volumes. 25ul-150ul of EDSc caused 14.0-19.3% aggregation respectively. No significant difference existed between the responses induced throughout the dose range used with EDSc.

A dose-related response was however obtained with EDSp throughout the dose range used (Fig. 22). The aggregation induced by ADP in the 100ul EDSp+WPS mixture was similar to that in PRSi obtained by interacting 100ul of parasitized erythrocytes with WPS. EDSc+WPS mixture did not give a sigmoid curve suggesting no dose-related-response relationship.

3.6.3. Effect of thrombin inactivation on EDS-WPS aggregation:

Thrombin was inactivated by incubating EDSc or EDSp overnight at 37°C (Kwaan et al 1972) before mixing them with freshly prepared WPS. Aggregation was induced with ADP (Table 12). No significant differences could be shown between the % aggregation before or after thrombin inactivation in both infected and control systems. This indicated that thrombin was not the priming activity in the EDSp supernatant.

3.6.4. Effect of Heat on platelet aggregation after incubation with EDSc or EDSp:

EDS was incubated for 1 hour in a 50°C waterbath after which the cooled EDS was interacted with freshly prepared washed human platelet suspension. Aggregation was induced with 0.25uM ADP. The result (Table 13) shows that there was no difference in % aggregation with addition of fresh or heated EDSp.

TABLE 11: Effect of interaction of washed platelets with different volumes of erythrocyte derived supernatants EDS (control or infected) on platelet aggregation.

Volume of EDS interacted with WPS (ul)	% EDSc Aggregation	% EDSp Aggregation
25	13.9 ± 3.8 (n=4)	4.3 ± 3.2 (n=4)
50	14.7 ± 5.1 (n=4)	14.3 ± 5.4 (n=4)
75	17.7 ± 3.6 (n=3)	60.7 ± 14.9 (n=3)
100	15.4 ± 6.1 (n=5)	91.4 ± 11.7 (n=8)
150	19.3 ± 2.7 (n=3)	98.6 ± 5.8 (n=3)

Interaction was for 1 minute only

0.25uM ADP in the presence of 0.04% fibrinogen was used to induce aggregation.

% Parasitaemia = 5.7.

Values represent Mean ± S.D.

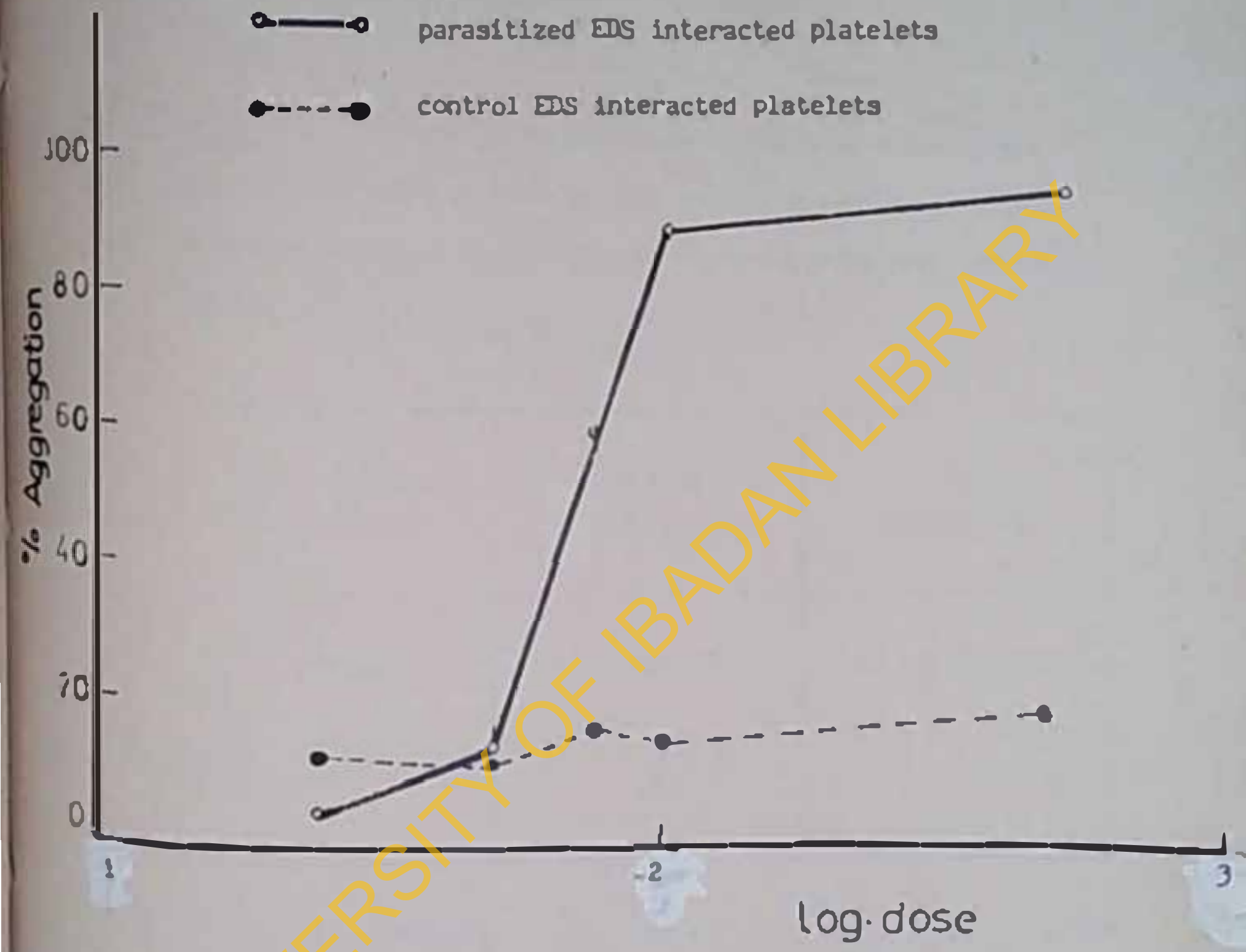


Fig. 22 A log-dose response curve obtained by interacting increasing volumes of EDS (control or parasitized) with WPS before inducing aggregation with ADP.

TABLE 12: Effect of thrombin inactivation on washed human platelet aggregation induced with control erythrocyte derived supernatant (EDSc) or parasitized erythrocyte derived supernatant (EDSp).

Heat treated Supernatant	% EDSc Aggregation	% EDSp Aggregation
BEFORE	18.4 ± 6.2 (n=3)	95.4 ± 5.2 (n=3)
AFTER	14.3 ± 4.7 (n=3) (NS)	91.7 ± 2.6 (n=3) (NS)

n = no. of experiments

8.1% P. falciparum infected red cells was used for this experiment.

0.25uM ADP was used to induce aggregation in the presence of 0.04% fibrinogen

NS = not statistically significant.

Values represent Mean ± S.D.

TABLE 13: Effect of heat at 56°C on platelet aggregation after incubation of WPS with EDSc or EDSp.

	Unheated Samples	Heated Samples	p
% EDSc+WPS Aggregation	22.1 ± 8.0 (n=3)	24.5 ± 6.7 (n=3)	NS
% EDSp+WPS	89.7 ± 6.5 (n=4)	90.2 ± 7.9 (n=4)	NS

n = no. of experiments

Parasitized erythrocytes had 8.1% parasitaemia

0.25uM ADP was used to induce aggregation in the presence of 0.04% fibrinogen.

P = students t-test; NS = not statistically significant.

Values represent Mean ±S.D.

3.7. SDS-PAGE ANALYSIS:

The result of the SDS polyacrylamidegel electrophoresis of PRSi or PRSc supernatants is shown on Plate 3. Lanes 1, 3 and 5 show the protein resolutions for PRSc supernatant, while lanes 2, 4 and 6 show protein band for PRSi supernatants, after ADP induced platelet aggregation. A distinct band was visible in the PRSi tracts indicating presence of a protein subunit not found in the PRSc tracts. Similarly, Plate 4 which shows protein resolution for supernatants of PRSi (lanes 1, 2 and 4) and PRSc (lanes 3 and 5), before ADP induced aggregation, had this band in the PRSi tracts but not in the PRSc.

