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Effects Of Allopurinol On Platelet Function In Man And Experimental Animals

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EFFECTS OF ALLOPURINOL ON PLATELET FUNCTION IN MAN AND EXPERIMENTAL ANIMALS

by

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ABSTRACT

Allopurinol was introduced in 1963 for clinical treatment of gout and/or hyperuricemia. As a xanthine oxidase inhibitor, it reduced the amount of uric acid produced, and thus provided an approach to treatment, other than that of uricosurics.

An association between thromboembolism and hyperuricemia has been described (Pearce and Aziz, (1969), and others) and allopurinol's role in purine metabolism has been thoroughly elucidated (Elion, Kovensky, Hitchings, Metz and Rundles (1966), and others).

One of the components of purine metabolism, adenosine diphosphate (ADP), has been shown to be a strong stimulus for the aggregation of blood platelets (Gaarder, Jonsen, Laland, Hellem, and Owren (1961), and others).

The influence of allopurinol on the blood platelets of rats, rabbits and man was investigated.

Pre-treatment of rabbits with intraperitoneal sodium urate (60 mg./kg.) followed in one hour by intravenous potassium oxonate (a uricase-inhibitor) at 60 mg./kg. resulted in an effective animal model of hyperuricemia, with a serum urate of 8 mg.-% (4x normal) 32 minutes after the oxonate injection. Treatment of this hyperuricemic model with intravenous sodium allopurinol (40 mg./kg.), given 20 minutes after the oxonate injection, not only lowered the-

serum urate to normal within 44 minutes, but also resulted in a significant decrease in ADP (3.3×10^{-6}M. final concentration)-induced platelet aggregation within 12 minutes ($p < .05$) of the allopurinol injection.

In rabbits orally pre-treated with allopurinol (40 mg./kg./day), however, ADP (3.3×10^{-6}M. final concentration)-induced platelet aggregation was increased ($p < .02$) within three weeks of such oral treatment. This finding coincided well with an increase in platelet ADP levels in those same rabbits during the same period ($p < .05$).

Further, platelets of nine patients receiving allopurinol for clinical conditions, demonstrated increased aggregation by ADP (4.1×10^{-6}M. final concentration) during month 1 ($p < .01$) of treatment.

Allopurinol in vitro (1.4×10^{-3}M. final concentration) also inhibited collagen-induced aggregation of rabbit ($p < .001$) and human ($p < .005$) platelets.

The evidence presented supports the hypothesis that allopurinol possesses a two-fold action on blood platelets:

1. A short-term direct effect, manifested by decreased platelet aggregation, and prevention of release of platelet constituents.
2. A longer-term effect, manifested by elevated levels of platelet ADP, resulting in increased platelet aggregation

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This thesis is dedicated to my father, Peter Alexander Wildeman (1919-1965) whose inspiration and encouragement have largely been responsible for my seeking higher education.

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INTRODUCTION

The first recognition of platelets as distinct, formed elements of the blood was Donné's (1842) description of "globulins". Hayem (1878a) is credited with the first description of the basic role of platelets in thrombus formation and hemostasis (Hayem, 1882). Although much of the work of these early investigators was later confirmed and clarified there was relatively little platelet research undertaken in the 60 years following 1890. During the last 25 years, research in this area has shifted from the study of blood coagulation to a consideration of platelet function. The latter has been characterized by the study of platelet morphology and kinetics as well as electron-microscopic observation of the hemostatic plug.

An extraordinary resurgence of platelet research followed the discovery (Hellem, 1958) and identification (Gaarder, et al., 1961) of adenosine diphosphate (ADP) as a component of erythrocytes which caused platelets to stick to one another. Additional stimuli to this research were the isolation of a contractile protein, "thrombosthenin", from platelets (Bettex-Galland and Lüscher, 1961), and the observation that platelets adhere to the connective tissue of the vessel wall (Bounameaux, 1959), later specifically identified to be collagen (Hugues, 1960). From these discoveries and from the results of a great deal of recent research by many investigators, it is now generally accepted that ADP, thrombin and collagen are the trigger substances

involved in platelet adhesion and aggregation in hemostasis and thrombus formation. Of these, ADP is believed to be the common mediating substance in all platelet adhesion and aggregation reactions.

Among the benefits of this acceleration of platelet research has been the application of many research techniques to clinical problems. Platelet defects have been observed in some hemorrhagic diseases, such as thrombastnenia (Caen, Castaldi, Leclerc, Inceman, Larrieu, Proust and Bernard, 1966), von Willebrand's disease (Weiss, 1968), and others, offering hope for solutions to these heretofore inexpicable conditions. Considerable evidence has also been accumulated which suggests changes in platelet function in post-operative thromboembolic complications (Ham and Slack, 1967), ischemic heart disease (McDonald and Edgill, 1959), peripheral vascular diseases (Murphy and Mustard, 1962), diabetes (Moolten, Jennings and Solden, 1963), homocysteinuria (McDonald, Bray, Field, Love and Davies, 1964), cancer (Moolten, Vroman, Vroman, and Goodman, 1949), multiple sclerosis (Caspary, Prineas, Miller and Field, 1965), hypercholesterolemia (Gertler, White, Cady and Whitier, 1964), hyperuricemia (Pearce and Aziz, 1969) and venous thrombosis, itself (Bygdeman, Eliasson and Johnson, 1966).

The recognition of the ominous significance of thromboembolic disease as a major public health problem requires consideration of only a few surveys and studies in this area. Even though most studies include thrombosis in the general category

of "heart disease", it should be remembered that thrombosis is a condition common to many other types of disease, including venous thrombosis in extremities and in congestive heart failure, arterial thrombosis in coronary or cerebral vessels, and thrombi seeded with cancer cells which permit metastases to become established (Wood, 1958).

A recent study (World Health Organization, 1967) indicates that heart disease was the leading cause of death in 22 countries in North America, Europe and Oceania in 1964, causing 32.5% of all deaths at all ages, and 36% of all deaths at ages 65 years and over. In 1963, cardiovascular diseases accounted for 54.8% of all deaths in the United States, claiming 993,300 lives (United States President's Commission on Heart Disease, Cancer and Stroke, 1965). "Cardiovascular disease" in Canada in 1965 was responsible for 50% of all deaths of all ages and approximately 62% of all deaths at age 45 and over (Dominion Bureau of Statistics, 1968). The observation of Freiman, Suyemoto and Wessler (1965) that 64% of 61 consecutive autopsies showed evidence of pulmonary thromboembolism, serves only to emphasize the seriousness of thromboembolic disorders.

The great significance and grave consequences of thromboembolic disorders have stimulated much research regarding the effects of new drugs (and drugs already in clinical use) on platelet aggregation, platelet adhesion, and experimental models of thrombosis. Decreased adhesiveness or aggregation of platelets has been reported with acetylsalicyclic acid (Morris, 1967), clofibrate (Symons, de Toszeghi and Cook, 1964), nialamide (Maschouf, Robinson

and LeBeau, 1964), sulfinpyrazone (Mustard, Rowsell and Murphy, 1966), intravenous dextran (Bygdeman, Eliasson and Gullbring, 1966), dicoumarol (Borchgrevink, 1961), heparin (Mustard and Murphy, 1963), corticosteroids (Bounameaux, 1955), antihistamines (Mitchell and Sharp, 1964), monoamine oxidase inhibitors (Shimamoto, 1961, reserpine (Salzman, 1962), prostaglandin E_1 (Kloeze, 1966), aspergillus enzyme (Bygdeman, 1967), phosphatidyl serine (Nishizawa, 1965), dipyridamole (Emmons, Harrison, Honour and Mitchell, 1965), cyclic-AMP (Marcus and Zucker, 1965), and "substance 86" (Bicher, 1970).

On the other hand, some agents induce or enhance platelet adhesion and aggregation, such as adrenaline (Mitchell and Sharp, 1964), noradrenaline (O'Brien, 1964), 5-hydroxytryptamine (Mitchell and Sharp, 1964), and oral contraceptives (Vessey and Doll, 1969).

It is becoming increasingly evident that more must be learned concerning the effects of drugs upon platelet function. A decrease in normal platelet activity may predispose to hemorrhage, particularly in patients with latent or overt coagulation defects. Conversely, hyperactivity of platelets could contribute to thrombotic disease. Drugs possessing such qualities might, however, be useful as adjuncts to the therapy of hemorrhagic or thrombotic disease. Numerous drugs have already been shown to potentiate or antagonize anticoagulant therapy (Hunninghake and Azarnoff, 1968; Moser, 1968 and others).

The research documented in this manuscript was undertaken in an attempt to determine the effects of allopurinol on platelet

adhesiveness and aggregation. Allopurinol (Zyloprim, Burroughs Wellcome and Co.) is a drug used clinically in the treatment of primary and secondary gout and secondary hyperuricemia. It and its dihydroxymetabolite, alloxanthine, competitively inhibit xanthine oxidase, an enzyme which mediates the conversion of hypoxanthine to xanthine, and xanthine to uric acid (Elion, et al., 1966). Adenosine diphosphate has been shown to be degraded, by a number of plasma enzymes, to hypoxanthine via adenosine monophosphate, adenosine and inosine (Holmsen and Rozenberg, 1963). The presence of platelets increases the formation of hypoxanthine without affecting the other reactions involved in the degradation of ADP (Ireland and Mills, 1964). It seemed possible, therefore, that the indirect effect of allopurinol on plasma levels of hypoxanthine and xanthine might also affect the metabolism of ADP, thus influencing platelet adhesiveness and aggregation.

The importance of determining the nature of such platelet effects has been outlined above. In addition, some information might be gained which would be of assistance in the understanding of platelet metabolism and its implications in disease processes.

HISTORICAL REVIEW

A. Early History

The earliest mention of platelets as formed elements of the blood was Donné's (1842) description of red and white globules, and little globules ("globulins") observed in his microscopic studies. This historic description of "globulins" launched an inquiry of research on platelets which has continued for over 130 years.

At about the same time as Donné's discovery, Zimmermann (1846) described some blood cells which he believed were precursors of red blood cells. He named these distinctive blood cells "Elementarbläschen" and noted their tendency to clump together. Zimmermann was one of the first investigators to use anticoagulants by collecting horse blood in equal parts of 6% magnesium sulfate.

Much of the very early investigative work was characterized by a generous portion of erroneous conclusions. Schultze (1865), for example, correctly observed that platelets have a tendency to clump and form granular masses ("Kugel"), but he incorrectly concluded that these small elements of the blood were formed as the result of the destruction of the white corpuscles, and that they were in no way similar to Zimmermann's "Elementarbläschen". This erroneous hypothesis of Schultze was later supported by Riess (1872), who named them "Zerfallskorperchen" (disintegration bodies) because he thought that they were fragments of disintegrated white corpuscles. Because

of the variations in platelet shape due to varying collection and handling techniques, it is understandable that they were sometimes described as bacteria (Osler and Schaefer, 1873).

Vulpian (1873) noted the presence of platelets sticking to a cover glass and observed platelet clumping, while in the same year, Ranvier, (1873) observed platelet masses in the centre of the fibrin network of coagulated blood, concluding that they were granulations of fibrin.

Using recently-killed young rats, Osler (1874) demonstrated for the first time that the "granular masses" of Schultze were really the aggregation of small blood cells which occurred as single units in the circulation.

Hayem (1878b), being aware of platelets, incorrectly believed that they were primitive red cells ("hemoblasts") whose functions included acceleration of blood coagulation, as well as the regeneration of the blood.

From his observations of the circulating blood of living animals, Bizzozero (1882) concluded that the "granular masses" of Schultze were not residues of white blood cells, nor were they fibrin granulations as suggested in 1873 by Ranvier. It was Bizzozero who, in 1882, firmly established the foundation of the present day concept of the platelet as a distinct blood cell ("Plättchen") which has a role in thrombosis. He was able to demonstrate that the white thrombus consisted almost exclusively of platelets which had accumulated subsequent to vessel wall injury or obstruction of blood flow. When accumulated in a thrombus, either *in vivo* or *in vitro*,

platelets exhibited changes in appearance and became unusually sticky, which he described as "viscous metamorphosis".

Bizzozero's findings aroused considerable controversy, with investigators such as Howell (1884) supporting his views, and workers such as Schmidt (1882) and Lowit (1885) expressing sharp opposition, the latter authors still believing that these "Plättchen" were nothing more than leukocyte fragments. In response to Weigert's (1887) criticism (in that Bizzozero's observations were really artifacts due to circulatory disturbances, vessel compression, and anesthesia), Bizzozero repeated his experiments on the intact vessels of a live, unanesthetized bat (Bizzozero, 1891) confirming his previous observations.

Hayem (1896), and others, developed platelet-counting techniques with sufficient proficiency to observe thrombocytopenia associated with certain hemorrhagic disorders.

At the turn of the century, scientific debate was still raging regarding the existence of platelets; and for those who believed that they did in fact exist, the origin of platelets was the subject of much speculation. It was Howell (1890), Dominici (1900), Wright (1910), and others, who re-affirmed Bizzozero's (1869) observation of megakaryocytes giving rise to platelets.

Deetjen (1901), using osmic acid fixation and other special techniques, demonstrated what he believed to be platelet nuclei, protoplasm and protoplasmic pseudopods. Dekhuyzen (1901), supporting Deetjen's views, and thinking that the mammalian platelets were homologous, in their function during bleeding, to the nucleated

spindle cells of invertebrates and oviparous animals, named the mammalian platelets "thrombocytes".

During most of the first half of this century platelet research was concentrated on thrombopoiesis, platelet morphology (Wolpers and Ruska, 1939), and blood-clotting and plasma-clotting factors with relatively little work being conducted on the mechanisms involved in platelet function.

Threat of nuclear war indicated a very practical need for research on the technological aspects of adequate platelet transfusions in anticipated radiation-induced thrombocytopenia, resulting in the establishment, in 1951, of research funds for the investigation of platelet biochemistry and physiology (Johnson, 1971). This funding, the recent prominence of electron microscopy in hematology, and other stimuli, launched a newly-accelerated period of research into platelet function which has continued inexorably for the last twenty-five years. This progression of interest and research in platelets has reached a level of activity at the present time that has resulted in several recent international symposia, societies, councils, etc., whose sole interests are platelets, and their many implications, in health and disease.

B. Development of Techniques for Evaluation of Platelet Function

In attempts to obtain a clearer understanding of the role of platelets in hemorrhagic and thrombotic clinical conditions, several techniques have been developed to evaluate platelet function. Henry (1971) has provided a thorough summary of in vivo techniques in

animals which attempt to simulate thrombotic conditions. These techniques include injection of thrombosing substances, mechanical trauma, perivascular applications of thrombosing substances, slowing of the circulation, intravascular insertion of foreign bodies, electric currents, and alterations in diet.

Of greater clinical interest has been the development of *in vitro* tests of platelet function which are applicable for use in testing platelets in relatively small samples of blood from patients.

One such *in vitro* test is Wright's (1941) rotator method, which involves the determination of the percentage of platelets, in heparinized blood, which adhere to the unsiliconized interior surface of a glass flask, rotating at 3.5 r.p.m. Although this test indicates abnormally-elevated adhesiveness data in post-operative thrombosis, in myocardial infarction and in homocysteinuria, it is not sufficiently reliable to differentiate between normal and abnormal individuals.

At approximately the same time as the discovery (Hellem, 1958), and identification (Gaarder, *et al.*, 1961), of ADP as the component of erythrocytes which was the cause of platelets sticking to one another, Hellem (1960) developed the Hellem glass bead column method of assessment of platelet adhesiveness. This test involves the passage of anticoagulated blood through a column of glass beads, noting the percentage of platelets retained in the column by adhesion to the glass beads or by adhesion to platelets which had already adhered to the glass beads. This test, as described above, and modifications of it (O'Brien, 1961; Hellem, Odegaard and Skalhegg, 1963) have been studied extensively but, unfortunately, their clinical

value has yet to be clearly established.

The Salzman glass bead column technique (Salzman, 1963) involves the passage of native blood, drawn directly from a venipuncture, through a column of glass beads, into a vacuum tube containing anticoagulant. In this test, the anticoagulant variable, and the time between venipuncture and glass bead exposure, have been eliminated or standardized. The blood flow rate, on the other hand, cannot be controlled, and decreases as the vacuum in the vacuum tube decreases. Platelet adhesiveness, using this technique, has been shown to be reduced in individual patients with von Willebrand's Disease (Strauss and Bloom, 1965) and shown to be unusually low in uremic patients (Salzman and Neri, 1966). O'Brien and Heywood (1967) subsequently modified the Salzman technique by forcing collected native blood, through the glass bead column, at a constant speed. Since glass is a foreign substance to platelets, any correlation between platelet adhesiveness to glass, and clinical conditions, may still be only coincidental. The Salzman technique has created such interest, however, that the Subcommittee on the Assessment of Platelet Function (of the International Committee on Haemostasis and Thrombosis) undertook a co-operative international study (Born and Mason, 1971) of the Salzman technique, concluding (Murphy, 1971) that it does not distinguish significantly between von Willebrand's disease and control subjects if adjustment is made for Factor VIII levels, bleeding time, hematocrit and age, all of which were found to be directly proportional to the Salzman adhesiveness, except for bleeding time, which was inversely proportional. The subcommittee further concluded, therefore, that further modification and standardization of the Salzman test is

necessary.