3.8. PHOSPHOLIPID ANALYSIS:

PRSc or PRSi cell-free supernatants before and after aggregation were analysed for their phospholipid contents. No phospholipid was detected in any of the samples analysed by the method used.

3.9. RESULTS ON PLATELET SURVIVAL STUDIES IN P. BERGHEI INFECTED SUCKLING RATS:

Initial recovery of injected ^{51}Cr-platelets in normal and P. berghei infected rats are summarized in Table 14. In this experiment homologous platelets labelled with ^{51}Cr-NaCrO4 were injected into control rats and those at various stages of P.berghei infection. Percentage parasitaemia ranged from 0.8% - 2.3% on the day of labelling. In control animals, at time 0, 81.2% of the injected labelled platelets was recovered in the general circulation while 66.8% was recovered in the infected rats. The difference in the recovery for both control and infected animals at time 0 hours, was however not statistically significant.

Plate 3: SDS-PAGE of PRSi and PRSc cell free supernatants after ADP-induced aggregation.
Lanes 1, 3, and 5 show protein resolutions for PRSc supernatants
Lanes 2, 4 and 6 show protein resolutions for PRSi supernatants.

Plate 4: SDS-PAGE of PRSc and PRSi cell free supernatants before ADP induced aggregation.
Lanes 1, 2 and 4, show protein resolutions for PRSi samples
Lanes 3 and 5 show protein resolutions for PRSc samples.

TABLE 14: Mean percent recovery of ^{51}Cr-labelled rat platelets in normal and *P. berghei* infected rats at sampling time.

SAMPLING TIME (HOURS)	MEAN % RECOVERY OF ^{51}Cr-LABELLED PLATELETS			P (CONTROL AND INFECTED)
	CONTROL (n=4)	INFECTED (n=7)	% PARASITAEMIA (n=7)	
0	81.2±19.8	66.8±11.1	1.4±0.6	NS(p>0.05;t=1.58)
17.0	61.7±22.2	38.8±20.0	8.5±4.2	NS(p>0.05;t=1.77)
42.5	41.4±15.2	7.9±3.1	11.6±3.0	p<0.005;t=4.82
66.0	26.8±15.7	2.77±4.1	14.1±2.5	p<0.005;t=3.99

Correlation between platelet recovery in infected samples and percent parasitaemia is significant at 5% (r=-0.9789).

Values represent Mean ± S.D.

There was a negative correlation between parasitaemia and percentage initial recovery of ^{51}Cr-labelled platelets in circulation ($r = -0.9789$: significant at 5% level). Statistically significant differences were observed between the recovery in both control and infected rats in the 42.5 and the 66th hours sampling times (Table 14).

Table 15 shows the recovery of ^{51}Cr-prelabelled platelets as a percentage of initial recovery. The survival pattern obtained for control and infected rats are shown in Fig. 23. This is a plot of percent platelet ^{51}Cr-recovery (Table 15) against time of sampling. The disappearance pattern in normal rats was very nearly linear with a slight curvilinear component over the first 24 hours. With P. berghei infected rats, the pattern was linear over the first 43 hours after which a "tail" became apparent. Mean platelet survival time for the control rats was 4.3 days while that for the infected rats was 2.01 days.

Table 16 summarizes platelet survival parameters determined in the experiment. A typical blood film of P. berghei infected rat erythrocytes just before labelling is shown in Plate 5.

3.10. DETERMINATION OF SIALIC ACID IN P. BERGHEI INFECTED AND CONTROL SUCKLING RATS:

Since the sialic acid content of plasma glycoproteins and the concentration of surface sialic acid of platelets, leucocytes and erythrocytes are important in determining their survival in circulation, it became necessary to investigate the total platelet sialic acid content during interaction with plasmodia infected red cells.

TABLE 15: Mean recovery of ^{51}Cr-labelled rat platelets at sampling time as a percentage of initial recovery (i.e. recovery at time 0).

SAMPLING TIME (HOURS)	RECOVERY AS A PERCENTAGE OF INITIAL RECOVERY	
	CONTROL	INFECTED
0	100.0	100.0
17	76.0	58.1
42.5	51.0	11.8
66.5	33.0	2.8

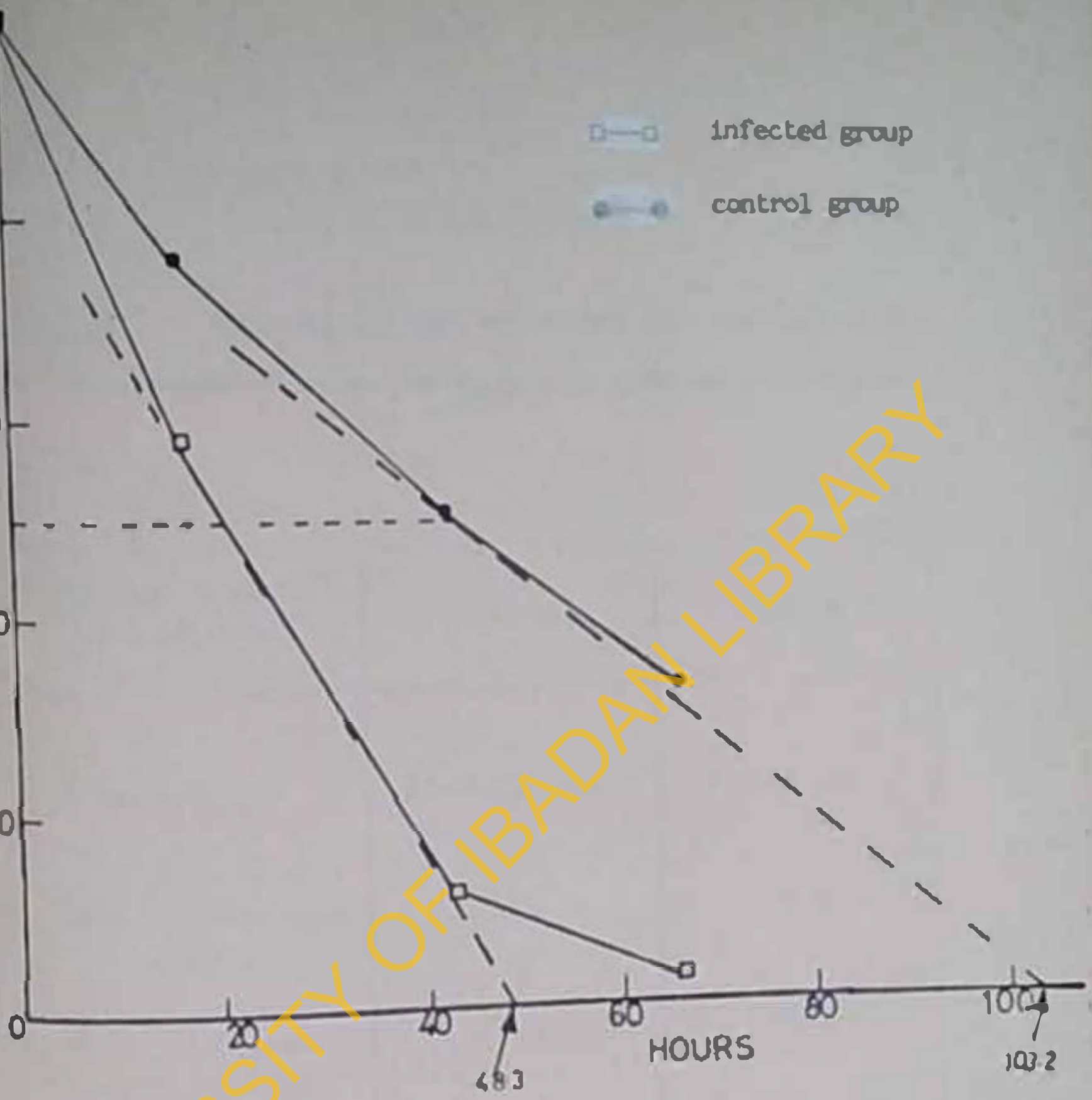


Fig. 23. Mean platelet survival curves using ^{51}Cr-labelled platelets in control and P. berghei infected rats.

TABLE 16: Platelet survival paraceters from survival studies in ^{51}Cr-labelled platelets in P. berghei infected and control rats.