A method not involving glass beads, is Chandler's (1958) tube technique in which native or anticoagulated blood is put into a plastic tube, and the two ends of the tube joined together with the blood occupying only the bottom one-third of the tube's capacity. This circular tube is then rotated at an appropriate speed such that the blood remains at the bottom of the tube, with a film of blood being lifted up one side of the tube and transferred to the other side. At the point where this film joins the pool at the bottom of the circular tube, a formation occurs which histologically resembles a fresh thrombus. Although this method is of considerable interest as a research technique, it is not yet used extensively as a clinical diagnostic test.

Since adheston of platelets probably involves electrostatic forces, Hampton and Mitchell (1966a) investigated the nature of electrostatic charges on platelets in what has become known as platelet electrophoresis. They were able to show that the movement of platelets under the influence of an electric current (electrophoretic mobility) was decreased by exposure to glass. This is a very interesting technique, but it has not become adopted as a routine diagnostic test.

Platelet aggregation was first reported as a useful technique by Born (1962) and by O'Brien (1962). This technique involves passing a light beam through platelet-rich plasma, adding an aggregating agent, and recording the increased transmission of light through the platelet-rich plasma, as the platelets clump together in aggregates. The aggregation response can be quantified by measuring

the slope of the curve, the maximum curve height, the area under the curve, the slope of the disaggregation curve, and the time for disaggregation to begin. Even though many investigators have made numerous modifications to this technique, it still has disadvantages, such as the variability in the preparation of platelet-rich plasma. Nevertheless, this technique has become so widely accepted and so extensively used that a symposium was devoted entirely to this subject (Vermylen, de Gaetano, and Verstraete, 1971). A study is currently underway, assisted by the Subcommittee on the Assessment of Platelet Function (Born and Mason, 1971), to attempt to standardize this technique. Platelet aggregation is undoubtedly the method of choice for the assessment of platelet function because of the increasingly significant relevance of these test data to clinical conditions (Vermylen, et al., 1971).

Some of the foregoing techniques for evaluation of platelet function have proven useful in the study of clinical conditions with which thrombotic or bleeding tendencies have been associated. Although these techniques are extensively used in research respecting specific diseases, they have not gained wide acceptance as diagnostic aids.

The association of arterial occlusion with diabetes mellitus, has been accepted for many years (Feldman and Feldman, 1954; and others). Recent evidence indicates that this association can be detected with in vitro platelet tests. Rathbone, Ardlie and Schwartz (1970), using the Chandler method, were able to demonstrate that thrombus formation was significantly increased in platelet-rich plasma from diabetics as compared with that from normal controls. Increased adhesiveness of platelets to glass beads was shown by Shaw,

Pegrum, Wolff and Ashton (1967) to occur in diabetes mellitus.

Using the Wright rotator method and the photometric (Born) method, Coccheri and Fiorentini (1971) found increased platelet adhesiveness and increased platelet aggregation, respectively, in hypertensive patients.

In the hemorrhagic disease, thrombasthenia, a platelet defect is suspected to account for the failure of these platelets to aggregate (with the Born method) and their failure to adhere to glass beads (adhesiveness test) (Hellem, 1968).

Synthetic substances (e.g. heart valve prostheses, etc.), when exposed to blood, develop a surface coat of adsorbed plasma constituents to which platelets may adhere (Salzman, 1971). Mustard, Glynn, Nishizawa and Packham (1967) reported that platelets adhered to gamma-globulin-coated surfaces, undergoing a release reaction, whereas fibrinogen-coated surfaces enabled platelets to adhere, without releasing their contents. Although anticoagulants, alone, do not effectively prevent fibrin deposition on heart valve prostheses (Douglas, 1971), there is evidence that anticoagulants combined with dipyridamole result in a sharp reduction in the incidence of thromboembolism in these cases (Sullivan, Harken and Gorlin, 1968). Dipyridamole, and other similar drugs, were first observed to inhibit platelet aggregation in the <u>in vitro</u> Born technique, before it was considered advisable to test their effectiveness for therapeutic prevention of thromboembolism. Drugs such as dipyridamole may also be useful in the prevention of rejection of transplanted organs, such rejection often being closely associated with accumulation of

platelet aggregates in the vasculature of the transplanted organs (Dempster, 1969). Several analogues of dipyridamole are currently being studied with in vitro platelet aggregation tests, because of their superior ability to inhibit platelet aggregation (Philp, Francey and Gibson, 1971).

C. Current Understanding of Platelet Physiology and Biochemistry

Until relatively recently, platelets were thought to be metabolically inert, simple cells. The work of many investigators, however, has shown that platelets contain all the metabolic capabilities necessary to living cells, with the exception of reproduction. A detailed discussion of the biochemical aspects of platelets has been presented by Seitz (1969).

1. Role of Some Biochemical and Physical Factors in Platelet Aggregation and Adhesion.

(i) Adenine Nucleotides

Subsequent to Hellem's (1958) observation that a substance from erythrocytes (later identified by Gaarder, et al (1961) as ADP) was capable of making platelets stick to one another, attention was focused on ADP and related compounds. ADP induces a change in shape in platelets from the normal disc shape to a spherical shape with pseudopodic protrusions (White, 1968), with the normally randomly distributed granules clustered in the centres of the aggregated platelets. There is a considerable

amount of disagreement regarding a possible platelet volume change in conjunction with this shape change (Born, 1971). Mannucci and Sharp (1967) and others have stated that the rapid platelet shape change recorded optically is associated with an increase in platelet volume. Born (1970, 1971) presented convincing evidence that the brief, increased, optical density following the addition of ADP to platelet-rich plasma, is entirely due to platelet shape change and that there is no platelet volume change in these circumstances.

Macmillan (1966) observed a "biphasic" platelet aggregation when induced by ADP or adrenaline, the first phase being reversible and the second phase being irreversible. The first phase of aggregation was thought to be due to the added ADP or adrenaline, whereas the second phase of aggregation has been associated with the release of ADP (Macmillan, 1966), 5-hydroxytryptamine (Zucker and Peterson, 1967) platelet factor 3 (Zucker and Peterson, 1967; Horowitz and Papayoanou, 1968) and platelet factor 4 (Niewiarowski, Lipiński, Farbiszewski and Poplawski, 1968; Niewiarowski and Thomas, 1969) from platelets. Zucker and Peterson (1968) have also shown that acetylsalicylic acid inhibited the second phase of platelet aggregation by interfering with the release of ADP from the platelets. Stabilizers of biologic

membranes, such as chlorpromazine, imipramine and desmethylimipramine were found to inhibit the second phase of platelet aggregation (Mills and Roberts, 1967).

The uptake into, the storage of and the release ("platelet release reaction") from platelets of substances such as ADP, have been the subjects of intensive research for some time, and have been discussed in excellent reviews (Holmsen, Day and Stormorken, 1969; Holmsen and Day, 1971). Holmsen (1971) has summarized this subject once again, outlining the "three-pool concept" of adenine nucleotides and has suggested the presence of a "fourth pool". Using radioactive adenine nucleotide precursors in vitro, Holmsen, Day and Storm (1969) showed that platelets subsequently exposed to release inducers, released non-radioactive nucleotides, and in fact, retained the radioactive nucleotides within the platelets. These findings suggested the presence of two distinct "pools" of adenine nucleotides in platelets. The non-radioactive nucleotides in these platelets are considered to be the "storage pool", comprising approximately 2/3 of the ATP and ADP of platelets. These adenine nucleotides in the platelet storage pool are released directly into the extracellular medium, during the platelet release reaction.

These storage pool adenine nucleotides are not labelled with radioactive orthophosphate, adenine, or adenosine and are thus considered to be metabolically inactive (i.e., not part of the "metabolic pool"). This "metabolic pool" is, on the other hand, labelled by these radioactive nucleotide precursors, and does not release its contents during the release reaction. During the release reaction, about 5 - 25% of the ATP in the metabolic pool is converted, via IMP, to hypoxanthine, suggesting that this consumption of energy enables the release reaction to take place. The ATP so converted has been designated the "release energy pool", the third "pool" in this "three-pool concept". A "fourth pool" was suggested (Holmsen, 1971) as being that portion of the metabolic pool of platelet adenine nucleotides which can be demonstrated to be protein-bound. This fourth pool increases during the release reaction indicating that it is in this fourth pool that ATP is converted to ADP.

Considering ADP to be the common mediating factor inducing platelet aggregation, many investigators attempted to explain the mechanism whereby ADP induces platelet aggregation. Gaarder and Laland (1964) and Hellem and Owren (1964)

postulated that ADP was bound to platelet membranes by hydrogen bonding and calcium complexation, thus effecting platelet aggregation, and that substances which were inhibitors of platelet aggregation acted as competitive inhibitors for the ADP receptor sites on the platelets. Born (1965) and Hampton and Mitchell (1966b), using different methods, determined that there are approximately $1x10^5$ such binding sites per platelet, at which ADP could become attached. Salzman, Chambers and Neri (1966), on the other hand, maintain that ADP inhibits a platelet membrane "ecto-ATPase" which normally is responsible for keeping the platelet membrane in an "unsticky" state. Platelets held together only by calcium bridges, has been suggested by Davey and Lüscher (1968), as a possible mechanism. Rozenberg and Holmsen (1968) proposed that adenosine inhibits platelet aggregation by uptake of adenosine by the platelets, this uptake requiring energy provided by ATP stored in the platelets, thus less ATP is available to induce, via ADP, platelet aggregation. The adenosine so taken up by the platelets, and phosphorylated by adenosine kinase, could also interfere stereochemically with myokinase, possibly thus inhibiting platelet aggregation. Rafelson and Booyse (1971) have shown immunohistochemically that interplatelet bridges

consist of thrombosthenin. These authors suggest that interplatelet bridges occur before aggregation occurs and become unidentifiably merged after platelet aggregation has occurred. They suggest, therefore, that ADP or thrombin induces the formation of interplatelet bridges of actin-like and myosin-like thrombosthenin moieties.

A great deal of other work has been carried out by many investigators on substances, such as sulfhydryl inhibitors, amino acids, local anesthetics, prostaglandins, adenosine, anti-inflammatory agents, and others, with a view to determining the mechanism of inhibition of ADP-induced platelet aggregation (Aledort, 1971). Of particular interest is the platelet aggregation inhibiting effect of increased levels of platelet cyclic AMP (3', 5'-adenosine monophosphate; cAMP). Stimulation of platelet adenyl cyclase activity, resulting in increased platelet cAMP, inhibits platelet aggregation. Stimulation of platelet phosphodiesterase activity, resulting in decreased cAMP, enhances platelet aggregation. Adrenaline, noradrenaline, serotonin, thrombin and collagen, all of which are substances which initiate the platelet release reaction, have been shown (Salzman, Rubino and Sims, 1970) to inhibit platelet adenyl

cyclase. Adenosine, which is a strong inhibitor of platelet aggregation, is capable of not only inhibiting phosphodiesterase, but also of stimulating adenyl cyclase, both effects resulting in elevated cAMP (Haslam and Lynham, 1972). ADP, which is capable of inducing first phase platelet aggregation, as well as initiating the platelet release reaction, stimulates platelet phosphodiesterase (Salzman, et al., 1970). Similarly, platelet cAMP is increased (and therefore platelet aggregation is inhibited) by prostaglandin E_1 (stimulates platelet adenyl cyclase) and caffeine (inhibits platelet phosphodiesterase) (Salzman, et al., 1970). The consistency of these observations suggests that cAMP may in some way provide a basic mechanism of platelet aggregation, common to most or all of the known inducers of platelet aggregation. Mills and Smith (1971) obtained similar results in which adenosine, 2-chloroadenosine, isoproterenol, prostaglandin E_1, and other drugs which inhibit phosphodiesterase, were shown to inhibit platelet aggregation, probably by a common mechanism involving cAMP.

(ii) Collagen

Even though Bizzozero had demonstrated in 1882 that a white, platelet thrombus accumulated at the site of vessel wall injury, it was not until 1959 that Bounameaux reported that fragments of vascular endothelium, when stirred with platelets, resulted in platelet aggregation. Hugues (1960) demonstrated that the portion of connective tissue which induced platelet aggregation was collagen. Hovig (1963) noted that platelets aggregated by tendon extract resulted in the supernatant having the ability to induce aggregation in other platelets, leading to the theory that ADP and other substances released from platelets in contact with collagen, and not collagen itself, are responsible for collagen-induced platelet aggregation. It is also known that platelets, as well as adhering to exposed collagen, also adhere to vascular endothelium and exposed basement membrane (Hovig, 1971; Warren and Vales, 1972). Aledort (1971) summarizes the considerable controversy still prevalent regarding the precise mechanism of action of collagen as an inducer of platelet aggregation. This controversy is probably understandable when the data of Legrand and Pignaud (1971), and others, are considered regarding the variability of collagen preparations

with respect to age and species of the collagen source material, the purity of the collagen suspension when prepared and the standardization of technique necessary for the proper use of collagen suspension. Although two collagen preparations are under study (Day and Hardisty, 1974), and one such preparation may become accepted as a standard, collagen variability is still a problem at the present time.

D. Gout and Hyperuricemia

Gout has been of concern to mankind since ancient times, when joint pain, gouty nodules and incapacitation were readily observable. Historically, it is recognized (McCarty, 1970) that Hippocrates (460-377 B.C.) associated gout with adulthood, that Galen (129-199 A.D.) recognized familial gout, and that von Leeuwenhoek (1632-1723) was the first to make microscopic observations, in 1679, of urate crystals from a gouty tophus. In 1848, Garrod contributed greatly to modern clinical diagnosis of gout by observing abnormally elevated quantities of uric acid in blood.

Salicylates were used (for their uricosuric effects) more than 1900 years ago in attempts to relieve the discomforts of gout. Colchicine's cathartic effect was considered beneficial to gout patients in the sixth century A.D. It was not until 1951 that probenecid, a by-product of penicillin research, was used clinically as a uricosuric. Sulfinpyrazone, a metabolite of phenylbutazone,

was found to have uricosuric effects and was used clinically for that purpose in 1957. The introduction, in 1963, of allopurinol, not a uricosuric, but a xanthine oxidase inhibitor, to the drug armamentarium for the treatment of gout and hyperuricemia undoubtedly heralded a new era for an ancient disease. This drug was found to be capable of inhibiting xanthine oxidase, an enzyme necessary for the formation of uric acid from hypoxanthine and xanthine.

Although gout has an incidence of about 0.1% of the population, hyperuricemia (with or without gouty signs) has an incidence of 3.5% of the population. Normal levels of urate in the human serum are 3-5 mg.-%, with the theoretical limit of solubility of monosodium urate in serum at approximately 6.3 mg.-%. Gout patients, therefore, having serum urate levels of 8-12 mg.-%, have a "super-saturated solution" of urate in their serum. The reported affinity of urates for connective tissue (Katz and Schubert, 1970), has been suggested as an explanation for the preferential precipitation of urates in joints.

Plasma glutamate levels have been found to be markedly elevated in gout, with reduced ammonia excretion leading to increased urinary acidity and development of urate stones in the kidneys (Pagliara and Goodman, 1969). It was postulated, therefore, that perhaps glutamine was being utilized to produce the urate found in abnormally high quantities in gout patients.

Paulus, Coutts, Calabro and Klinenberg (1970) reported that 20% of hyperuricemic, hospitalized patients studied were hyperuricemic due to decreased urate excretion associated with

diuretic therapy.

Hyperuricemia has also been shown to be associated with many diseases and states (Scott, (1969); Bluhm and Riddle (1973); and others) such as lacticacidemia, stress, congestive heart failure, hypertension, hemorrhagic shock, cancer and thrombosis.

Of experimental significance is the work of Johnson, Stavric and Chartrand (1969) and Stavric, Vera, Johnson and Salem (1973) in which they demonstrated that potassium oxonate inhibition of uricase in rats resulted in hyperuricemia, hyperuricosuria, elevated blood urinary nitrogen, and multiple kidney stones in rats, which could then be considered an animal model for these conditions or diseases.

E. Purine Metabolism

1. Purine Catabolism, and Effects of Allopurinol

Purines, important constituents of nucleic acids, are catabolized in a variety of ways, depending upon the species (Harbers, Domagk and Müller, 1968). The end product of purine catabolism in humans, higher apes, reptiles and birds, is uric acid, usually excreted via the kidneys. Many animals, however, by the action of uricase, are able to catabolize uric acid to allantoin. All purines are catabolized in man to a common product, xanthine, which in turn is catabolized to uric acid. Of particular interest are the precursors to xanthine which are hypoxanthine, inosine, adenosine, AMP, ADP and ATP, respectively. Xanthine oxidase, which mediates

the conversion of hypoxanthine to xanthine, and xanthine to uric acid, is inhibited by allopurinol and by the metabolite of allopurinol, alloxanthine. This enzyme inhibition by allopurinol and alloxanthine interferes with the production of uric acid, thereby lowering serum urate levels.

2. Purine Biosynthesis and Feedback Inhibition

The biosynthesis of purines involves, firstly, the utilization of ribose-5-phosphate, ATP, glutamine, glycine, carbon dioxide and formate resulting in the formation of inosinic acid (IMP). The IMP is then transformed into guanosine monophosphate (GMP) and adenosine monophosphate (AMP). Subsequent phosphorylation of AMP results in the formation of ADP and ATP.

Even though allopurinol has proven to be an effective drug for controlling purine catabolism by interfering with uric acid production, it might be more efficient to control purine biosynthesis at an early stage, thus reducing the quantity of urate precursors. Some known physiological "feed-back" controlling mechanisms have been outlined by Seegmiller (1970). The rate-limiting step in purine biosynthesis is that in which 5-phosphoribosyl-1-pyrophosphate (PRPP) is utilized in the formation of inosinic acid. Inosinic acid, along with guanylic acid and adenylic acid act as feed-back inhibitors of PRPP. PRPP is also involved in the conversion of guanine to guanylic acid, and of hypoxanthine to inosinic acid, both

reactions being mediated by hypoxanthine-guanine phosphoribosyltransferase (HG-PRT). HG-PRT is therefore an enzyme which is acting as an indirect feed-back inhibitor of PRPP. Of interest here, are the findings of Lesch and Nyhan (1964) in which certain individuals with neurological abnormalities and self-destructive traits, demonstrated markedly-elevated serum urate levels. This condition, the Lesch-Nyhan Syndrome, was subsequently shown to be due to a deficiency of HG-PRT resulting in elevated urate levels due to inefficient conversion of hypoxanthine to inosinic acid, and thus inefficient feed-back inhibition of PRPP by inosinic acid (Seegmiller, Rosenbloom and Kelly, 1967). Kogut, Donnell, Nyhan and Sweetman (1970) reported finding patients with only a partial HG-PRT deficiency who are normal neurologically and who exhibit no self-mutiliation, even though they have elevated serum urate levels. The severity of this condition may, therefore, be proportional to the extent of the HG-PRT deficiency. In both total and partial HG-PRT deficiency, use of allopurinol has been beneficial.

Pomales, Bieber, Friedman and Hitchings (1963) showed that allopurinol, by inhibiting xanthine oxidase, resulted in a greater accumulation of xanthine and hypoxanthine, and caused a greater re-incorporation of hypoxanthine into nucleotides and nucleic acids.

The findings of Kelley and Wyngaarden (1970a; 1970b) are indicative of the continued interest in the role of allopurinol in feed-back control of purine biosynthesis. Using cultured human fibroblasts as an *in vitro* model, these authors found that allopurinol and alloxanthine (oxipurinol) inhibited purine synthesis *de novo*. They postulated three possible mechanisms for these effects:

1. Allopurinol or oxipurinol, acted upon by HG-PRT could result in allopurinol-or oxipurinol-ribonucleotide, which in turn would inhibit PRPP.
2. Allopurinol, by inhibiting xanthine oxidase, provides increased quantities of xanthine and hypoxanthine, the latter resulting in increased production of inosinic, adenylic and guanylic acids, which would inhibit PRPP.
3. Since both allopurinol and hypoxanthine require PRPP in the formation of allopurinol ribonucleotide and hypoxanthine ribonucleotide, perhaps the formation of these ribonucleotides causes a depletion of intracellular PRPP which may decrease purine biosynthesis.

The third possibility above was confirmed by Fox, Wyngaarden and Kelly (1970) when they reported that the use of allopurinol in gout patients resulted in a significant reduction in their erythrocyte PRPP content. They were also able to demonstrate, *in vitro* and *in vivo*, that this depletion of PRPP resulted from the enzymatic conversion of allopurinol to allopurinol ribonucleotide, a reaction which required PRPP and therefore lowered the PRPP levels in erythrocytes.

METHODS

A. Hematological Techniques and Experimental Subjects

All syringes used in these techniques were either plastic disposable or siliconized glass. All glassware was siliconized.

1. Collection of Blood Samples

(i) Rats

Rats (male, Sprague-Dawley, 150-250 gm.) were anesthetized with 50 mg./kg. of pentobarbital sodium (Nembutal Sodium, Abbott) in order to expose surgically the inferior vena cava from which 4.5 ml. of blood were collected using a 22 gauge, 1 in. needle and a 5 ml. syringe containing 0.5 ml. of 3.8% sodium citrate (i.e. 9:1, blood to sodium citrate solution). After adequate mixing by gentle inversion, 3 ml. of blood were transferred into a test-tube for platelet-counting and for the preparation of citrated, platelet-rich plasma (CPRP). The remaining 2 ml. of blood were used to determine platelet adhesiveness.

(ii) Rabbits

(a) Cardiac Puncture

Rabbits (male, California or New Zealand White, 2.0-3.0 kg.) were anesthetized with 35 mg./kg. of pentobarbital sodium injected into a marginal ear

vein with a sterile, 26 gauge, 1/2 in. needle and a sterile, 5 ml. syringe. The chest area of the rabbit was clipped and was swabbed with 70% ethyl alcohol. Using an 18 gauge, 1 1/2 in. needle, aseptic cardiac puncture was effected through the third, left intercostal space, 4 mm. from the sternum (Schermer, 1967). A stethoscope was held against the left side of the chest to confirm left ventricular cardiac puncture. Approximately 10-15 ml. of blood were withdrawn and mixed, as before, with sodium citrate for the preparation of citrated, platelet-rich plasma (CPRP), platelet counts and the determination of platelet adhesiveness.

(b) Intravenous Cannulation.

Rabbits were anesthetized with 20% ethyl urethane (ethyl carbamate) in saline, given intravenously via the marginal ear vein until a suitable surgical level of anesthesia was obtained (usually approximately 2 gm./kg.). The trachea was cannulated to ensure unobstructed respiration. The right, jugular vein and the right, carotid artery were cannulated with Intramedic, polyethylene tubing (Clay-Adams) PE 160. Each segment of tubing, with an 18 gauge needle attached, was filled with saline prior to installation in the blood vessel. This

tubing was kept patent by periodic withdrawals of 1 ml. of blood with subsequent saline flushing. Seven milliliter blood samples were collected via the cannulated carotid artery at 2 min. before and at 2, 16, 32, 64, 120, 180, and 240 min. following the intravenous injection of a drug. A portion of each sample was left at room temperature to clot. The remainder was anti-coagulated with sodium citrate, as before, for determination of platelet counts, packed cell volume (PCV), and platelet aggregation in CPRP.

(iii) Human Subjects

(a) Oral Therapy with Allopurinol

Approximately 5 ml. of blood were drawn from the cephalic vein in the cubital fossa using a sterile, 18 gauge, 1 1/2 in. needle and a plastic 10 ml. syringe. After disengaging the needle from the syringe, this first sample was discarded. Without removing the needle from the vein, two, 10 ml. samples were collected in separate syringes. A further 5 ml. blood sample was similarly collected to be used for serum urate determination. The blood was anti-coagulated as before and transported to the laboratory in an insulated carrying case at approximately 20°C. Aliquots were prepared for platelet counting, platelet adhesive-

ness determinations and aggregation studies on CPRP.

(b) Blood Donors for *in vitro* Studies

A single sample of approximately 20 ml. of blood was collected and anticoagulated, as described in (a) for the preparation of CPRP.

Blood donors were all healthy young adult male and female volunteers who had not ingested acetylsalicylic acid or any other platelet-affecting drugs during the seven days preceding the blood donation.

2. Packed Cell Volume (PCV) Determination

Microhematocrit tubes containing samples of citrated, whole blood were flame-sealed at one end, placed in transparent segments and centrifuged at approximately 3000 r.p.m. for 10 min. in a Microhematocrit Centrifuge (Measuring and Scientific Equipment Ltd.). Packed Cell Volumes (PCV) were measured with a Microhematocrit Reader (Measuring and Scientific Equipment Ltd.).

3. Blood Platelet Counts

Blood samples were diluted 200 times in Adams, red cell pipettes using the Wright (1941) modification of Reese-Ecker's diluting fluid. Platelets were counted visually in Spencer-Bright Line, improved, Neubauer, hemocytometer chambers.

4. Platelet Adhesiveness Determination

A modification of the glass-bead method introduced by Hellem (1958), and later fully described (Hellem, 1960), was used to measure the adhesiveness of platelets in whole blood. In this procedure, citrated, whole blood is driven vertically through a column 8 mm. in diameter containing 5 gm. of glass beads (B. Braun, 0.5 mm. diameter, Canlab) at a constant rate of 1 ml./min. Platelet adhesiveness was defined as the percentage of the platelets in the original blood sample which adhere to the glass beads:

$$\text{Percent-Adhesiveness} = \frac{(\text{Original Count} - \text{Final Count}) \times 100}{\text{Original Count}}$$

5. Preparation of Citrated Platelet Rich Plasma (CPRP)

Citrated, whole blood was centrifuged at 750 g. for 90 sec. in an International Clinical Centrifuge, Model CL. The supernatant, CPRP, was drawn off and the CPRP platelet count determined in preparation for its dilution.

6. Preparation of Diluted CPRP

The CPRP obtained as outlined above was diluted, to a predetermined platelet concentration, by using as the diluent, cell-free citrated plasma obtained by centrifuging a portion of the original, citrated, whole blood sample at 750 g. for 10 min. in an International Clinical Centrifuge, Model CL. A platelet count was then determined on the diluted CPRP to verify the desired platelet concentration.

The diluted CPRP was then pipetted, in aliquots of 0.3 ml. for rat and rabbit samples, and 0.35 ml. aliquots for human samples, into aggregometer cuvettes, each containing a siliconized stainless steel magnetic stirrer, in preparation for the platelet aggregation determination.

7. Platelet Aggregation Determination

Platelet aggregation was measured by a method based upon that introduced by Born (1962), and by O'Brien (1962), and subsequently modified by Cuthbertson and Mills (1963) to permit automatic recording.

A Bryston Aggregometer, connected to a Bausch and Lomb VOM5 chart recorder, was used to measure changes in the optical density of stirred rat and rabbit CPRP upon the addition of adenosine diphosphate (ADP) or collagen. Before each experiment was begun, the aggregometer was standardized by setting the base-line with an optically-dense standard (O.D. = 1.6) and by setting the sensitivity with a standard of low optical density (O.D. = 0.57).

All rat and rabbit platelet aggregation determinations were undertaken at room temperature with the CPRP stirring speed maintained at 1100 r.p.m. A high degree of accuracy was achieved in the addition of ADP, or collagen suspensions, by the use of an "Agla" Micrometer Glass Syringe and a Micrometer Syringe Driver (Burroughs Wellcome, and Co.).

Platelet aggregation in human CPRP was carried out in a manner similar to that described above except that in this instance the aliquots were warmed to 37°C. and held at that temperature for 5 min. prior to their installation in the aggregometer. The Born Aggregometer (Department of Pharmacology, Royal College of Surgeons, London, England), connected to a Rikadenki Kogyo Electronic Recorder (Model B-141) was used for the aggregation determinations in human CPRP. This equipment employs a water-jacketed cuvette housing, maintaining the temperature at 37°C. during the aggregation determination.

8. Serum Urate Determination

Serum urate levels in human blood were measured in the Department of Laboratory Services, Westminster Hospital, with an autoanalyzer employing a modification of the orthophosphotungstic acid, colorimetric method of Folin and Denis (1912).

Serum urate levels in rabbit blood were determined in this laboratory by first centrifuging the clotted blood samples at 15,000 r.p.m. in a Sorvall SS-3 Superspeed centrifuge for 10 min. The supernatant serum was removed and assayed for urate content according to the phosphotungstic acid colorimetric method of Wells (1968), employing a Zeiss MM12 Spectrophotometer connected to a Zeiss PMQII Recorder. All urate levels were subsequently read from a previously-determined curve of standard urate and optical density readings.

9. Determination of Total Nucleotides, Adenosine Triphosphate (ATP), and Adenosine Diphosphate (ADP)

For the adenine nucleotide assays, rabbit blood collected by cardiac puncture was anticoagulated with 0.5 ml. of 0.077 M. disodium ethylenediamine tetraacetate (EDTA) (Fisher, A.C.S.) solution to which was added 6.5 ml. of blood. This mixture was mixed as previously described and refrigerated for 15 min. at +4°C. All centrifuging details described below were carried out in a Sorvall Superspeed, RC-2B automatic, refrigerated centrifuge at +4°C. The platelet re-suspending procedure outlined below is a modification of the method described by Haslem (1964) and that of the firefly luciferase assay of adenine nucleotides is a modification of the method described by Holmsen, Holmsen, and Bernhardsen (1966). Details of the re-suspending fluids, standards, enzyme preparations, luciferase preparation, etc. are outlined in Appendix I. The following briefly describes the procedure employed in these adenine nucleotide assays:

(1) The EDTA-anticoagulated, cooled, blood samples were centrifuged at 120 g. for 10 min. after which the platelet-rich plasma (EPRP) was removed.

(2) The EPRP was centrifuged at 75 g. for 5 min. to separate the remaining few erythrocytes from the EPRP, after which this "refined" EPRP (EPRP(R)) was removed and the platelet count determined.

(3) The EPRP(R) was centrifuged at 3000 g. for 10 min. to separate the platelets from the plasma, after which the plasma was removed and an equal volume of Suspending Fluid No. 1 (S.F.No.1) was

added to the platelets.

(4) The platelets were resuspended in S.F.No.1 by agitation in a Vortex Test Tube Mixer (Scientific Industries, Model K-500-4) at "high" speed. Agitation was continued until the platelets were uniformly resuspended (approximately 2 min.). This resuspension was then centrifuged at 3000 g. for 10 min. to remove the platelets from S.F.No.1.

(5) The S.F.No.1 was removed and an equal volume of Suspending Fluid No. 2 (S.F.No.2) was added to the platelets following which they were resuspended by agitation as described above. The platelet count in S.F.No.2 was determined and then this resuspension (in S.F.No.2) was frozen.

(6) Samples in S.F.No.2 were frozen and thawed three times, in total, to lyse the platelets.

(7) The platelet lysates were finally thawed and centrifuged at 12,000 g. for 10 min. to remove the cellular debris. Two milliliters of the supernatant (S_1) were removed and added to 2 ml. of 95% ethyl alcohol, previously chilled on ice.

(8) This supernatant-alcohol mixture was centrifuged at 3000 g. for 10 min. to remove any alcohol-insoluble, proteinaceous or other matter, following which this supernatant (S_2) was used to assay for ADP and ATP.

Assay for ADP

(a) 1.0 ml. of S_2 was added to 3.0 ml. of activated phosphoenolpyruvate-pyruvate kinase mixture ($PEP\text{-}PK_{(A)}$).

(b) The mixture in (a) above was warmed at 80°C. for 6 min. and then cooled on ice.

Assay for ATP

(a) 1.0 ml. of S_2 was added to 3.0 ml. of inactivated phosphoenolpyruvate-pyruvate kinase mixture ($PEP\text{-}PK_{(I)}$).

(b) The mixture in (a) above was warmed at 80°C. for 6 min. and then cooled on ice.

(d) 0.5 ml. Firefly Lantern Extract (FLE) (Luciferase) was added to 1.0 ml. of the mixture in (b) above and the light emission measured as outlined below.

(c) 0.5 ml. Firefly Lantern Extract (FLE) (Luciferase) was added to 1.0 ml. of the mixture in (b) above and the light emission measured as outlined below.

(9) Light emission was measured in a Beckman standard silica cell in a specially-constructed light-proof cell housing. FLE was added to the test sample with a 1.0 ml. glass tuberculin syringe attached to a 19 gauge, 2 in. needle, through a hole in the cell housing cover. The light emission was received by a photo-multiplier tube (type 931-A) which was also contained in the cell housing. The photomultiplier tube was connected to a Photomultiplier Microphotometer (American Instrument Co. Inc., Model 10-213) with meter multiplier setting at 0.01 and sensitivity setting at 50 (maximum). The scale deflections displayed on the Photomultiplier Microphotometer were recorded on a Bausch and Lomb (VOM5) Recorder set at 0.1 volts D.C. and chart speed of 1 in./min.

The extent of the initial deflection was measured (in mm.) in all test samples with subsequent conversion to "%-Transmission" and "ug. ATP/ml. of sample", using a previously determined ATP Standard Curve.

Unknown test samples were always tested in conjunction with Standard ADP and ATP test samples in order to routinely reaffirm the accuracy of the ATP Standard Curve.

Subsequent calculations employing the platelet count in S.F.No.2, and the determined ug. ATP/ml. of test sample, resulted in the uMoles ATP/10^{11} platelet values.

B. Preparation and Storage of Aggregation-Inducing Agents

1. Adenosine Diphosphate

Adenosine diphosphate (ADP) (Calbiochem) was stored in the dry, frozen, powdered state. Using accurately-weighed small quantities of ADP, a 2.5×10^{-4} M. solution was prepared. This was divided into several, small aliquots and frozen for storage. Prior to each aggregation experiment, one of these aliquots was thawed, and used only after it had warmed to room temperature.