Platelet Survival Parameter	Control	Infected
% Recovery	81.2±19.8 (n=4)	66.8±11.1 (n=7)
Mean Survival Time (dnys)	4.3	2.01
Half-disappearance time ($t_{1/2}$) hours	43.3	21.0

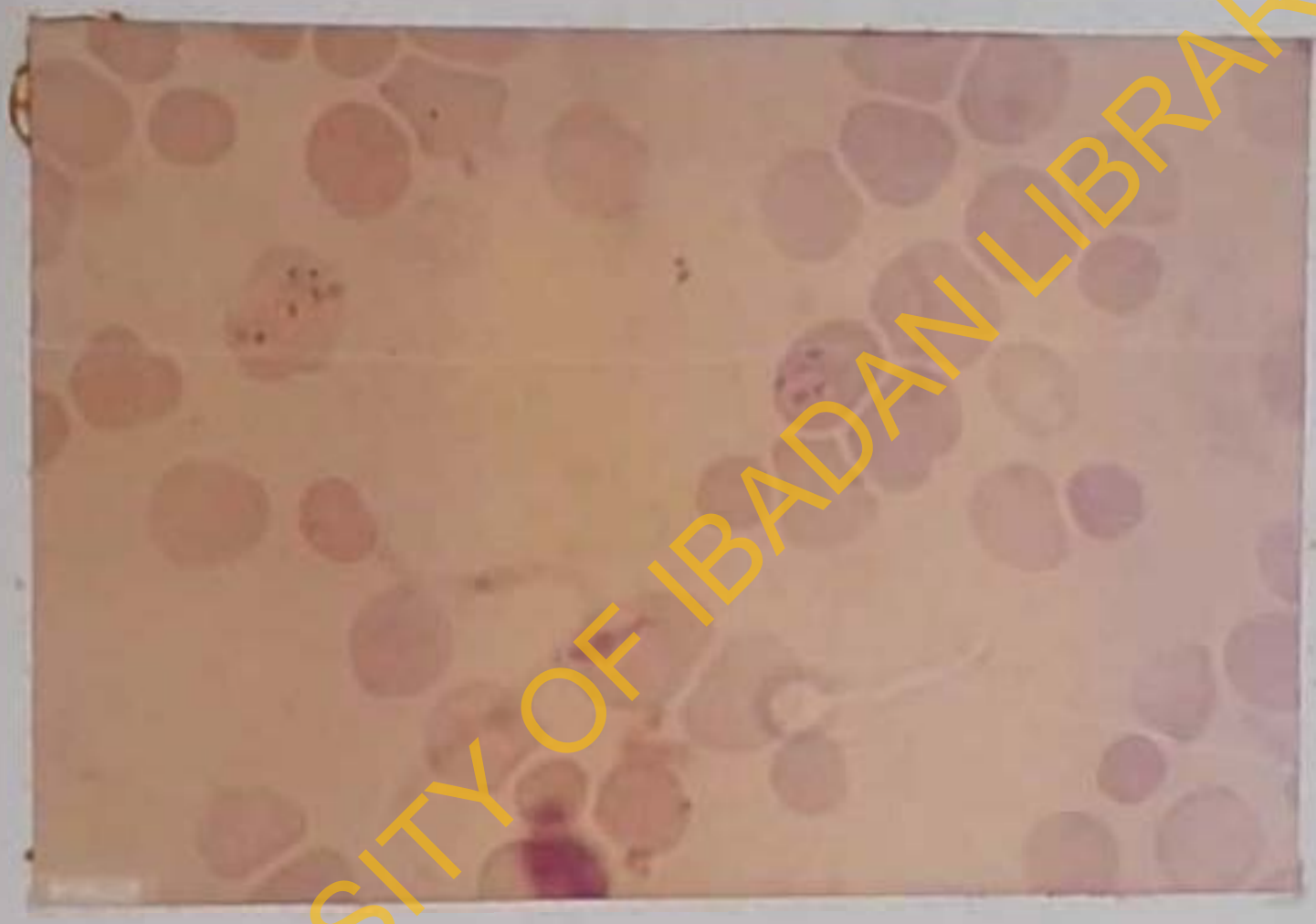


Plate 5: A blood film of P. berghei infected rat erythrocytes. (x 1000)

Total platelet sialic acid was determined essentially by the methods of Warren (1959) and Aminoff (1961) and results summarized in Table 17 show that there was significantly decreased total sialic acid content of platelets at all levels of parasitaemia. The difference from control values became significant with % parasitaemia of 14 and above. Fig. 24 is a histogram showing variation in platelet sialic acid with parasitaemia. A negative correlation, significant at the 5% level existed between the platelet sialic acid levels and parasitaemia ($r = -0.9510$).

TABLE 17: Total platelet sialic acid content of normal *P. berghei* infected rats.

GROUPS	NO. OF EXPTS.	SA nM/ 0.5ml WPS	Protein mg/ 0.5ml WPS	nM Platelet SA/mg Platelet Protein	Statistical test (t-test)	% Parasitaemia
CONTROL	4	1.51	0.132	11.43± 1.3	NS	0.0
INFECTED	5	1.31	0.136	9.63± 2.04	NS	8.73± 2.04
INFECTED	5	0.80	0.114	7.02± 1.87	p 0.005 t =4.19	14.01± 3.0
INFECTED	5	0.504	0.116	4.34± 1.92	p 0.005 t =6.63	16.92± 6.77

% Parasitaemia shows mean reading from 10 animals.

Sialic acid determinations were carried out in duplicate.

* Statistical test (t-test) compares total sialic acid content in control platelets and infected platelets at various levels of parasitaemia.

Negative correlation exist between parasitaemia and sialic acid content of the platelets (r = -0.9510; significant at 5% level).

Values represent Mean ± S.D.