2. Collagen Suspensions

Fine-particle suspensions of collagen were prepared by pulverizing lyophylized, shredded, bovine achilles tendon (General Biochemicals) in an Omni-Mixer (Sorvall, Type OM) for 25 min., suspending the minced tendon in 0.9% sodium chloride and centrifuging at 750 g. for 4 min. to remove the larger particles. The fine-particle suspension was removed and stored at $+4^{0}$C. and warmed to room temperature before being used. Despite rigorous efforts to maintain a standardized technique of preparation and to standardize the optical density of the collagen suspensions, considerable variation of the aggregating potency occurred from one batch of collagen suspension to another. Experimental results were therefore compared only when a given batch of collagen suspension was used throughout that experiment.

C. Preparation and Storage of Allopurinol Preparations

Allopurinol for oral use in rabbits was accurately weighed into hard gelatin capsules (Lilly No.1) and stored at room temperature. Identical capsules containing a comparable quantity of lactose were prepared for the control group of rabbits.

Sodium allopurinol for intravenous use in rabbits was prepared by the addition of 10 ml. of distilled water to 500 mg. of lyophilized, sterile sodium allopurinol resulting in a 50 mg./ml. solution.

For in vitro studies, the re-constituted sodium allopurinol described above was further diluted with distilled water to a concentration of 4.44×10^{-2}M.

D. Comparison of Aggregation Curves

I. ADP-Induced Platelet Aggregation at 23°C

(i) Aggregation Index

As indicated by Woods and Clarke (1971), the decrease in optical density associated with ADP-induced platelet aggregation (as determined by the Born (1962) method) does not obey Beer's Law if the platelet count is above 270,000/mm.^{3}. In an attempt to overcome this and other problems associated with quantification of ADP-induced platelet aggregation curves, a transformation technique was developed whereby an expression of density (Density Units) was found to be proportional to the reciprocal of the time (seconds) after the

addition of ADP. The results of this transformation and the subsequent calculations yielded a value which was termed the Aggregation Index. The value of the Aggregation Index, thus, is a method of integration of the rate and extent of ADP-induced platelet aggregation. The Aggregation Index is directly proportional to the rate and/or the extent of aggregation. The method for calculation of the Aggregation index is described fully in Appendix II. Cronberg (1971) has described a method of quantitation of platelet aggregation curves, similar to that described above, in which he demonstrated that the percentage of light beams passing through PRP was inversely proportional to the reciprocal of time, using a semilogarithmic plot.

(ii) Rate of Rise and Curve Height

The rate and extent of ADP-induced platelet aggregation were sometimes determined independently by measuring the rate of rise and curve height of the aggregation curve. The rate of rise of the aggregation response induced by ADP was determined by measuring the angle (in degrees) to the horizontal, of a line drawn from the point of addition of ADP to a position tangent to the aggregation curve. The extent of the ADP-induced aggregation response was determined by measuring (in mm.) the height of the aggregation curve at the highest point.

2. ADP-Induced Platelet Aggregation at 37°C.

In the case of each subject's CPRP, a final concentration of ADP was selected at which a "biphasic", ADP-induced, platelet aggregation curve was obtained. This biphasic platelet aggregation curve was characterized by a "first phase" platelet aggregation (due to the added ADP) which occurred within a few seconds of adding ADP, and a "second phase" platelet aggregation (due largely to ADP released from the platelets) which was greater in extent and occurred later, than the first phase platelet aggregation. The height of the second phase platelet aggregation curve was measured (in mm.) 5 min. after the addition of ADP. The height of the first phase platelet aggregation curve (with the second phase portion inhibited) was measured (in mm.) at the highest point.

3. Collagen-Induced Platelet Aggregation

Aggregation of platelets induced by collagen suspension differs from that induced by ADP in that there is a considerable delay between the addition of collagen and the onset of aggregation as well as the fact that the aggregation reaction proceeds at a much slower rate.

Therefore, for purposes of comparison, the time (in seconds) from the addition of collagen, to the commencement of platelet aggregation was measured, and termed the "Time to Onset". The extent of aggregation was determined by measuring the height of the aggregation curve (in mm.) 3 min. after the onset of

aggregation, and was termed 'Curve Height'.

4. Experiments Using Intravenous Sodium Allopurinol in Rabbits

1. Allopurinol-Lowering of Oxonate-Induced Hyperuricemia

Eighteen male, California rabbits, weighing 2.8-3.2 kg. were randomly divided into three groups:

(i) Hyperuricemic Group - receiving sodium urate intraperitoneally (60 mg./kg.) followed one hour later by intravenous potassium oxonate (60 mg./kg.) to produce a high serum urate level. Potassium oxonate is an inhibitor of the enzyme, uricase, which converts uric acid to allantoin.

(ii) Allopurinol-Treated Hyperuricemic Group - similar to the Hyperuricemic Group except that intravenous sodium allopurinol (40 mg./kg.) was given 30 min. after the potassium oxonate injection.

(iii) Control Group - undergoing the same procedures as the above two groups, except that saline injections were given instead of drugs.

Each of the rabbits in these three groups was studied with respect to PCV, serum urate, and platelet aggregation in PRP induced by ADP (3.3×10^{-6} M., and 1.25×10^{-5} M., final concentrations) and collagen suspension.

2. Allopurinol in Normouricemic Rabbits

In order to investigate the possible effects on platelet aggregation of intravenous sodium allopurinol (40 mg./kg.) alone, 12 male California rabbits, weighing 2.8-3.2 kg. were randomly divided into two groups.

(i) Intravenous Sodium Allopurinol Group - receiving treatment only with sodium allopurinol (40 mg./kg.) intravenously.

(ii) Control Group - receiving only saline intravenously.

Each of the rabbits in these two groups was studied with respect to serum urate, PCV, and platelet aggregation in CPRP induced by ADP (3.3×10^{-6}M., and 1.25×10^{-5}M.; final concentrations) and collagen suspension.

I. In Vitro Experiments with Sodium Allopurinol

1. Rabbit CPRP Samples

CPRP samples were obtained by cardiac puncture, as previously described, from normal, male, New Zealand White rabbits weighing 2.5-3.0 kg. Each CPRP sample was diluted with autologous plasma to yield 300-500 $\times 10^3$ platelets/mm.^{3}.

Platelet aggregation in these CPRP samples was carried out at 23^{0}C. Sodium allopurinol was added to the CPRP aliquots, individually, while each aliquot was being stirred in the Bryston aggregometer. One minute after the sodium allopurinol addition, either ADP or collagen was added to the CPRP aliquots.

2. Human CPRP Samples

CPRP samples were obtained from normal male and female adults, each having given prior written consent, currently on no medication and on a normal diet. Each CPRP sample was diluted, if necessary, as previously described, to a diluted CPRP level of approximately 200 x 10^3 platelets/mm.^{3}. Each CPRP aliquot was warmed at 37°C. without stirring, for five minutes just prior to the aggregation testing procedure. After permitting each CPRP aliquot to stablize for one minute in the Born Aggregometer, with stirring, ADP was added to induce platelet aggregation. Since each person's platelet sensitivity to ADP is different, several attempts were necessary to determine the final concentration of ADP which would result in a "biphasic" platelet aggregation curve. Sodium allopurinol was added to the CPRP aliquot, in the Born aggregometer, 30 seconds before adding the previously determined quantity of ADP.

G. Experiments Using Oral Allopurinol in Animals

1. Rats.

Thirty-six male Sprague-Dawley rats, 150-250 gm., were randomly divided into a control group and a treated group, each consisting of 18 rats. The control group received Purina Lab Chow and the treated group received the same diet, but containing allopurinol, 280 mg./kg. of diet, or 19.2 ± 0.2 (S.E.M.) mg./kg. body wt./day based upon measurements of daily food intake and regular weighing of the rats. All rats received water ad libitum

After five weeks all rats were anesthetized as previously described and platelet counts, platelet aggregation induced by ADP, adhesiveness of platelets to glass beads, and PCV values were determined.

In order to determine the effects of allopurinol on rats on a varied duration of treatment, a similar experiment was undertaken for nine weeks using double the previous allopurinol dosage. Sixty-two male, Sprague-Dawley rats, 150-250 gm., were randomly divided into a control group and a treated group, each consisting of 31 rats, the treated group receiving allopurinol (41.3 ± 0.7 (S.E.M.) mg./kg. body wt./day) as in the previous experiment. Again, all rats received water ad libitum. At weekly intervals, three rats from each group were anesthetized as before and the same studies were conducted.

To further extend these experiments, 36 male, Sprague-Dawley rats, 150-250 gm., were randomly divided into a control group and a treated group, each consisting of 18 rats. The treated group received allopurinol in the diet at a level of 33.0 ± 0.6 (S.E.M.) mg./kg. body wt./day, for 12 weeks. Weekly, from the eighth week to the twelfth week of the experiment three rats from each group were anesthetized and blood was collected to perform the studies described above.

2. Rabbits

(i) Allopurinol and Platelet Aggregation

Blood was collected by cardiac puncture three times at seven-day intervals from 42 rabbits, 2.0-3.0 kg., and

platelet counts, platelet adhesiveness to glass beads; PCV values, and platelet aggregation in response to ADP and collagen were studied. Twenty-five rabbits were given allopurinol (40 mg./kg. body wt./day) orally, in gelatin capsules, and the remaining 17 rabbits received similar capsules containing lactose. Blood was then collected weekly for four weeks and the same tests were performed. It should be noted that, because it was not practical to test more than three or four rabbits on a given day, this experiment extended over a period of several months. Moreover, not all rabbits survived the total of seven, consecutive, weekly cardiac punctures.

(ii) Allopurinol and Determination of Total Nucleotides, ATP, and ADP in Platelets

Ten, male New Zealand White rabbits, 2.5-3.0 kg., were randomly assigned numbers 1 to 10. Rabbits No. 1, 5, and 10 received daily lactose orally, and served as controls, while the other seven rabbits received allopurinol (40 mg./kg./day) orally. Each rabbit underwent weekly cardiac punctures for up to a total of five punctures. The experiment was stopped after 40 days, some of the rabbits not having fully completed the five weekly bleedings. In each group, the initial blood sample served as a control, before allopurinol or lactose was given. Allopurinol treatment was started at different dates for each rabbit, such that when all the data were

collected, there was data for 28 consecutive days of allopurinol treatment, from days 1-28. This treatment regimen is fully outlined in Appendix III.

Platelet adenine nucleotide content, platelet aggregation induced by ADP (3.3×10^{-6}M., and 1.25×10^{-5}M., final concentrations) and collagen suspension, inhibition of platelet aggregation by *in vitro* sodium allopurinol, whole blood platelet counts, CPRP counts, diluted CPRP counts, and PCV values, were studied for all blood samples. The weight of each rabbit was recorded at weekly intervals.

H. Studies on the Clinical Use of Allopurinol

Male patients, with a mean age of 69 ± 4 (S.E.M.) years, with primary gout or with asymptomatic hyperuricemia secondary to renal disease (and without gout) were selected by M. Harth (personal communication), from his clinical practice. Primary gout was defined (M. Harth, personal communication) as the presence of any three of the first six criteria listed below, or the presence of the seventh criterion alone:

1. At least two typical attacks in joints other than the big toe.
2. One attack in a joint other than the big toe with a characteristic response to colchicine.
3. One typical attack in the big toe.
4. Typical urate crystals in synovial fluid.
5. Hyperuricemia (more than 6 mg.-%).

6. Characteristic radiological change of tophaceous gout, or a clinical mass suggestive of a tophus.
7. Tophi, proven to be such by analysis of their contents.

After diagnosis and prior to commencing treatment with allopurinol, blood was collected with the consent of each patient, and platelet counts, PCV values, platelet adhesiveness to glass and platelet aggregation induced by ADP and collagen were studied. Treatment consisted of 100 mg. allopurinol in tablet form (Zyloprim, Burroughs Wellcome and Co.) taken orally in doses of 100-500 mg./day. The hematological studies were repeated at monthly intervals for six months of treatment. Not all patients completed the study and not all were available for tests in each month.

RESULTS

A. Effects on Platelet Aggregation of Alteration of Urate Levels in Rabbits

1. Oxonate-induced Hyperuricemia and Intravenous Allopurinol

Within 2 min. of the intravenous potassium oxonate (60 mg./kg.) injection (Fig. 1), the serum urate had more than doubled, continuously rising to four times normal at 32 min. and nearly five times normal at 64 min., as compared with the control group. Administration of intravenous sodium allopurinol (40 mg./kg.) 20 min. after the oxonate injection prevented a further rise in urate levels and resulted in values similar to those of the control group (Fig. 1) at 64 - 102 min. This effect was detectable within 15 min. of the sodium allopurinol injection.

Platelet aggregation induced by ADP or collagen was not significantly altered in either the saline-treated control group or in those rabbits receiving pre-treatment with intraperitoneal sodium urate (60 mg./kg.) plus intravenous potassium oxonate. The group receiving urate, oxonate, and allopurinol, however, had statistically significant decreases in ADP-induced platelet aggregation at 12 min. ($p < .05$) and at 82 min. ($p < .005$) after the injection of allopurinol (Fig. 2a) as well as significantly longer delays (time to onset of aggregation) in response to collagen (Fig. 3) at

Fig. 1.

Effect of Allopurinol on Serum Urate Levels in Oxonate-Induced Hyperuricemic Rabbits.

Pre-treatment with intraperitoneal sodium urate (60 mg./kg.), followed one hour later by intravenous potassium oxonate (60 mg./kg.) resulted in a near-linear rise (---) in serum urate in rabbits. Similar pre-treatment with urate and oxonate followed by intravenous sodium allopurinol (40 mg./kg.) produced a subsequent reduction (.——.——.) in serum urate to levels comparable to those of the control group. The serum urate levels of the control group were found to gradually rise (——) during the 120 min. period. Each of the three groups consisted of six rabbits, with blood samples taken from each rabbit at the times indicated.

FIGURE I

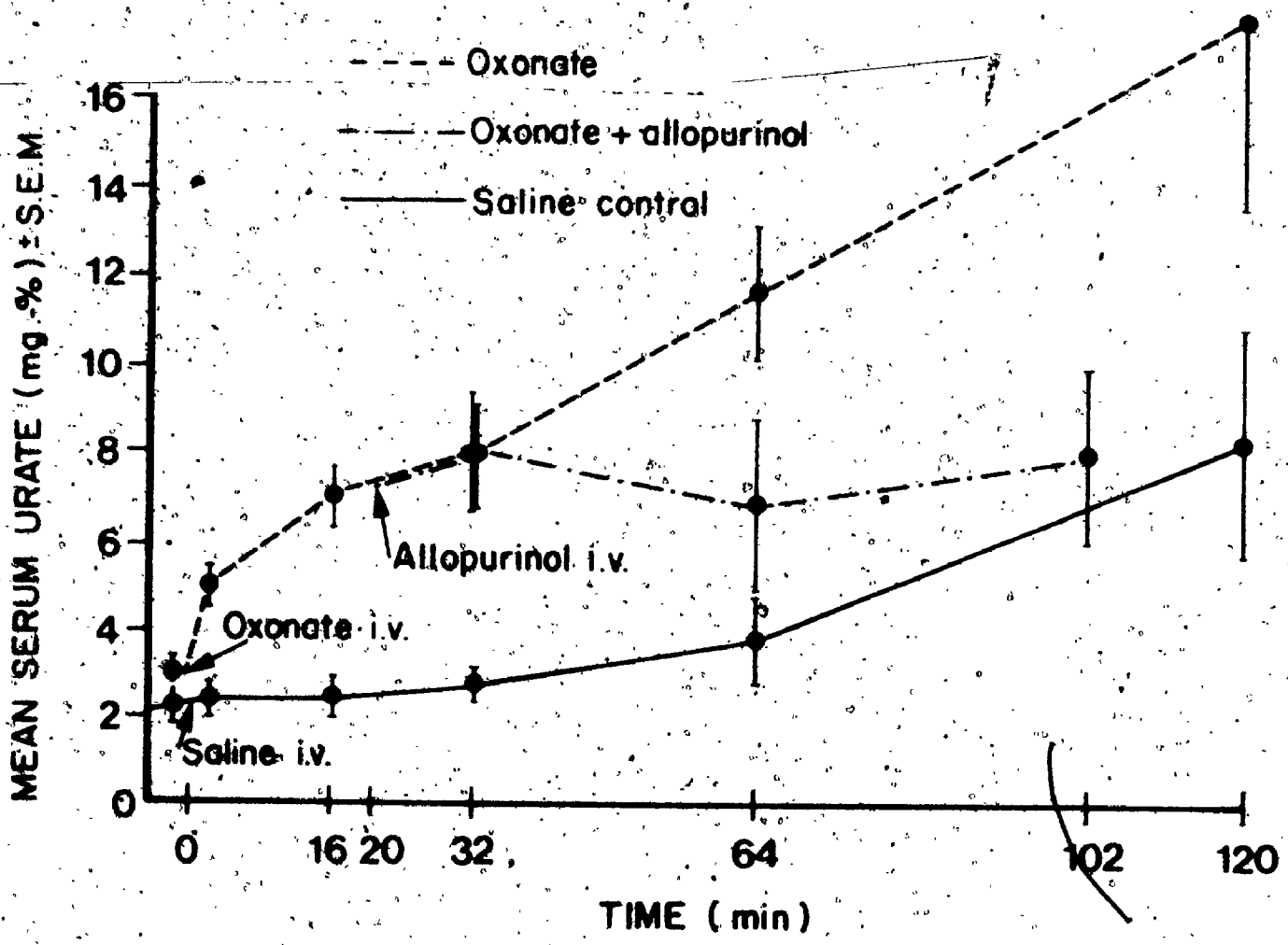

Oxonate
Oxonate + allopurinol
Saline control
MEAN SERUM URATE (mg.%) ± S.E.M.
16
14
12
10
8
6
4
2
0
Allopurinol i.v.
Oxonate i.v.
Saline i.v.
0
16
20
32
64
102
120
TIME (min)

Fig. 2

Inhibition by Intravenous Allopurinol of ADP (3.3×10^{-6} M. final concentration)-Induced Platelet Aggregation in Rabbit CPRP.

(a) Oxonate-Induced Hyperuricemic Rabbits

Platelet aggregation was significantly reduced within minutes of receiving intravenous sodium allopurinol (40 mg./kg.). The p-values shown were obtained by student's t-test using paired data.

(b) Normouricemic Rabbits

Similarly, intravenous allopurinol produced a reduced platelet aggregation in normouricemic rabbits, this reduction being statistically significant at 180 and 240 min. after allopurinol was given. The p-values shown were obtained by student's t-test using paired data.

Each of the above groups (a and b) consisted of six rabbits, with blood samples taken from each rabbit at the times indicated.

FIGURE 2(a)

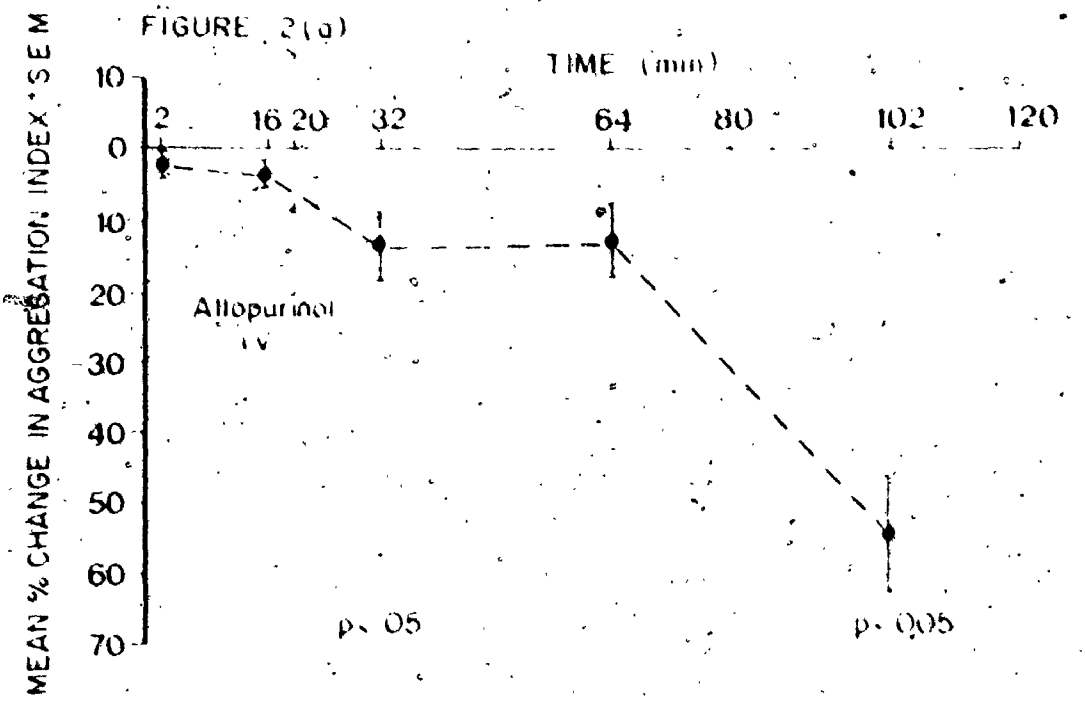


FIGURE 2(b)

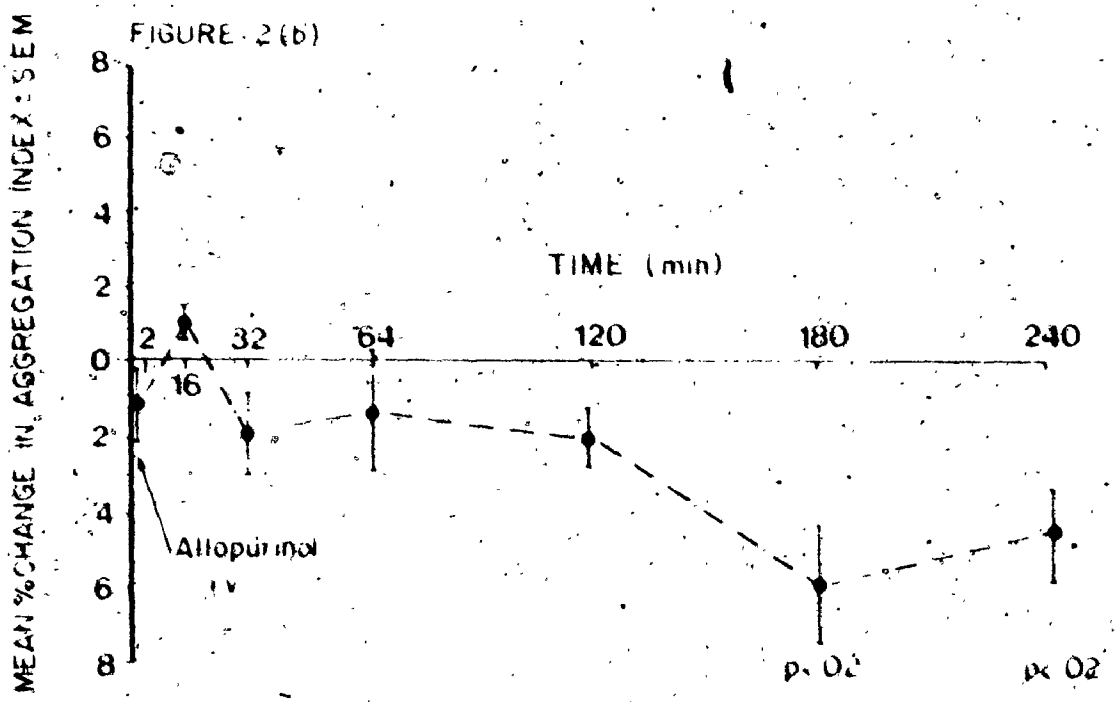
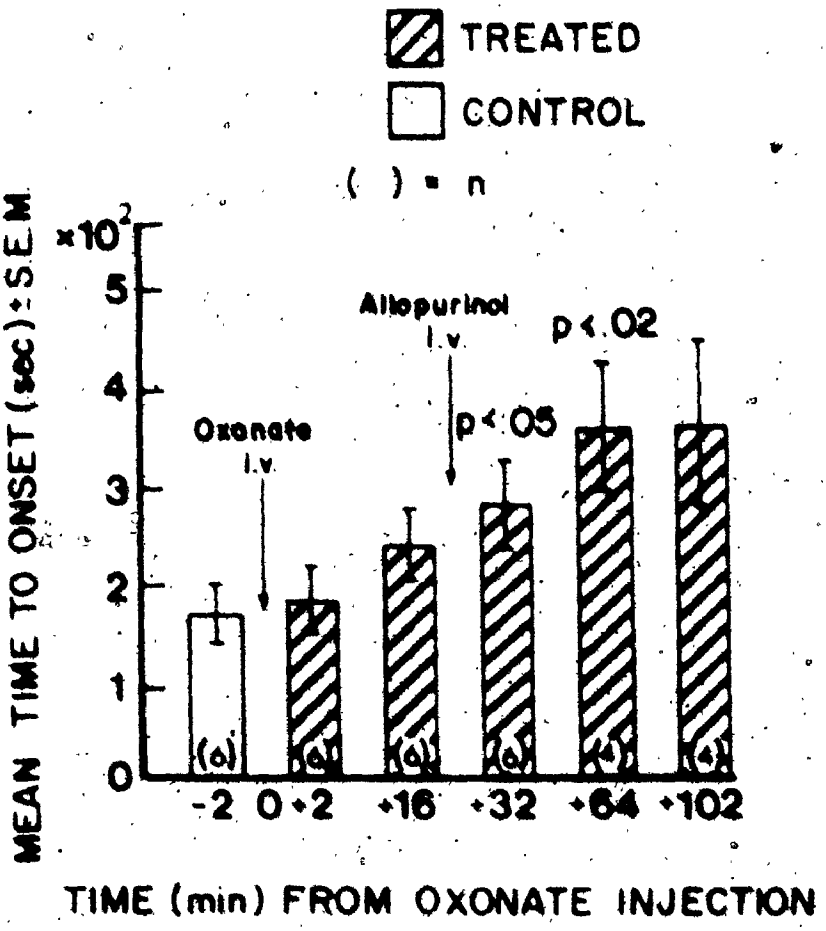


Fig. 3

Inhibition by Allopurinol of Collagen-Induced Platelet Aggregation in Hyperuricemic Rabbits.

Sodium allopurinol (40 mg./kg.) given intravenously to oxonate-induced hyperuricemic rabbits resulted in significant increases in mean time to onset of collagen-induced platelet aggregation, comparing each value obtained, with a pre-injection control value. The p-values shown were obtained by student's t-test using paired data. The group consisted of six rabbits with blood samples being obtained at the time intervals shown.

FIGURE 3


TREATED
CONTROL
( ) = n
MEAN TIME TO ONSET (sec) ± S.E.M.
×10²
5
4
3
2
1
0
Oxonate I.v.
Allopurinol I.v.
p < .05
p < .02
(6)
(6)
(6)
(6)
(4)
(4)
-2
0
+2
+16
+32
+64
+102
TIME (min) FROM OXONATE INJECTION

120 min. ($p < .05$) and at 240 min. ($p < .02$) after the allopurinol injection. No statistically significant differences, however, could be demonstrated in the collagen curve heights in these experiments.

Normouricemia and Intravenous Allopurinol

Treatment of six normouricemic rabbits with intravenous allopurinol (40 mg./kg.) was followed by statistically significant, but less marked, decreases (Fig. 3b) in ADP-induced platelet aggregation at 180 min. ($p < .02$) and 240 min. ($p < .02$) as compared, by individual rabbit, to pre-treatment control values. A control group of normouricemic rabbits, treated similarly except that intravenous saline was given instead of allopurinol, showed no significant changes in ADP-induced platelet aggregation. Allopurinol also caused greater delays in the onset of aggregation in response to collagen in CPRP of normouricemic rabbits, but these differences were not statistically significant. However, significant decreases in the curve heights of collagen-induced platelet aggregation in CPRP of normouricemic rabbits (Fig. 4) were observed in 120 min. ($p < .05$) and 240 min. ($p < .001$) after the administration of intravenous allopurinol (40 mg./kg.). The ability of intravenous allopurinol to lower serum urate in rabbits was illustrated in these normouricemic rabbits--by significant reductions (Fig. 5) at 15 min. ($p < .05$), 30 min. ($p < .01$), 60 min. ($p < .001$), 120 min.

Fig. 4

Inhibition by Allopurinol of Collagen-Induced Platelet Aggregation in Normouricemic Rabbits.

Within minutes of the intravenous sodium allopurinol (40 mg./kg.) injection, the collagen-induced platelet aggregation curve height was reduced below the pre-injection level, significantly so at 120 and 240 min. post-injection. The p-values shown were obtained by student's t-test using paired data. The group consisted of six rabbits with blood samples being obtained at the time intervals shown.

FIGURE 4

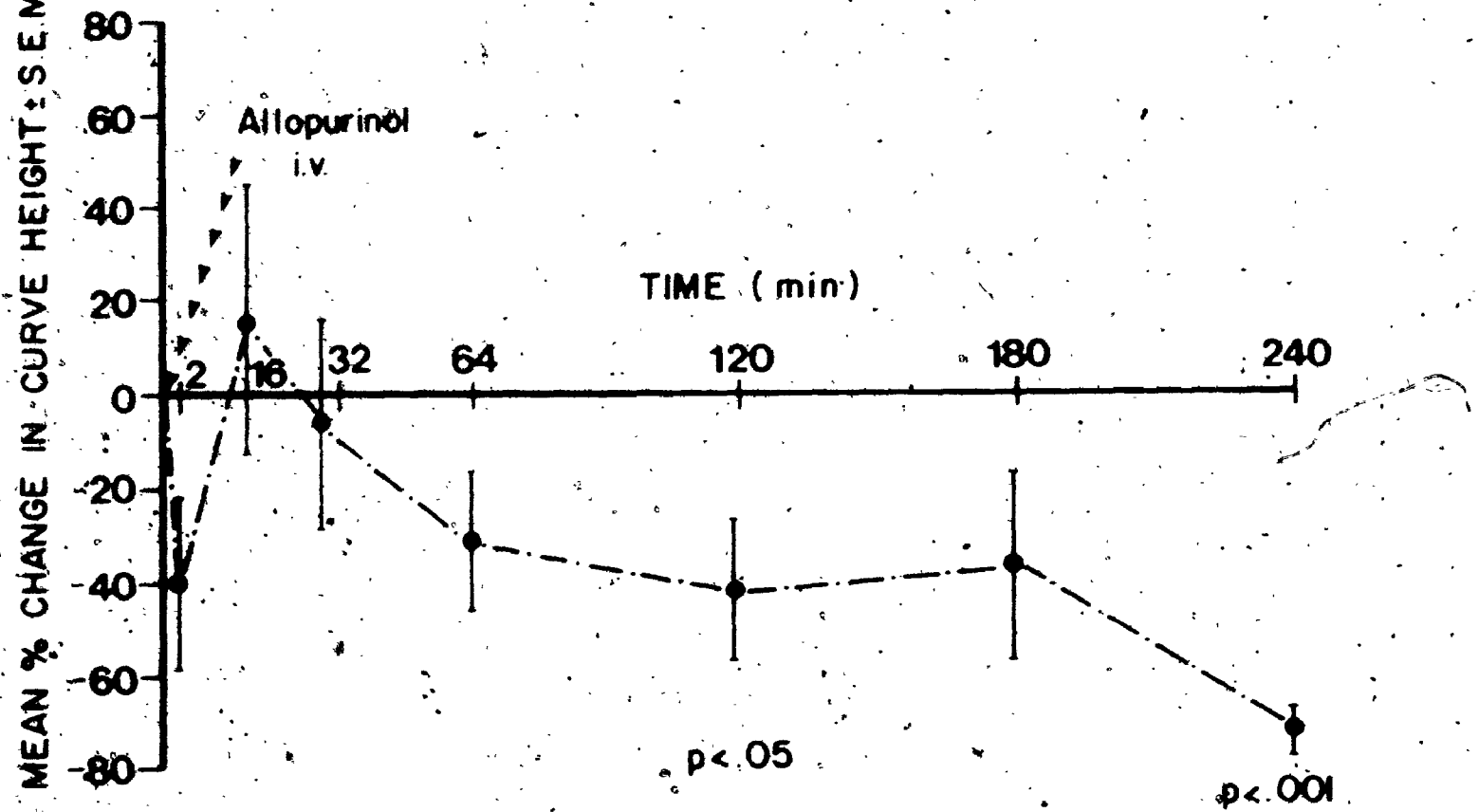

MEAN % CHANGE IN CURVE HEIGHT ± S.E.M
80
60
40
20
0
-20
-40
-60
-80
Allopurinol
i.v.
TIME (min)
2
16
32
64
120
180
240
p<.05
p<.001

Fig. 5.

Serum Urate-Lowering Capacity of Allopurinol in Normouricemic Rabbits.

Compared with a control group, those rabbits receiving intravenous sodium allopurinol (40 mg./kg.) exhibited a significantly reduced serum urate level which persisted for 180 min. post-injection. The p-values shown were obtained by student's t-test using non-paired data. The group consisted of six rabbits with blood samples being obtained at the time intervals shown.