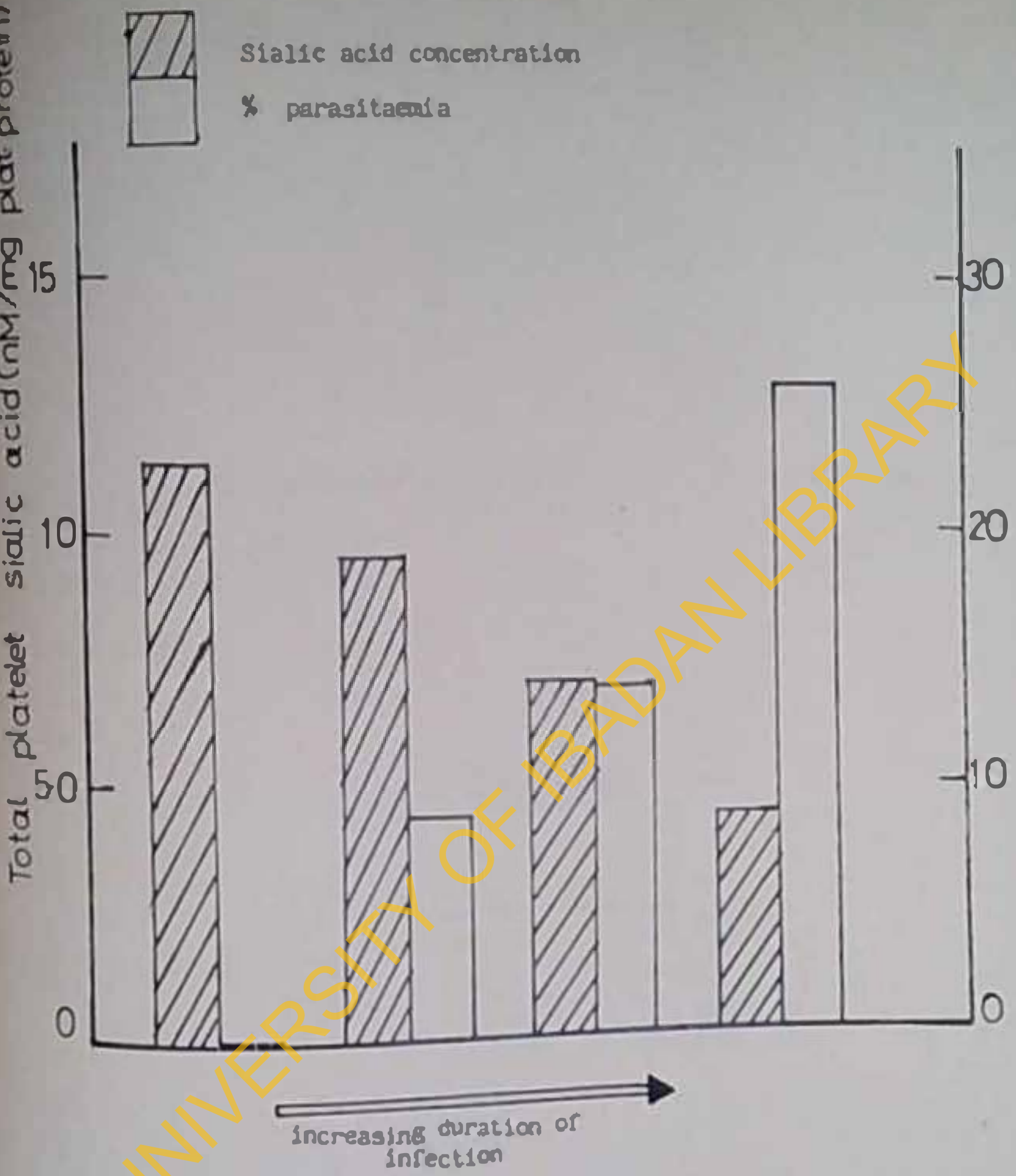


Fig. 24. Platelet sialic acid variation with rise in parasitaemia in *P. berghei* infected rats.

CHAPTER FOUR

DISCUSSION

D I S C U S S I O N

It is shown in these studies that thrombocytopaenia set in rapidly in Swiss albino mice experimentally infected with P. berghei. This confirms further, a previous observation by Singer (1954) in mice, and Essien and associates (1984) in golden hamsters. In the group of animals in this study that were treated with 20mg of chloroquine per kg. body weight of mice for 7 days (Group A) (see table 4), the fall in platelet count by the 11th day was not as pronounced as with the group that was treated with chloroquine for only 4 days (Group B). In the third group of P. berghei-infected mice that were not treated at all, platelet count was very low by the 5th day (96 to $107.4 \times 10^9/l$ cf $888.6 \pm 36.2 \times 10^9/l$ in normal mice) and all the animals had died by the 7th day (our unpublished data). However the treated groups of mice survived for longer than 27 days, by which time platelet count in those treated for 7 days had returned to baseline levels whereas, the counts still remained below normal level in those that were treated for only the conventional 4 days. This was due to the fact that parasitaemia of 0.2-2.4% was still observed in this group.

Chloroquine has besides its antimalarial actions, some anti-inflammatory properties (Carter et al 1971). At concentrations that can be achieved in vivo chloroquine and its analog hydroxychloroquine, can inhibit platelet aggregation induced by ADP or collagen and the release of adenine nucleotides induced by low concentrations of thrombin or collagen (Jobin and Tremblay 1969, Carter et al 1971). Carter and associates (1971) and Carter and Eban (1974) have reported that the administration of hydroxychloroquine to patients undergoing major surgery caused a significant reduction in the incidence of venous

thrombosis and pulmonary embolism possibly by platelet function inhibition. As far as I am aware there are no reports of direct antithrombocytopaenic effect of chloroquine. Although this effect observed in this report can be explained by the clearance of the parasite P. berghei from the blood stream, it is also possible that the stabilizing effect of chloroquine on platelet membrane as on other biological membranes could also have contributed to rise in the platelet count. This stabilizing effect on membranes has been reported but at high concentrations (Weissman 1964, 1965; Warhurst and Hockley 1967) and it was suggested to be responsible for the local anaesthetic effect of the drug.

The accumulation of chloroquine by platelets (DaPrada and Pletscher 1975, Berquist and Doucet-Lyterg 1983) could be a mechanism geared towards attaining the drug concentration at which such membrane stabilizing effects in platelets are exhibited. Thus, this quinidine-like effect could have protected the platelets from increased lysis reported in acute malaria (Essien and Ebhota, 1983) and suggested as a mechanism of reduced platelet count in acute malaria infection (ibid). However, some school of thought contend that at least in some patients with malaria, the thrombocytopaenia is caused by immune mechanisms with specific IgG binding to platelet-bound malaria antigen and subsequent lysis (Kelton et al 1982).

Factors responsible for the increased leucocyte counts observed in this and in earlier reports in animal malarial infection (Singer 1954, Wellde et al 1972, and Essien et al 1984) remain unclear. Eperythrozoan cocoides and Haemobartonella muris, which are concomitant infections in wild malaria parasitized rodents, cause either reduced plasmodium parasitaemia or early death of the animals.

However, viruses and rickettsial organisms hardly have any noticeable effect on the white blood cell count in laboratory animals during concomittal infections (Cox 1978).

Leucocytosis had been found in rats and mice with P. berghei infection mainly because of an increase in peripheral blood monocyte count (Singer 1954). Wellde and associates (1971 a, b, 1972) suggested that leucocytosis reported in malaria infections correlated with stimulation of recticuloendothelial system and that its role was to handle malaria pigments and the products of accelerated destruction of infected and non-infected erythrocytes which is a prominent feature of the disease.

In this study, leucocytosis was recorded in the experimental groups (A and B) and in the placebo control group (C) (see tables 2, 3 and 4). Results in group C, support the suggestion that exogenous erythrocytes in peritoneal space also give rise to leucocytosis, although the mechanism involved was not examined further. In the experimental group that received 7 days of chloroquine treatment (group A), WBC count fell during therapy ($14.35 \pm 1.04 \times 10^9/l$ on day 4 to $11.16 \pm 1.2 \times 10^9/l$ on day 11) and attained baseline values on the 14th post-infection day. In group B animals, treated conventionally with chloroquine for 4 days, WBC counts rose from $7.27 \pm 0.9 \times 10^9/l$ to $15.50 \pm 4.2 \times 10^9/l$ by day 7 (last day of treatment) although it later fell gradually to baseline values by the 27th day. In both experimental groups, the leucocytosis seems to have been a combined effect of both the presence of the erythrocytes and of the parasites, as well as breakdown products. The results also show that treatment with chloroquine for 7 days was more effective in bringing down the elevated white cell count than treatment for 4 days, as normal leucocyte counts had been attained

in the 7 day treatment group by the 14th day whereas this was achieved only on the 27th day in the 4 day treatment group.

Blood coagulation parameters determined in both the infected and the control animals gave results which suggested coexisting consumptive coagulopathy in the infected animals. Besides the significant prolongation in both PT and APTT in the infected animals compared to the control animals a strong negative correlation existed between parasitaemia and fibrinogen depletion in the infected animals. In addition, a significant fall in clotteble fibrinogen was recorded in the infected mice (174.7±58.1mg/dl cf control 253.9±41.5mg/dl). Similar results had been obtained by others in man (Dennis et al 1966 a, b, Devakul et al 1966).

Disseminated intravascular coagulation (DIC) as a mechanism of the thrombocytopaenia in acute malaria infections had earlier been suggested in infections with P. falciparum, P. malaria and P. vivax in man (Devakul et al 1966, Dennis et al 1967, Bronchowitz et al 1970). However, results of more recent studies (Essien et al 1976, 1979; Horstman et al 1981) did not support such a view in man. By contrast increased platelet lysis was observed in association with hypersensitivity to exogenous ADP during acute P. falciparum infection (Essien and Ebhota 1981). Although the detailed mechanism(s) involved remained to be clarified, it was suggested that these findings as well as later ones which showed that the platelets were sensitized in vivo (Essien and Ebhota 1983) could adequately account for the reduction in circulating platelets regularly observed in man. Unlike in other reports, severe thrombocytopaenia was obtained only in 5% of the group studied (Essien et al 1979). Thus the mechanism(s) of thrombocytopaenia observed in acute malaria of man and animals remain uncertain.