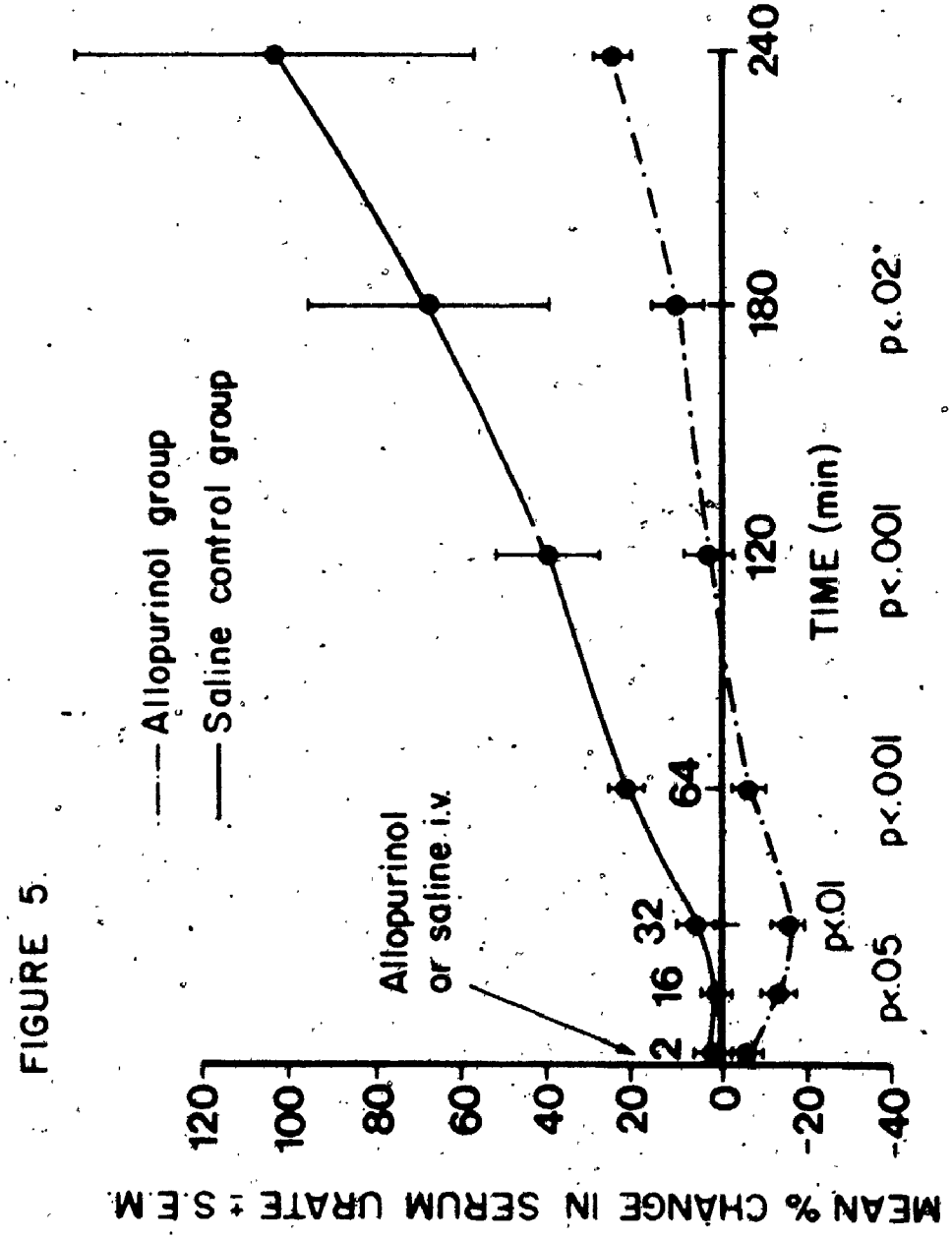

MEAN % CHANGE IN SERUM URATE ± S.E.M
120
100
80
60
40
20
0
-20
-40
2
16
32
64
120
180
240
TIME (min)
Allopurinol or saline i.v.
Allopurinol group
Saline control group
p<.05
p<.01
p<.001
p<.001
p<.02*

FIGURE 5

($p < .001$), and 180 min. ($p < .02$) after the injection of allopurinol comparing the treated group with a control group. Throughout these experiments a group of six saline-treated control rabbits showed no significant changes in ADP-induced, or collagen-induced platelet aggregation but did show (Fig. 5) a progressive rise in serum urate.

B. Effects of *in vitro* Sodium Allopurinol on Platelet Aggregation

1. Rabbit Platelets at 23°C

Prior addition *in vitro* of sodium allopurinol (2.1×10^{-3}M. final concentration) to CPRP samples obtained weekly from three control rabbits, each given lactose orally for 1-28 days, did not result in a significant change in ADP (3.3×10^{-6} M. final concentration)-induced platelet aggregation (Fig. 6b). In CPRP obtained weekly from seven rabbits treated orally with allopurinol (40 mg./kg./day) for 1-28 days, however, there was a statistically significant ($p < .02$) reduction (Fig. 6a) in ADP (3.3×10^{-6}M. final concentration)-induced platelet aggregation following *in vitro* addition of sodium allopurinol (2.1×10^{-3}M. final concentration).

Effects similar to the above were obtained when a higher concentration of ADP (1.25×10^{-5}M. final concentration) was employed (Fig. 7a) (Fig. 7b). In six, normal rabbits undergoing the cardiac puncture procedure only once, and not subjected to oral lactose pre-treatment for 1-28 days, there

Fig. 6.

Effect of *in vitro* Sodium Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP.

(a) In CPRP of rabbits treated with allopurinol (40 mg./kg./day, orally) for 1-28 days, a significant reduction in ADP (3.3×10^{-6}M. final concentration)-induced platelet aggregation was revealed when the CPRP aliquots were pre-treated with *in vitro* sodium allopurinol (2.1×10^{-3}M. final concentration). The p-value shown was obtained by student's t-test using paired data.

(b) In CPRP of a control group, no significant changes were revealed by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated.

Similarly, the control group consisted of three rabbits, with blood sampling procedures similar to the treated group.

For details, see Appendix III.

FIGURE 6(a)
GROUP RECEIVING
ALLOPURINOL ORALLY

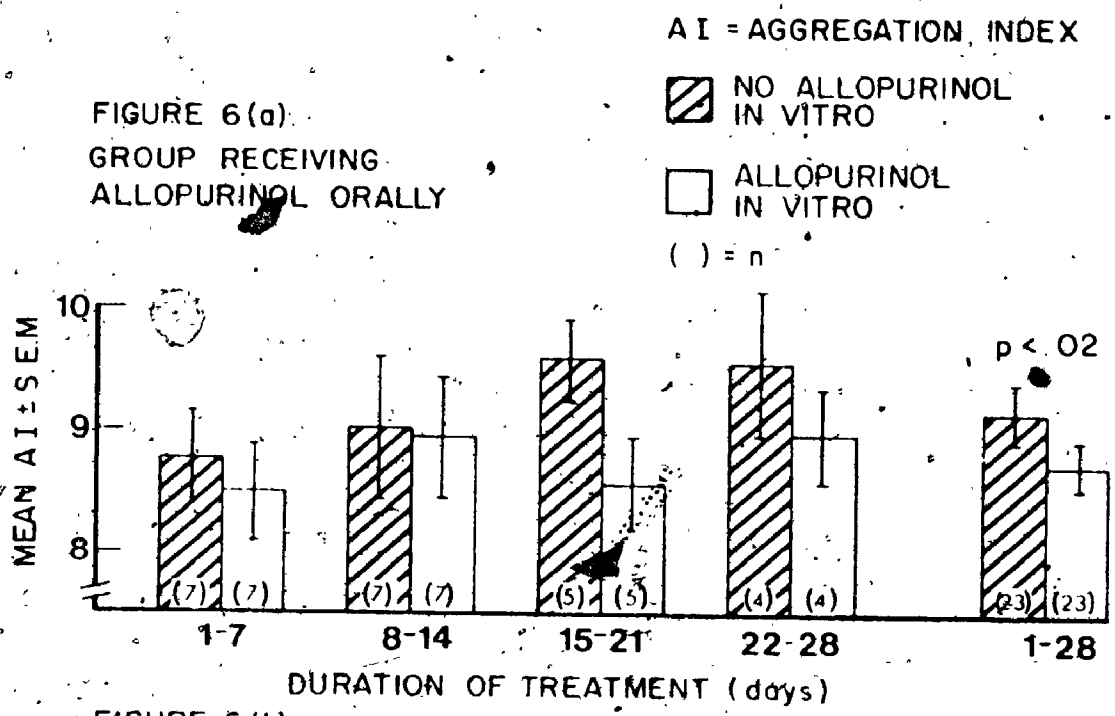


FIGURE 6(b)
CONTROL GROUP

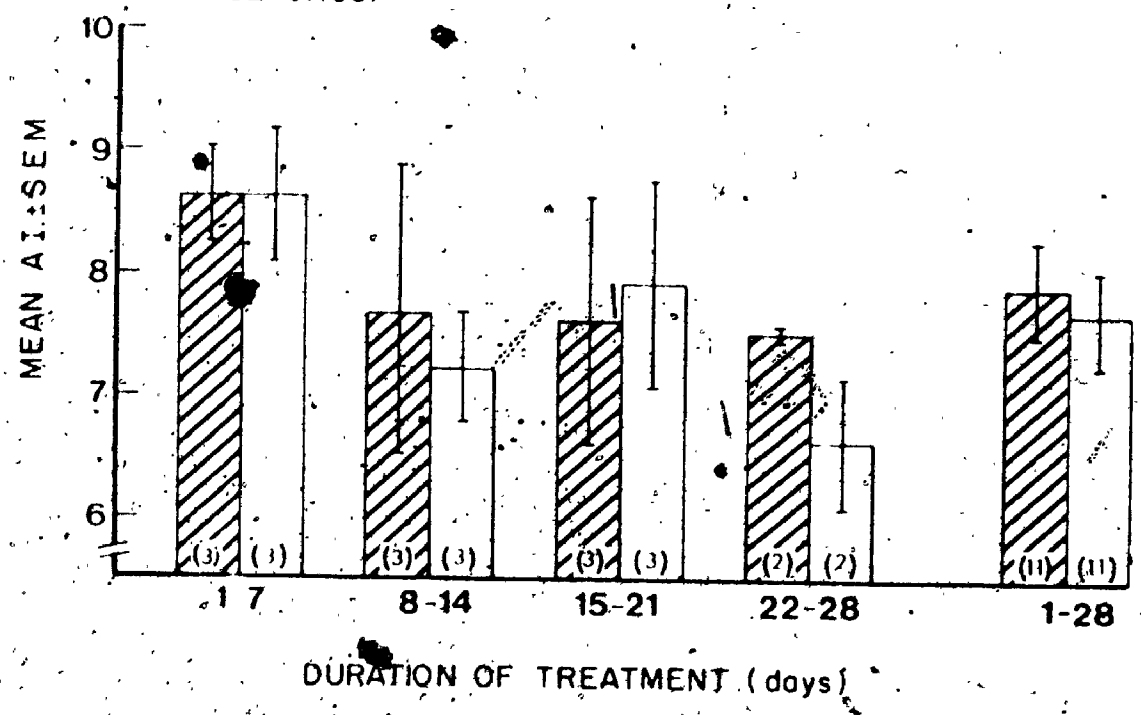


Fig. 7.

Effect of *in vitro* Sodium Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP, Using a Higher Concentration of ADP.

(a) In CPRP of rabbits treated with allopurinol (40 mg./kg./day, orally) for 1-28 days, induction of platelet aggregation with a higher concentration of ADP (1.25×10^{-5}M. final concentration) showed a significant reduction, (similar to that in Fig. 6 (a)) in Aggregation Index (A.I.) when the CPRP aliquots were pre-treated with *in vitro* sodium allopurinol (2.1×10^{-3}M. final concentration). The p-value shown was obtained by student's t-test using paired data.

(b) In CPRP of a control group, using this higher concentration of ADP, no significant changes were found with student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated.

Similarly, the control group consisted of three rabbits, with blood sampling procedures similar to the treated group. For details, see Appendix III.

FIGURE 7(a)
GROUP RECEIVING
ALLOPURINOL ORALLY

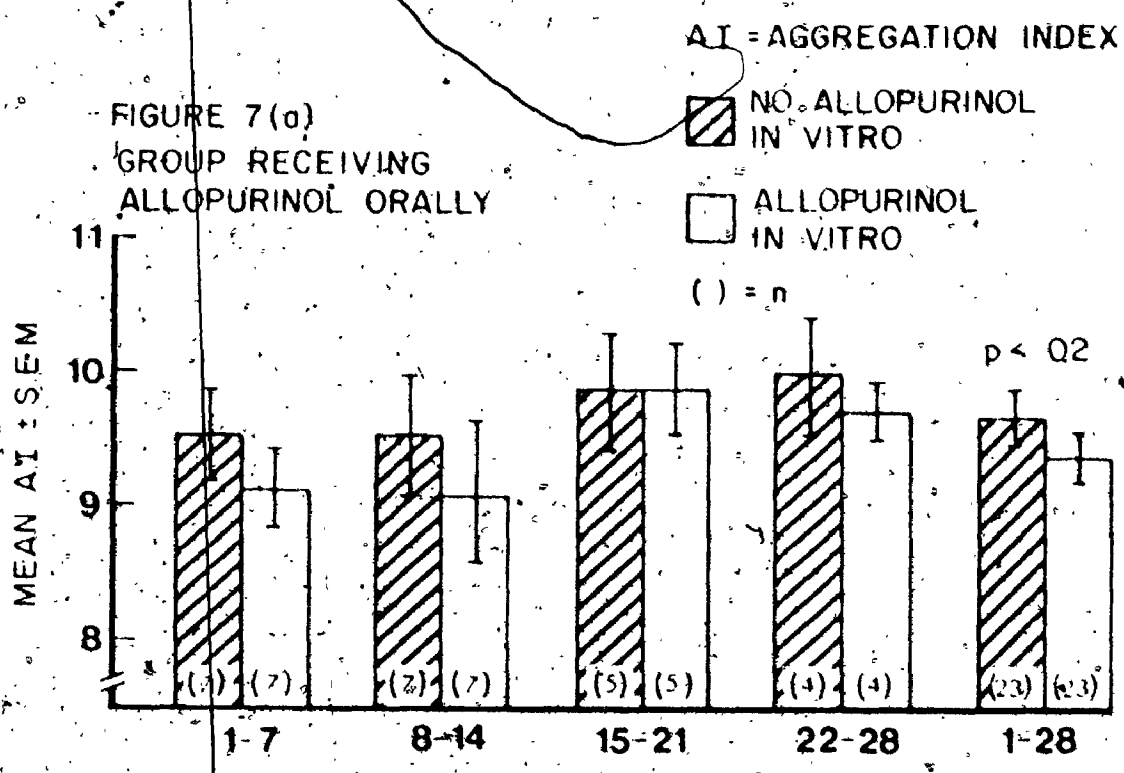
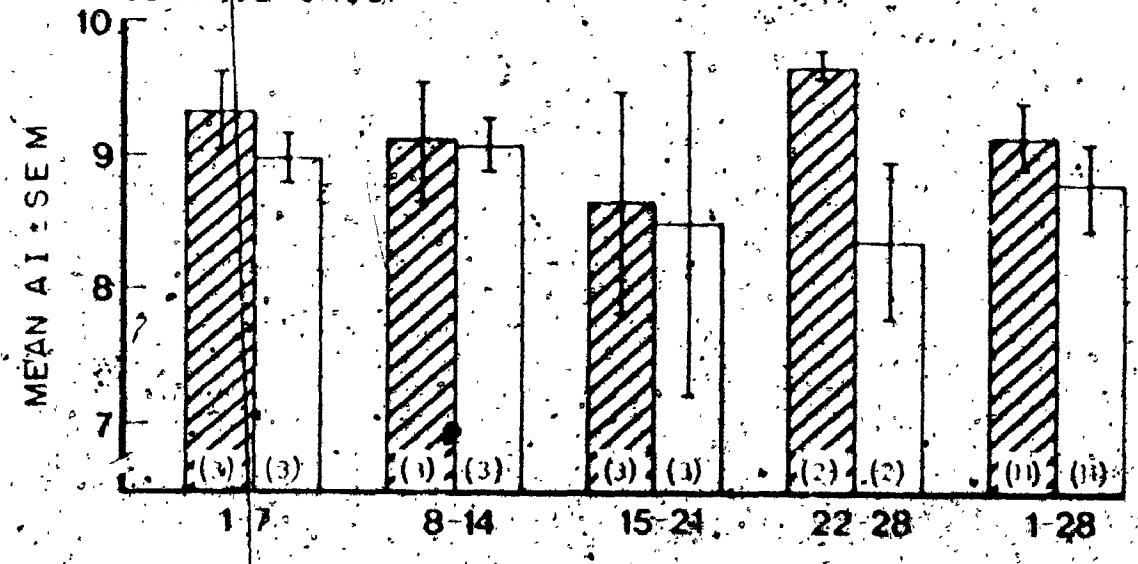


FIGURE 7(b)
CONTROL GROUP



was no significant change in platelet aggregation with lower (3.3×10^{-6}M. final concentration) or higher (1.25×10^{-5}M final concentration) concentrations of ADP following in vitro sodium allopurinol (2.1×10^{-3}M. final concentration.

In CPRP from normal rabbits in vitro sodium allopurinol (1.4×10^{-3}M. final concentration) resulted in complete inhibition of collagen-induced platelet aggregation in nearly all cases. In CPRP from six rabbits so tested, the collagen curve height was significantly ($p < .01$) reduced (Table 1a) and the collagen curve time to onset of aggregation was significantly ($p < .001$) prolonged (Table 1b).

2. Human Platelets at 37°C

In vitro sodium allopurinol ($1.2-2.4 \times 10^{-3}$M. final concentration) caused a complete inhibition of ADP ($1.6 \times 10^{-6} - 3.3 \times 10^{-5}$M. final concentration)-induced "second phase" platelet aggregation in human CPRP at 37°C, which can also be expressed as a 35.4 ± 6.7 (S.E.M.)% reduction ($p < .005$) in curve height (Table 2).

In all of the preceding in vitro experiments, the addition of sodium allopurinol did not affect the normal pH of the CPRP.