As earlier demonstrated with P. falciparum infection in man (Essien et al 1981), P. berghei infected mice platelet (as PRP) also manifested hypersensitivity response to low concentration (0.5%) of exogenous ADP when compared to the response from control animals. Higher concentrations of ADP (1.0-2.0%) or other agents such as collagen failed to evoke a comparable response from / P. berghei infected mouse platelets. When dense granule secretory functions were monitored using ^{14}C-serotonin uptake and release as parameters, no difference in uptake and release could be detected between platelets from P. berghei infected mice and those from normal mice/ (see table 6). This result was similar to those seen in human platelets (Essien and Ebhota 1983); and thus it was concluded that serotonin release (from mice PRP), as an index of dense granule secretion remained essentially normal during P. berghei infection in mice. Mice dense granule secretion stimulated by ADP was low. This was similar to results earlier reported by Nunn (1981) and Rosenblum et al (1983) although collagen still induced 44.1-52.3% release in platelets from both normal and infected mice.

Changes in some platelet functions such as hypersensitivity to low concentration of exogenous ADP during malaria parasitization are probably not the result of a single cellular defect but result from a series of molecular and biochemical alterations. Platelet hypersensitivity had been reported in diverse clinical conditions such as cardiovascular disorders, diabetes mellitus, acute renal failure, peripherial vascular diseases, sickle cell disease and nephrotic syndrome amongst others. (These have been reviewed by Packham 1978; Mustard and Packham 1984).

These reports are based on results of in vitro tests of platelet function and are taken to reflect in vivo platelet status with some unquantified artifacts. Well defined mechanisms of platelet aggregation are through (a) ADP release and (b) synthesis of thromboxane A2 (TxA2), an arachidonic acid metabolite. Another ill-defined mechanism has been suggested by Vargaftig et al (1981). The enhanced aggregatory response to low concentrations of exogenous ADP has been observed with human citrated PRP as well as PRP from *P. berghei* infected mice. In both instances, serotonin release has remained normal. A similar aggregation response to collagen had not been demonstrated probably because sufficiently low collagen concentration had not been tested. One probable explanation for the response to ADP could be pre-exposure of platelets to ADP in circulation likely resulting from red cell lysis during malaria infection (Seed and Kreier 1980). However such exposure should desensitize the platelets (Born and Cross 1963; Holme and Holmsen 1975). The observation of hypersensitivity in this acute malaria could also result from increased activity of the TxA2 pathway. This is supported by the finding of enhanced TxB2 in *P. berghei* infected hamsters (Essien et al 1984).

A main conclusion from the mouse model is that the Swiss albino mouse is another suitable animal model which can be used in the study of platelet - malaria parasite interactions in addition to the golden hamsters earlier described (Essien et al 1984). The golden hamsters however proved to be more expensive and difficult to obtain and breed successfully in required numbers in circumstance when they were needed.

However, the problems presented by the use of the mouse model include the limited animal blood volume (0.5-0.8ml per animal) which was overcome by pooling blood from a number of animals. In addition

enhanced erythrocyte fragility which caused relatively mild anaemia but adequate platelet changes were still observed.

Platelet hypersensitivity in acute malaria could also lead to shortened platelet survival which could result in reduced platelet counts reported by several workers. Because of the limitations (already listed) encountered with mice or golden hamsters, the rat malaria model was used to study platelet survival in plasmodium infection. Earlier studies had shown that the thrombocytopaenia recorded in P. berghei infected mice and hamsters, and P. falciparum infected Aotus monkeys (Singer 1954, Volle et al 1972) suggested rapid platelet clearance from the circulation. In this study a similar finding was observed in suckling rats following P. berghei infections (see table 6) (our unpublished data). It was observed that there was an 81.2 % initial recovery of ^{51}Cr-labelled platelets in normal rats, similar to results reported by others (Aster 1969, Aster and Ginsburg 1969). The mean platelet survival in normal animals was 4.3 days, a result similar to those of Winocour and associates (1982, 1983). By contrast in the infected animals, the mean platelet survival time was 2.01 days and represented a 53.3% reduction in platelet survival from normal. This result represents, as far as I am aware, the first study of ^{51}Cr-labelled platelet survival study in rats during acute malaria infection.

In addition to shortened survival decreased platelet production in acute malaria could also be responsible for the lowered counts observed in these and in other studies. Since no evidence of decreased platelet production in malaria has been reported (Skudowitz et al 1973) excessive splenic pooling and decreased platelet life span have been suggested as mechanisms involved in the lowered platelet counts observed.

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In humans, rodents and non-human primates, proliferative changes in the spleen were particularly prominent (Aikawa, Suzuki and Gutierez 1980) but, only moderate changes in the reticuloendothelial system were detected in P. falciparum infected Aotus monkeys (Jervis et al 1972). Quinn and Wyler (1979) showed that ^{51}Cr-labelled P. berghei infected erythrocytes were more rapidly cleared from circulation than uninfected erythrocytes. This accelerated clearance of infected rbcs appeared to result from greater splenic uptake of the red cells even in rats rendered immune by prior infection. Similarly, Ginsburg and Aster (1969) while studying the mechanism of "hypersplenic" thrombocytopaenia in rats, concluded that splenic platelet pooling and the associated shortening of platelet life-span were sufficient to explain the thrombocytopaenia observed in splenomegalic animals.

It is suggested that decreased platelet recovery observed in the infected rats in this study, could result from increased splenic pooling. There was no facility to test this suggestion and this aspect was therefore not examined further. Our results are taken to indicate the fate of ^{51}Cr-labelled platelets in rats previously infected with P. berghei malaria parasites. Further work is indicated in this area to study the kinetics of platelets during malaria parasitization as this could help point out the stage of the acute disease at which splenic sequestration starts during infection.

Normal platelet senescence involves, decrease in circulating platelet density, reduction in platelet size and loss of surface sialic acid, the latter change marks the cell for removal from the circulation by the spleen (Rand, Greenberg, Packham and Mustard 1981). Furthermore, removal of surface sialic acid from platelets by neuraminidase

for viruses with neuraminidase-like activity such as influenza: Greenberg et al 1975, Scott et al 1978), or proteolytic enzymes such as trypsin, pepsin or chymotrypsin (Parkham et al 1977 a, b) has been shown to be an important factor in determining platelet survival in circulation. These reports therefore necessitated a study of platelet total sialic acid during P. berghei infection in rats. Platelet sialic acid comes mainly from glycoproteins and hematoside, having about 60% located on platelet surface. The result in Table 17 clearly demonstrates a significant decrease in the total sialic acid content of the platelets during P. berghei infection. The total platelet sialic acid content of the uninfected control suckling rats (71.43μM sialic acid/mg platelet protein) obtained in this study, is in close agreement with values reported by Winocour and associates (1953). It is shown for the first time in P. berghei malaria infection in rats that a highly negative correlation exists between total platelet sialic acid and percentage parasitaemia (r = -0.95).

This result further confirms the role of sialic acid in determining platelet life span earlier described by several workers (Terrada et al 1966, Scott et al 1978 and Rand et al 1981). Shortened platelet survival in viral infections involving viruses with neuraminidase activity can be attributed to the removal of sialic acid from platelet membrane glycoprotein (Scott et al 1978). While no information on plasmodial neuraminidase-like activity had previously been reported, an increased plasma concentration of proteolytic and lipolytic enzymes have been demonstrated in malaria infections (Homewood 1978; Sherman 1979; Sead and Kreier 1980). These elevated enzyme activities might have been responsible for the cleavage and loss of platelet membrane sialic acid, and subsequently reduced total platelet sialic acid reported

here. However, although total platelet sialic acid was determined in this study, it is known that only surface sialic acid from platelet membrane glycoproteins would be most readily affected (Choi et al 1972; Greenberg et al 1977, 1979; Scott et al 1978) as no study has as yet implicated reduced granule sialic acid content with shortened platelet life-span.

There is still considerable controversy on the effects of reduced (or increased) surface sialic acid and enhanced platelet aggregation (Mester et al 1972; Greenberg et al 1975; Pearschke and Zucker 1978; Bunting et al 1978 and Rand et al 1980). In this study total platelet sialic acid and platelet hypersensitivity have been demonstrated during acute infection with P. berghei (the latter result in rats was unpublished). Our results which demonstrated enhancement of platelet aggregation as well as reduced total platelet sialic acid during acute malaria infection suggest that both are related.

For logistic reasons, suckling rat model was used in some of our studies as it was difficult for P. berghei to grow in adult rats. (Obi and Okonko working independently here have reported similar experiences / personal communications/). Zuckerman and Yoeli (1958) had observed that with advancing age, intact rats became less suceptible to P. berghei infections. By contrast MacGraith and co-workers (1952) employing the same strain of P. berghei as used by Zuckerman and Yoeli reported that there was a high mortality rate in mature rats infected with the P. berghei obtained by intraperitoneal blood passage into rats. The difference in susceptibility in these two reports has been attributed to the fact that the latter workers passaged their strain in rats, whereas Zuckerman and Yoeli, maintained theirs by mouse passage, thus selecting a strain with reduced virulence for rats

although it consistently killed all the recipient mice if untreated (Zuckerman and Yoeli 1958). This question was not considered to be relevant and was therefore not examined further.

Changes in platelet function during plasmodium infection in an in vitro system have been achieved in this study. The objective was to provide an experimental model in which well known limitations associated with in vivo models can be avoided or minimized. It was also thought that it would be easier to isolate and investigate the mechanism(s) responsible for the platelet changes such as enhanced platelet sensitivity observed.

As in human PRP from P. falciparum malaria patients (Essien and Ebhota 1981) and mice PRP with P. berghei reported earlier (Inyang, Okpako and Essien, in press), platelet rich supernatant obtained after interaction of washed normal platelets with infected erythrocytes (PRSi) showed significantly enhanced aggregation response to low concentrations of exogenous ADP than platelets which interacted with normal red cells (PRSc). However, unlike the response reported in human PRP, from malaria patients, there was also enhanced serotonin secretion in PRSi compared with controls (PRSc). Although the reason for the difference could not be readily obtained, it is suggested that part of the reason for the observed differences could be in measurement procedures such as the use of imipramine in the in vitro model to check serotonin reuptake. This procedure was not adopted in the in vivo studies. Secondly, the higher % parasitaemia attained in the in vitro system compared with what is normally obtained in patients could have increased the sensitivity of the model. Failure to obtain a similar serotonin release in mice with higher % parasitaemia than was obtained in the in vitro systems may be due to species differences

between human and mice platelets with respect to this parameter as well as the fact that PRP samples were used in both human and mice studies.

Enhanced TxA2 production has been reported in P. berghei infected hamsters (Essien et al 1984). A mechanism of the TxA2 production was in all probability platelet activated interaction with parasitized rbcs and the parasites, in addition to production from monocytes. A similar platelet mechanism almost certainly operated in this system.

One great advantage of the in vitro system is that it greatly simplified investigations into the mechanisms of the hypersensitivity reaction in acute malaria infections. The accepted mechanism of platelet aggregation are via ADP, thromboxane A2 (TxA2) and a third ill-defined pathways. It has recently been shown (Reimers et al 1995) that sheared rbcs release ADP which enhances platelet aggregation. RBC lysis by rupture is a continuous and prominent feature of acute malaria infection. It is a well known mechanism of anaemia of acute malaria. In addition to the ruptured erythrocytes, the infected ones, having increased permeability and fragility, release ADP and this was therefore thought to be one of the likely mechanisms of the enhanced platelet aggregation in the system. It possibly acted by priming the platelets. The effect of continuous presence of ADP in the medium on platelets is not known, but the platelets were not refractory to subsequent exogenous ADP even when the additions were made within the interval (30-60 minutes) when the platelets were expected to be refractory. Although measurement of adenine nucleotide levels in the PRS was not done for logistic reasons, it was demonstrated in Fig. 21 that the concentration of ADP and other agonists in the system were not adequate to induce spontaneous aggregation. The serotonin released in the PRSi

samples (see results) must have acted synergistically with the ADP (Baumgartner and Born, 1966) released from ruptured red cells together with TxA2 from activated platelet (Essien et al 1984) to induce the maximal platelet aggregation recorded.

It is suggested that these three factors were the contributing factors which overcome the platelet refractory state normally expected in the presence of ADP (O'Brien 1962; Rosenberg and Holmsen 1969). It is also suggested that these factors were responsible for the observed hypersensitive response of the PRSi platelets to low exogenous ADP concentrations.

Results of experiments designed to test these suggestions support the conclusions reached. For instance, the results obtained from addition of creatine phosphate/creatine phosphokinase (CP/CPK) or pyruvate kinase/phosphoenulpyruvate (PK/PEP) to the PRSi (table 9) shows that aggregation of platelets from PRSi samples was significantly reduced by about 75% compared to usual response without the enzyme systems. The controversy surrounding its mechanism notwithstanding (Huang and Detwiler 1981; Munn and Chamberlain 1983), CP/CPK when added to platelet suspension is believed to inactivate extracellular ADP prior to platelet activation by exogenous ADP. Apyrase also performs the same function. Apyrase was added to both the platelet washing and suspending fluid in which both washed infected and control rbcs were resuspended. PK/PEP was also used to study the role of secreted ADP in some experiments. There was also a reduction in the PRSi platelet response in the presence of PK/PEP to exogenous ADP stimulation. PK/PEP enzyme system has been shown to be quite effective on endogenous (secreted) ADP inactivation (Haslam 1964) but quite ineffective against exogenous ADP (Saniabadi et al 1984). Thus the results obtained here

suggests that some of the ADP in the melieu was secreted ADP from the platelets possibly during interaction with infected red cells or during activation. Although the relative concentrations of secretable and erythrocyte released ADP fractions were not measured in our experiment, result using ^{14}C-5HT release showed that there was considerable dense granule release reaction in the PRSi samples. The 5-HT release was taken as an index of dense granule secretion and indirectly pointed to a higher ADP release, as the latter shares the same storage granule with 5-HT.

The extent of TxA2 activation in this system was determined by measuring the extent of inhibition of ADP-induced PRSi aggregation using Dazoxiben, a thromboxane synthetase inhibitor, in the system. An 18.5 % reduction in ADP-induced aggregation in PRSi samples was obtained (see table 10). However, dazoxiben significantly reduced aggregation of PRSi samples when it was induced by a more potent agonist, collagen. Thus, as was reported in hamsters, the thromboxane pathway was also activated in the in vitro system.

It is concluded from this experimental model that the platelet hypersensitive reactions to exogenous ADP during acute malaria infection was due to platelet activation by synergistic effects of released ADP, from infected erythrocytes, 5-HT secreted from activated platelets and increased TxA2 production by the platelets. It is also suggested that these factors acting simultaneously overcame the platelet refractory state which would have been induced by the presence of ADP in the system.

The model also showed that upto 20 %of the platelet aggregation inducing activity could not be accounted in the two establisied pathways. Since the aggregation-inducing activity was located in the

supernatant after platelet interaction with infected red cells (PRSi) the preliminary characteristics of this activity were investigated. It was found that the platelet priming activity occured rapidly and reached maximal levels within one minute of incubation. It was also found to be heat-stable at 56°C for one hour, the activity was still present after thrombin inactivation and could also be detected in parasitized erythrocyte derived supernatants (EDSp) in approximately equal amounts as in PRSi. On its own the "factor(s)" present in the EDSp did not induce platelet aggregation but sufficiently primed the platelets to give maximal aggregation in the presence of low ADP (0.25uM) concentrations. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed a band only in the PRSi cell free supernatant samples obtained before and after ADP aggregation. The presence of this band in the samples before ADP aggregation, suggests, that this "factor" is not a product of the aggregation process, but could have been derived either from the interaction between platelets and infected red cells, or from the infected red cells themselves. In addition phospholipid analysis of the samples obtained before and after ADP aggregation, yielded negative results. Thus the "factor" in question is not a phospholipid.

Although the model described leaves a number of questions concerning certain aspects of the plasmodium-platelet interaction unanswered, it is none-the-less a very useful, flexible and practical system that has yielded results that are similar to those obtained in man and mice and probably in hamsters and rats, too.