C. Effects of Oral Allopurinol on Platelet Aggregation in Animals

1. Rats.

(1) Five-week Experiment with Terminal Assessment

In rats treated with allopurinol in the diet (13 mg./kg. body wt./day) for five weeks, it was

Table I:

Effect of in vitro Sodium Allopurinol (1.4×10^{-3}M., final concentration) on Collagen-Induced Platelet Aggregation in PRP from Normal Rabbits.

In vitro sodium allopurinol added to normal rabbit PRP resulted in a complete inhibition of collagen-induced platelet aggregation in nearly all cases as reflected by significantly decreased curve heights ($p < .01$), shown in Table I(a), and significantly increased times to onset of platelet aggregation ($p < .005$) as shown in Table I(b). The p-values indicated here were obtained by student's t-test using paired data.

Table I(a)

Curve height (mm.)

Rabbit number	In vitro Sodium Allopurinol	In vitro Sodium Allopurinol

Table I(b)

Curve to chart (sec.)

Rabbit number	In vitro Sodium Allopurinol	In vitro Sodium Allopurinol

Table 2

Effect of in vitro sodium Allopurinol on ADP Induced Second Phase Platelet Aggregation in Human PRP at 37°C.

In vitro sodium allopurinol (1.2×10^{-4} M, final concentration) completely inhibited (p < .005) the second phase platelet aggregation induced by ADP-(1.6×10^{-6} M, final concentration). The p-value indicated here was obtained by student's t test using paired data.

noted that ADP (2.0×10^{-6}M, final concentration)-induced platelet aggregation was lower (but not significant at the 5% probability level) than the control group.

(11) Nine-Week Experiment with Weekly Assessment, With Double the Previous Allopurinol Dosage

To determine if any of the effects of allopurinol might occur earlier or later than five weeks of treatment, this nine-week experiment using allopurinol in the diet (41 mg./kg. body wt./day) was undertaken. Due to technical difficulties, data for the second week could not be used.

Each week, CPRP from two or three rats was pooled to provide sufficient CPRP for aggregation studies. With the exception of weeks three and eight, the Aggregation Index of the treated group was higher, with peaks at weeks one and nine (Table 3). In this instance, this data was more clearly assessed statistically by measuring, independently, the rates of rise (rate of aggregation) and the curve heights (extent of aggregation). Platelet aggregation in the treated group occurred at a significantly faster rate (Fig. 8a) than in the control group at weeks one ($p < .005$), six ($p < .05$) and nine ($p < .005$) and achieved significantly greater curve heights (Fig. 8b) at weeks one ($p < .05$) and nine ($p < .05$).

Table 3

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Rat CPRP.

ADP(2.0×10^{-6} - 1.7×10^{-5} M final concentration)-induced platelet aggregation in CPRP from rats fed allopurinol (41 mg./kg./day) in the diet for nine weeks, was noticeably higher than that of the control group in weeks one and nine.

Each group consisted of 31 rats. At weekly intervals, three rats from each group were anesthetized and blood samples taken.

TABLE 3

Week	Aggregation Index		Difference from Control Group	%-Difference from Control Group
	Control Group	Treated Group		
1	9.53	10.93	+1.40	+14.69
2	-	-	-	-
3	6.82	6.77	-0.05	-0.73
4	9.93	10.36	+0.38	+3.80
5	11.18	11.51	+0.33	+2.95
6	10.08	10.53	+0.45	+4.46
7	11.42	11.45	+0.03	+0.26
8	10.44	10.39	-0.05	-0.47
9	9.13	10.47	+1.34	+14.67

Fig. 8.

Effect of Allopurinol on ADP-Induced Platelet Aggregation (as assessed by rate of rise and curve height) in Rat CPRP.

(a) During nine weeks of treatment with allopurinol (41 mg./kg./day) the rate of rise of the ADP (2.0×10^{-6} - 1.7×10^{-5}M., final concentration)-induced platelet aggregation curve was significantly higher in the treated group than in the control group at weeks one, six, and nine. The p-values shown were obtained by student's t-test using non-paired data.

(b) Similarly, the treated group curve heights were significantly higher than those of the control group at weeks one and nine. The p-values shown were obtained by student's t-test using non-paired data.

Each group consisted of 31 rats. At weekly intervals, three rats from each group were anesthetized and blood samples taken.

FIGURE 8.(a)
RATE OF RISE

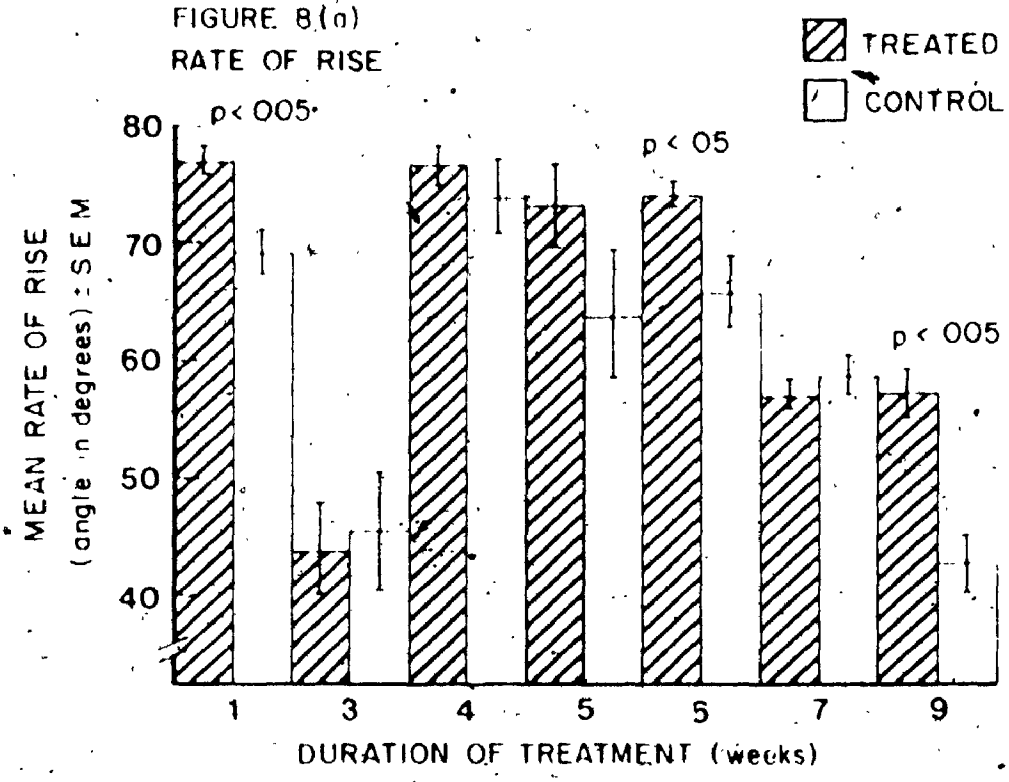
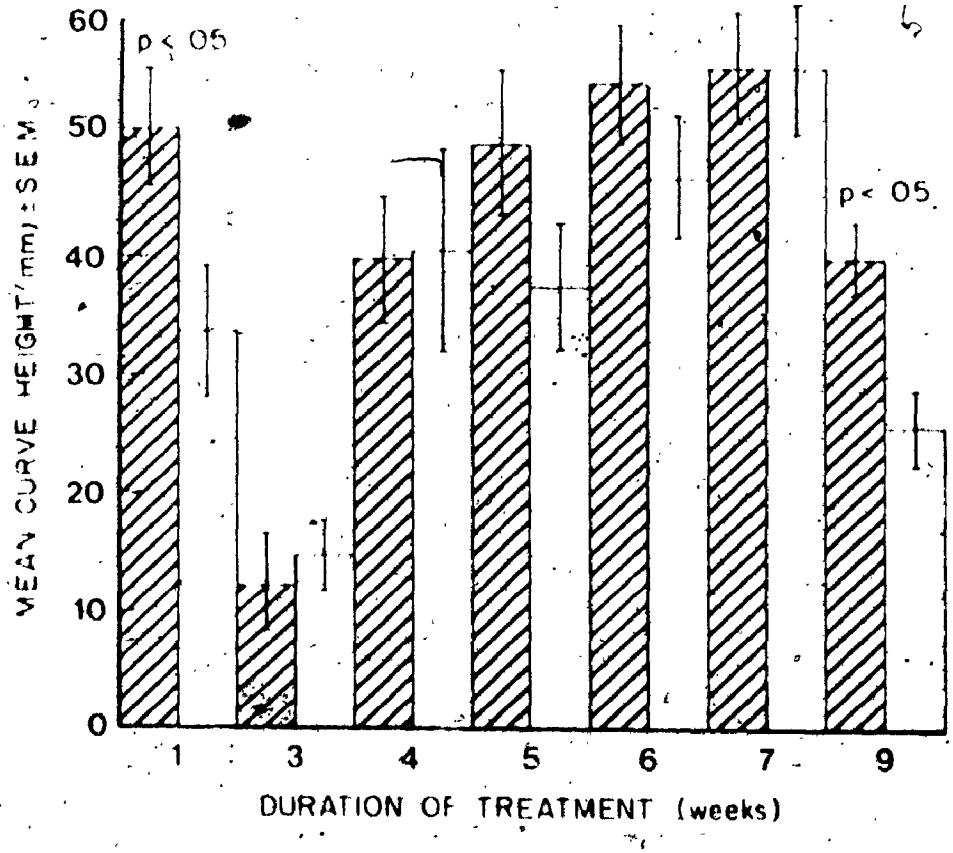


FIGURE 8-(b)
CURVE HEIGHT



(iii) Twelve-Week Experiment, With Weekly Assessment Beginning in the Eighth Week of Treatment

To examine the possibility that the tendency toward increased platelet aggregation might be more evident beyond the ninth week of treatment, a further experiment was conducted with allopurinol in the diet (33 mg./kg. body wt./day), for 12 weeks. In addition to platelet aggregation, platelet adhesiveness to glass was also studied.

Comparing the allopurinol-treated group with the control group, no statistically significant difference was observed in either platelet aggregation or platelet adhesiveness to glass during weeks 8-12.

2. Rabbits

(i) Platelet Aggregation and Adhesiveness

Weekly measurement of platelet adhesiveness in rabbit blood for two weeks prior to the administration of oral allopurinol (40 mg./kg./day) and during the subsequent four-week treatment period, revealed no significant changes in platelet adhesiveness. Similarly, no significant differences in platelet adhesiveness were found when the rabbits undergoing allopurinol treatment were compared with a saline-treated control group.

The aggregation of rabbit platelets by ADP(3.3×10^{-6}M., final concentration) disclosed

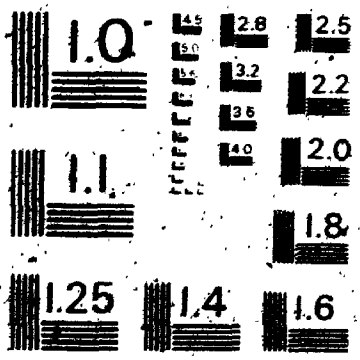
a trend in which the mean Aggregation Index of the allopurinol-treated rabbits was higher than those of the control group (Fig. 9), the difference being statistically significant ($p < .02$) in the third week of treatment.

When the curve heights and rates of rise were examined independently, the mean rate of rise was found to be significantly greater in the allopurinol-treated group than in the control group, in the third ($p < .05$) and fourth ($p < .01$) weeks (Fig. 10a). Moreover, the mean curve height was higher, though not significantly so, in the treated group than in the control group during all four weeks of the experiment (Fig. 10b).

At a higher concentration of ADP (1.25×10^{-5} M., final concentration), the increased platelet aggregation with allopurinol treatment was even more pronounced and consistent. The Aggregation Index was significantly greater (Fig. 11) in the treated group than in the control group in weeks two ($p < .02$), three ($p < .001$) and four ($p < .01$). When the curve heights and rates of rise were examined independently, the mean rate of rise in the treated group was found to be significantly greater (Fig. 12a) in weeks

2 2

OF/DE



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS - 1963

AEC - 374

Fig. 9.

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP

It was observed that ADP(3.3×10^{-6}M., final concentration)-induced platelet aggregation in CPRP of rabbits treated with allopurinol (40 mg./kg./day, orally), was greater than that of the control group for the first three weeks, being statistically significant in the third week. The p-value shown was obtained by student's t-test using non-paired data. The allopurinol-treated group consisted of 25 rabbits, and the control group consisted of 17 rabbits. Blood samples were obtained on a weekly basis.

FIGURE 9

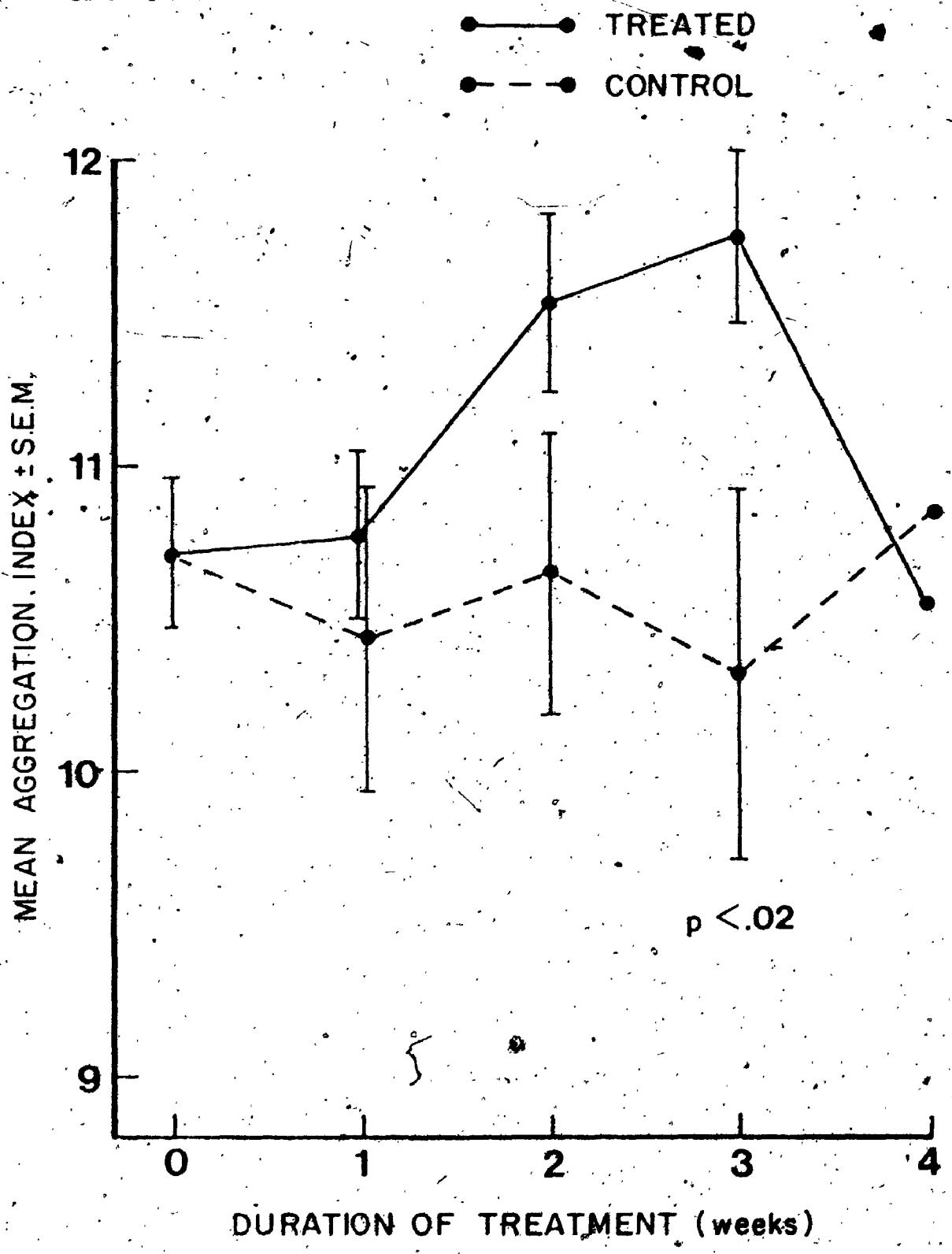

TREATED
CONTROL
12
11
10
9
MEAN AGGREGATION INDEX ± S.E.M.
p <.02
0
1
2
3
4
DURATION OF TREATMENT (weeks)

Fig. 10.

Effect of Allopurinol on ADP-Induced Platelet Aggregation Curve Rate of Rise, and Platelet Aggregation Curve Height in Rabbit CPRP.

(a) The mean rate of rise of the ADP(3.3×10^{-6}M., final concentration)-induced platelet aggregation curves of allopurinol (40 mg./kg./day, orally)-treated rabbits was greater than that of the control group, the difference being statistically significant in the third and fourth weeks of treatment. The p-values shown were obtained by student's t-test using non-paired data.

(b) Similarly, the mean platelet aggregation curve height was higher in the treated group throughout the four weeks of treatment, though not significant at the 5% probability level.

The allopurinol-treated group consisted of 25 rabbits, and the control group consisted of 17 rabbits. Blood samples were obtained on a weekly basis.