The platelet hypersensitivity reaction during acute malarial infection is a multidimensional phenomenon. It involves increased dense granule and alpha granule secretory reactions. It has been

shown here to involve the ADP and thromboxane pathways, as well as a third "factor" that has not been fully characterized. The detailed mechanism of this platelet priming that results in hyperactivity has not been investigated further. Calcium mobilization from dense tubular system into the cytoplasm forms the common pathway for platelet activation by most agonists (Detwiler, Charo and Feinman 1978). Investigations of calcium metabolism in acute malaria needs to be examined. This is even more necessary as it has been shown that during P. berghei infections in mouse, there is enhanced $^{45}Ca^{2+}$ uptake into the erythrocytes associated with increased glucose uptake (Meame and Homewood, 1975, Krung Kai and Yuthavong, 1983). A similar increase in Ca^{2+} and glucose uptake in P. berghei infected mouse platlets could result in enhanced platelet activation, a situation previously shown to do so (Massini and Luscher 1974). Furthermore, this could be the mechanism of the postulated platelet lysis in plasmodia infection (Essien and Ebhota 1983) as Romero and Whittman (1971) have shown that haemolysis accompanies increased Ca^{2+} uptake into erythrocytes.

CHAPTER FIVE

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

i) Thrombocytopaenia and mild leucocytosis accompany P. berghei infection. In cases where the mouse were not treated there was severe thrombocytopaenia and death by the 7th day. However in the treated groups of animals, they survived and the platelet count returned to the preinfection levels by the 27th day if treatment was given for 7 days (group A) or remained suboptimal by the 27th day if treatment was for 4 days only (group B). Mild leucocytosis was observed with respect to the infected groups but values returned to baseline count by the 14th day in the group A animals and slightly later in the group B ones. The leucocytosis was partially due to the presence of foreign red cells in the peritoneal space of the animals and partly due to the infection. This study showed that treatment of P. berghei infection in mice with the standard dose of chloroquine sulphate for the standard 4 days was not adequate to eradicate the parasites completely and platelet count did not return to preinfection levels, unlike treatment with same dose for 7 days. The beneficial effect of chloroquine in protecting the platelets has been attributed to its membrane stabilizing effects.

ii) Blood Coagulation tests carried out showed that there was a generalized derrangement of the blood coagulation systems in this animals (mice) during malaria. The results were consistent with a diagnosis of disseminated intravascular coagulation (DIC), a finding that has been reported by some workers in patients but not by others.

iii) Platelet hypersensitivity to exogenous ADP reported earlier in man was also observed in mice.

iv) Platelet survival studies showed that there was significant (↓ 53.3%) shortening of platelet life-span in suckling rats during P. berghei infection. Platelet recovery and half-life were also determined. Platelet recovery became significantly different in the infected rats only 42 hours after labelling when compared with the control rats. A negative correlation existed between parasitaemia and total platelet sialic acid content. This latter result suggests that shortened platelet life span was a consequence of changes in platelet membrane glycoprotein sialic acid. The reduced platelet sialic acid level during malaria could partially be responsible for the observed platelet hypersensitivity observed at least in this model.

(v) In this study, a model for studying platelet - plasmodium interaction which reproduces observed effects in vivo was successfully developed and successfully used to investigate the mechanisms of platelet-malaria interactions. It revealed that ADP, serotonin and thromboxane, a synergism of all, and another probable but undefined "factor" were involved in the platelet hypersensitivity reactions observed. Attempts to identify the additional "factor" involved showed that the "activity" was present in the supernatant from washed infected red cells after interaction with platelets before and after ADP induced aggregation. It was heat stable and was still present after thrombin-inactivation.

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APPENDICES

APPENDIX TABLE 1: Results of Prothrombin time test (control and infected mice) alongside platelet counts (P.C.) and % parasitemia (for the infected mice only)

	CONTROL		PARASITISED		
	TIME sec	P.C. ($10^9/l$)	TIME sec	P.C. ($10^9/l$)	% PARA-SITAEMIA
1	10.0	840	13.5	370	36.4
2	7.0	982	8.0	780	18.2
3	6.0	1130	7.0	485	28.6
4	9.0	799	14.5	360	39.5
5	5.0	861	10.5	565	8.1
6	10.5	820	12.5	590	53.4
7	8.5	773	13.0	410	31.6
8	9.0	698	14.5	719	40.7
9	-	-	11.5	480	40.8
10	-	-	12.0	650	41.0
11	-	-	-		
	8.13±1.94	862.8±134.9	11.70±2.6	534.9±135.1	33.8±13

APPENDIX TABLE 2: Results of APTT test (for control and infected mice) alongside platelet count (P.C.) and % parasitemia (for infected mice only)

SAMPLE NO.	CONTROL		PARASITISED		
	Time (sec)	P.C. (sec)	Time (sec)	P.C. (x106/ml)	% Parasitemia
1	36.0	840	34.5	370	36.4
2	27.5	982	54.5	702	18.2
3	20.0	1130	45.5	485	28.6
4	23.5	798	30.5	360	39.5
5	37.5	820	69.5	590	53.4
6	40.0	751	66.5	719	40.8
7	30.0	863	47.5	650	41.0
8	24.0	1005	50.0	407	73.5
9	30.0	603	40.0	418	44.6
10	32.0	811	45.0	526	62.6
11	18.0	977	36.0	599	12.7
12	-	-	30.0	726	18.6
13	-	-	42.0	579	42.4
MEANS	28.95± 7.2	878.2± 130.5	45.5± 12.4	548.5± 131.9	39.4± 17.5

APPENDIX
TABLE 3. Fibrinogen clot weight estimation in normal and in *P. berghei* infected mice

SAMPLE NO.	NORMAL	PARASITIZED	
	Fibrinogen clot wt. (mg/dl)	Fibrinogen clot wt.	% Parasitemia
1	200	240	6.1
2	300	200	43.5
3	280	160	44.6
4	200	236	7.6
5	260	200	12.7
6	300	180	42.4
7	280	245.1	5.4
8	200	180	18.6
9	280	100	62.6
10	233	140	53.4
11	-	240	28.2
12	-	122	34.8
13	-	144	28.4
14	-	144	31.3
15	-	171	26.7
16	-	248	3.8
17	-	50	34.3
Means	253.3±41.5 (n = 10)	174.7±58.1 (n = 17)	28.5±17.6 (n = 17)

APPENDIX TABLE 4: Determination of Hematocrit Tube radius.

	Empty Weight (gm)	Weight when filled water(gm) water	Difference in weight (gm)	Height of water column (mm)	Radius in cm
1	0.1175	0.1420	0.0245	41	0.0436
2	0.1340	0.1590	0.0250	39	0.0452
3	0.1306	0.1596	0.0290	44	0.0458
4	0.1276	0.1470	0.0194	31	0.0447
5	0.1220	0.1430	0.0210	35	0.0437
6	0.1380	0.1544	0.0164	25	0.0457
7	0.1269	0.1634	0.0365	58	0.0448
8	0.1227	0.1532	0.0255	41	0.0445
9	0.1220	0.1505	0.0285	46	0.0444
10	0.1219	0.1455	0.0236	39	0.0439
11	0.1289	0.1566	0.0277	42	0.0459
12	0.1350	0.1500	0.0150	22	0.0466

Mean Radius = 0.0447 ± 0.00095 (n = 12)

Density of water was given at $25^{\circ}C = 0.99823$ gm/ml

$30^{\circ}C = 0.99707$ " "

(Ref.: Book of Physical and Chemical Constants).

Vol. of a cylinder (V) $= \pi r^2 h$

$\therefore r = \left(\frac{V}{\pi h}\right)^{1/2}$

APPENDIX TABLE 5: Data from platelet survival.

0 hrs. 5-6-84	GROUP	CODE NO.	Vol. of labeled platelet injected (ml)	Total Radio-activity injected into animal (estimated from standard)	Length of blood column counted in (mm)	Vol. of blood column counted in (Ul)	Counts per minute of sample	Count per ml of blood	% Recovery	Mean % Recovery	% Parasitaemia	
	Control	1	0.2	23940	28	17.72	182	10271	99.1	81.2 ± 19.82	-	
		3	0.375	44888	40	25.32	306	12085	62.2		-	
		6	0.45	53865	32	20.26	460	22705	97.4		-	
		7	0.45	53865	36	32.79	350	15358	65.9		-	
	Infected	1	0.45	53865	32	20.26	311	15350	65.8	66.8 ± 11.13	1.4	1.44 ± 0.64
		2	0.45	53865	39	24.69	344	15933	59.8		2.1	
		3	0.50	59850	40	25.32	508	20063	77.4		1.26	
		5	0.45	53865	40	25.32	348	13744	58.9		1.7	
		6	0.45	53865	36	22.79	287	12593	54.0		0.6	
		7	0.375	44888	36	22.79	379	16630	85.6		0.73	
		8	0.375	44888	48	30.38	389	12805	65.9		2.3	

APPENDIX TABLE 5 (Contd.)

17 hrs. 6-6-84	GROUP	CAGE NO.	Vol. of labelled platelet injected (ml)	Total Radio-activity injected into animal (estimated from standard)	Length of blood column counted in (cm)	Vol. of blood column counted in (Cl)	Counts per minute of sample	Count per ml of blood	% Recovery	Mean % Recovery	% Parasitaemia
	Control	1			44	27.98	152	5433	52.4	61.7 ± 22.2	-
		3			38	24.17	247	10219	52.6		-
		6			32	20.35	450	22113	94.8		-
		7			39	24.80	271	10927	46.9		-
	Infected	1			37	23.53	98	4165	17.9	38.8 ± 20.0	14.5	8.46 ± 4.12
		2			22	13.99	188	13438	57.6		6.5	
		3			33	20.99	393	18723	72.3		3.4	
		5			38	24.17	114	2717	20.2		11.5	
		6			29	18.44	118	6399	27.4		8.5	
		7			39	24.80	186	7500	38.6		3.9	
		8			34	21.62	159	7354	37.8		10.9	

APPENDIX TABLE 5 (Contd.)

42.5hrs. 7/6/84	GROUP	CODE NO.	Vol. of labelled platelet injected (ml)	Total Radio-activity injected into animal (estimated) from standard	Length of blood column counted in (mm)	Vol. of blood column counted in (Ul)	Counts per minute of sample	Count per ml of blood	% Recovery	Mean	% Parasitaemia	
	Control	1			34	21.62	120	5550	53.6	41.4 ± 15.2	-	
		2			32	20.35	120	5897	30.3		-	
		3			38	24.17	148	6123	26.3		-	
		4			39	24.80	320	12903	55.3		-	
	Infected	1			39	24.80	20	807	3.5	7.9 ± 8.1	10.4	11.6 ± 2.98
		2			33	20.99	26	1239	5.3		9.7	
		3			38	24.17	164	6785	26.2		17.3	
		5			34	21.62	34	1573	6.7		11.1	
		6			33	20.99	22	1048	4.5		11.9	
		7			38	24.17	26	1076	5.5		7.9	
		8			42	26.7	19	712	3.7		12.9	

APPENDIX TABLE 5 (Contd.)

66 hrs. 8/6/84	GROUP	CODE NO.	Vol. of labelled platelet injected (ml)	Total Radio-activity injected into animal (estimated) from standard *	Length of blood counted in (mm)	Vol. of blood column counted in (Ul)	Counts per minute of sample	Count per ml of blood	% Recovery	Mean **	% Parasitaemia
	Control	1			30	18.99	92	4845	46.7		-
		2			45	28.49	48	1685	8.7		-
		3			28	17.72	97	5474	23.5		-
		4			37	23.42	155	6618	28.5		-
	Infected	1			-	-	-	-	-		14.6
		2			-	-	-	-	-		12.3
		3			30	19.0	53	2789	10.8		18.4
		5			40	25.3	30	1186	5.1		14.2
		6			-	-	-	-	-		13.8
		7			40	25.3	17	672	3.5		10.3
		8			-	-	-	-	-		15.1

* Standard counts from washed platelet suspension (0.1ml) = 11970
Vol. of blood in haematocrit tube ($\pi r^2 h$) is calculated from $\pi r^2 = 0.633 m^2$
Values in empty columns remain same as for that at 0 hr. sampling.

** Mean recovery value is estimated for 7 animals.

APPENDIX: METHODS

Total Phospholipid determination (by the modified method of Fiske and Subarrow (1925):

Principle: The phospholipids and proteins in the plasma (or any other medium) are precipitated by addition of 10% Trichloro-acetic acid (T.C.A.). This also destroys the action of enzymes which add to the value of phospholipids in plasma. The phospholipids are digested using 60% $HCLO_4$ (Perchloric acid) to release the phosphate. The phosphate thus released is complexed with molybdate forming phosphomolybdate. This complex is reduced to molybdenum blue with methol. The resulting colour intensity is measured spectrophotochemically.

Procedure: 0.2ml of plasma was added to 1.0ml of water in a digesting tube and 5.0ml of T.C.A. (10% solution of T.C.A.) was added and the mixed thoroughly. The mixture was centrifuged at 3,000 rpm for 10 minutes at room temperature. The supernatant was poured away and the tube was drained on a filter paper.

0.5ml of 60% $HCLO_4$ was added to the tubes (including 3 empty tubes; 2 for standard and 1 for blank) and digested on a heater for 45 minutes. Tubes were then removed and cooled to room temperature. The sides of all the tubes, except the 2 for the standard were then washed down with 5.0ml of distilled water.

5.0ml of standard solution (Standard Phosphate (PO4) solution of KH2 PO4 /Potasium dihydrogen phosphate/ was prepared from the dessicator pre-dried salt at a concentration of 1mgP/ml by dissolving 2.193gms of KH2 PO4 in distilled water) was used to wash each of the 2 tubes for standard. 0.4ml of 5% Ammonium molybdate was added to all the tubes and mixed well.

0.2ml of the reducing agent, metol (1% solution of metol was prepared using 3% sodium metabisulphite as diluent) was added to the tubes, mixed thoroughly and allowed to stand at room temperature for 30 minutes.

The optical density was measured at 700nM using a blank (distilled water) to set to zero.

Calculation:

$$\text{Concentration (X)} = \frac{\text{Test}_{\text{reading}}}{\text{Standard}_{\text{reading}}} \times 250\text{mg phospholipid per 100ml plasma.}$$

Note: Working concentration was made up of 2ml of the stock diluted to 500ml with distilled water (4×10^{-3}mgP/ml).

Percentage Phosphate (PO4) in phospholipids = 4

i.e. in a ratio of 1 : 25.

∴ concentration (X) is multiplied by 25 to convert to phospholipids.

Cultivation of Erythrocytic Stages of Malaria by the petri-dish-candle jar method (Jensen and Trager 1977, Trager 1980):

Appropriate amounts of 6 or 6% red blood cells were placed in clean, sterile petri dishes of any desired size to give a 2-3mm depth of medium. Culture medium (described below) was changed once daily by gently tipping the dish, drawing off the clear fluid and replacing it with fresh medium.

A gas phase of 2-3% CO_2 and 15-18% O_2 was provided by burning a candle in a dessicator jar holding the dishes until the flame goes out, when the dessicator stop cock is closed. Cultures started with an initial parasitaemia of 0.1% reach 5% in four days. If started at 1% they reached 5% two days, that is in one cycle. Higher parasitaemias was attained by changing the medium twice or thrice daily.

Medium, serum and cells: The medium used was RPMI 1640 with 25mM HEPES buffer. Human homologous serum AB, A or O was used. Freshly collected serum was stored at -20°C.

The medium was prepared from the powdered RPMI 1640 with glutamine but without bicarbonate. To prepare 1 litre of medium, 10.4g of RPMI 1640 powder was dissolved in 900ml of glass-redistilled water. To this 5.94g of HEPES buffer was added, the solution made up to 960ml and sterilized by filtration through a Millipore filter of 0.45uM porosity. The medium was completed just before use by the addition of 5% $NaHCO_3$ solution (sterilized by Millipore filtration) at a rate of 4ml per 96ml of the RPMI 1640 medium with HEPES buffer. Serum was then added in appropriate amounts. Complete medium was stored at 4°C and used within one week.

Human erythrocytes were obtained as a unit of blood collected in acid-citrate-dextrose or citrate-phosphate-dextrose and could be used for up to 4 weeks of storage at $4^{o}C$. The red cells were prepared by centrifugation and washed twice in complete medium without serum, with removal of the buffy layer after each of the three centrifugations. The cells were finally suspended at desired concentrations in complete medium with serum.