FIGURE 10(a)
RATE OF RISE

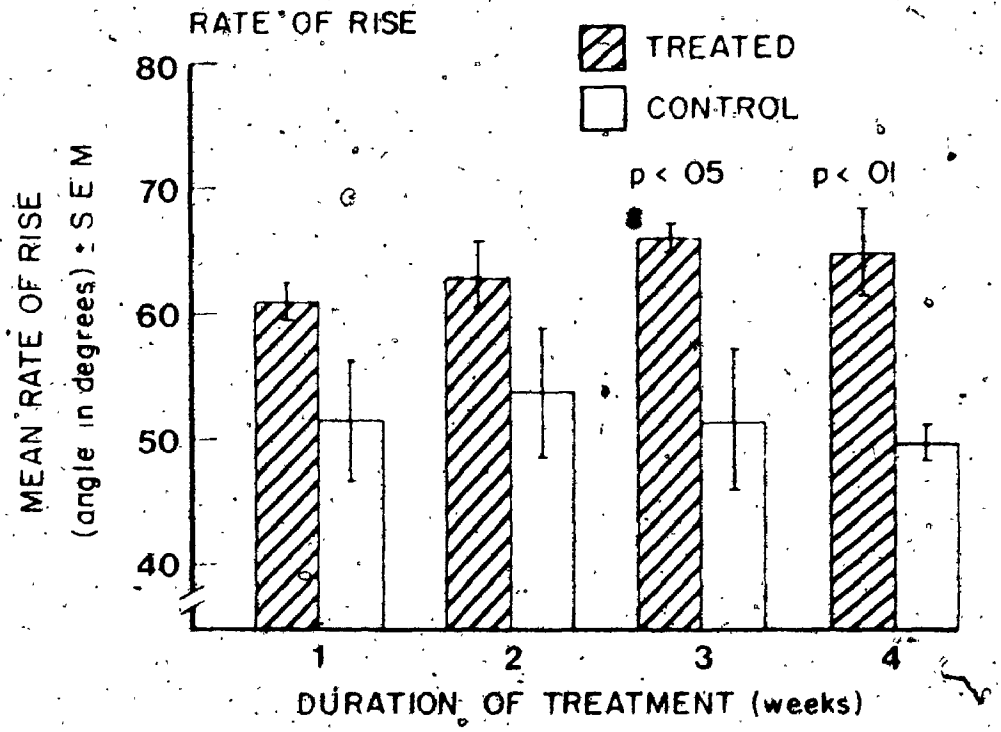


FIGURE 10(b)
CURVE HEIGHT

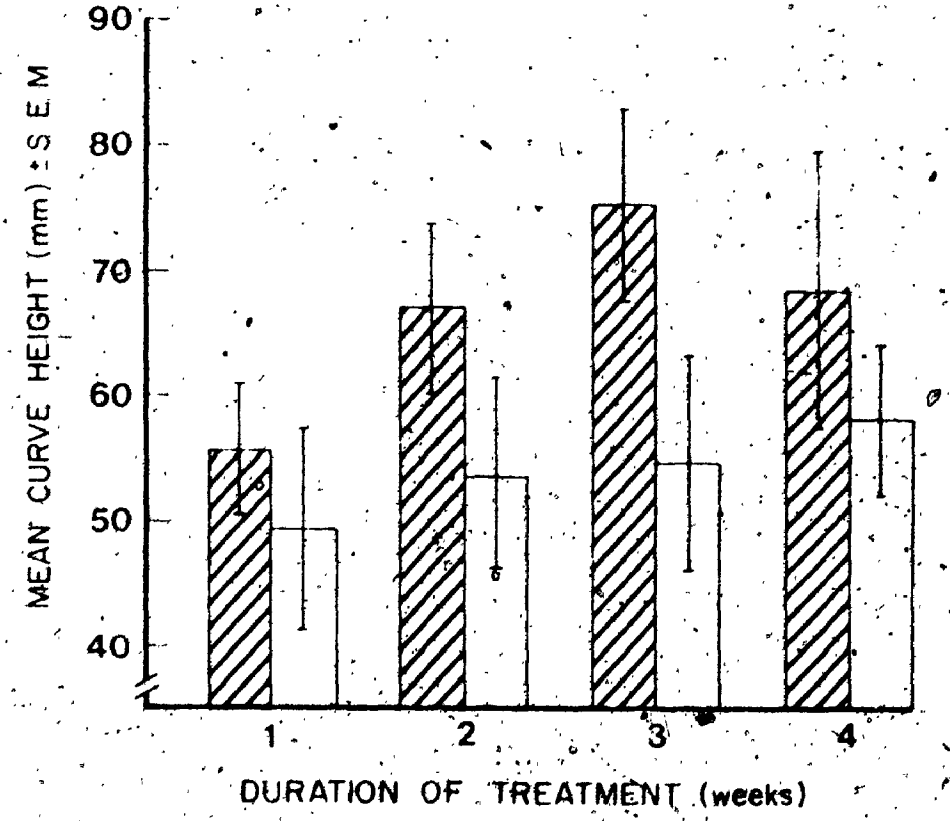


Fig. 11.

Effect of Allopurinol on Aggregation of Rabbit Platelets Induced by a Higher Concentration of ADP.

Use of a higher concentration of ADP (1.25×10^{-5} M., final concentration) to induce platelet aggregation, resulted in a significantly elevated Aggregation Index in the allopurinol (40 mg./kg./day, orally)-treated group in weeks two, three and four, as compared to the control group. The p-values shown were obtained by student's t-test using non-paired data.

The allopurinol-treated group consisted of 25 rabbits, and the control group consisted of 17 rabbits. Blood samples were obtained on a weekly basis.

FIGURE 11.

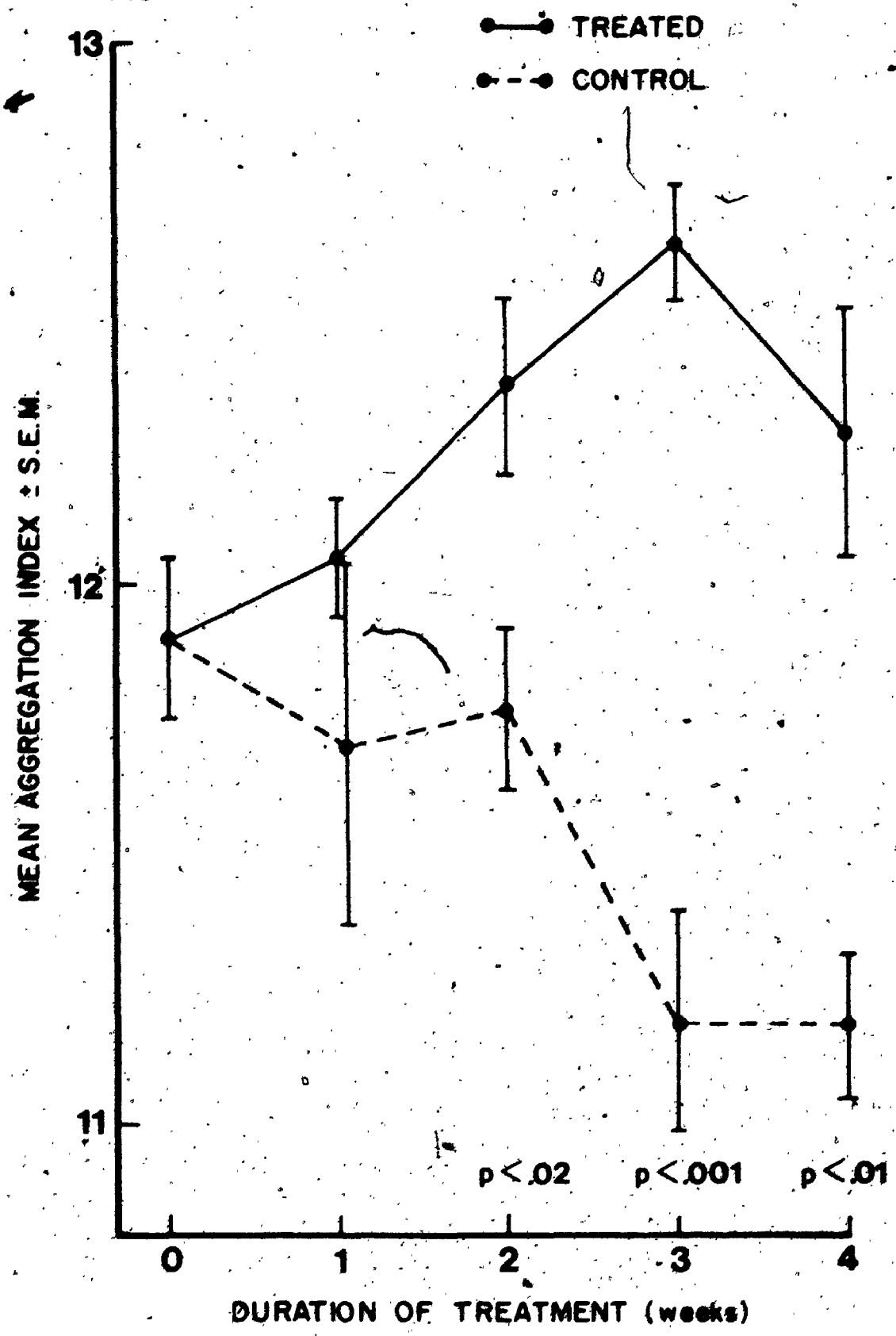

TREATED
CONTROL
13
12
11
MEAN AGGREGATION INDEX ± S.E.M.
p<.02
p<.001
p<.01
0
1
2
3
4
DURATION OF TREATMENT (weeks)

Fig. 12.

Effect of Allopurinol on Platelet Aggregation Curve Rate of Rise, and Platelet Aggregation Curve Height in Rabbit CPRP, Using a Higher Concentration of ADP.

(a) Use of a higher concentration of ADP(1.25×10^{-5}M., final concentration) to induce platelet aggregation revealed a significantly greater mean aggregation curve rate of rise in the treated group, than in the control group, in weeks three and four. The p-values shown were obtained by student's t-test using non-paired data.

(b) Similarly, the treated group mean aggregation curve height was significantly greater in the third week. The p-value shown was obtained by student's t-test using non-paired data.

The allopurinol-treated group consisted of 25 rabbits, and the control group consisted of 17 rabbits. Blood samples were obtained on a weekly basis.

FIGURE 12(a)
RATE OF RISE

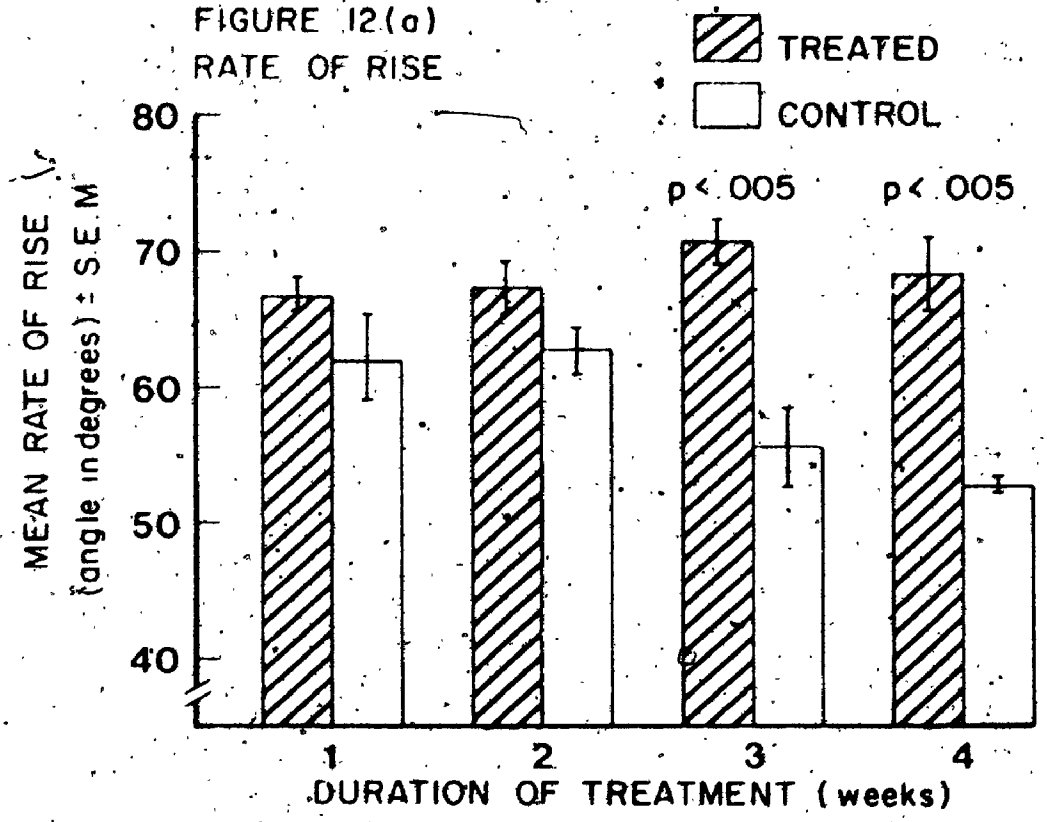
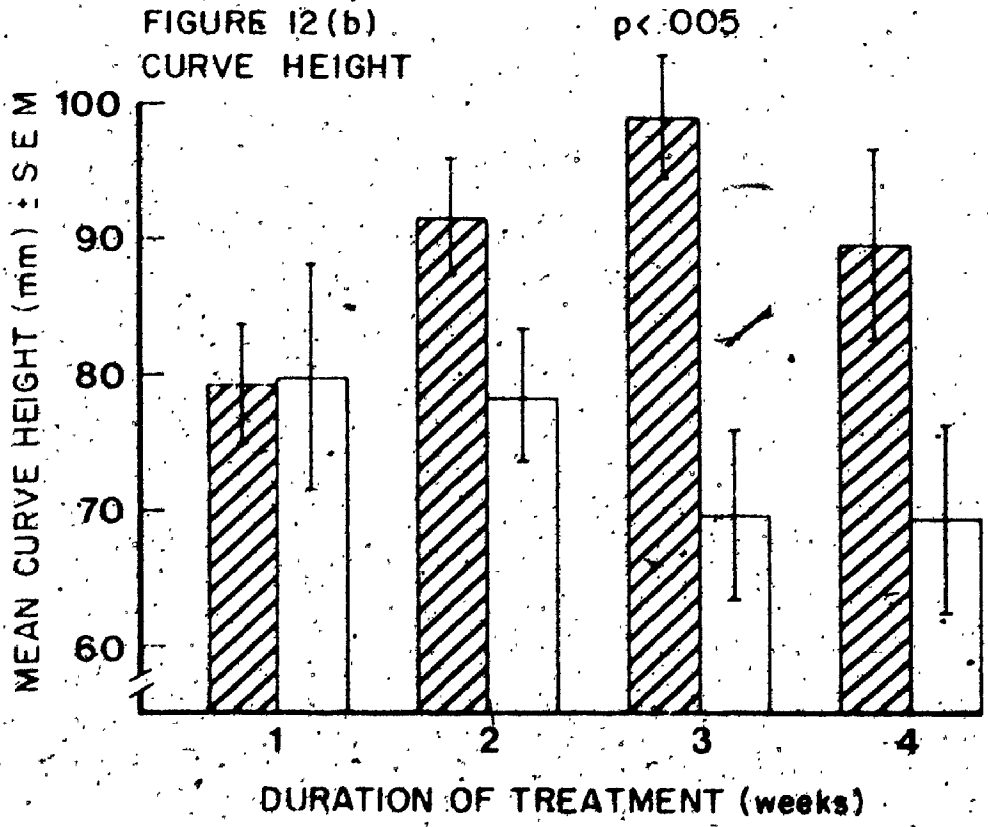


FIGURE 12(b)
CURVE HEIGHT



three ($p < .005$) and four ($p < .005$) and the mean curve height was significantly greater in week three ($p < .005$) (Fig. 12b).

Induction of aggregation of rabbit platelets by collagen suspension did not reveal any significant difference with respect to the mean collagen curve height, (Fig. 13a) between allopurinol (40 mg./kg./day, orally)-treated and control rabbits but there was observed a significantly lower time to onset of collagen-induced platelet aggregation (Fig. 13b) in the treated group in weeks one ($p < .02$) two ($p < .025$) and four ($p < .05$) as compared with the control group.

(ii) Platelet Levels of Total Nucleotides, ATP and ADP.

Measurement of adenine nucleotide levels in platelets of rabbits treated with allopurinol (40 mg./kg./day, orally) for 1-28 days revealed some significant facts.

Total adenine nucleotide levels in platelets were relatively unchanged in days 1-7 of treatment with allopurinol, but were elevated in each of the 8-14, 15-21, and 22-28-day periods. When the data from the first seven days of this experiment were omitted from the analysis, the 8-28 day period revealed a significantly ($p < .02$) elevated total adenine nucleotide level as compared with the pre-treatment

Fig. 13.

Effect of Allopurinol on Collagen-Induced Platelet Aggregation in Rabbit CPRP.

(a) No significant difference was observed between allopurinol (40 mg./kg./day, orally)-treated and control groups with respect to collagen-induced platelet aggregation curve height.

(b) There was observed, however, a significantly reduced mean time to onset of collagen-induced platelet aggregation in the allopurinol (40 mg./kg./day, orally)-treated group in weeks one, two and four, as compared with the control group. The p-values shown were obtained by student's t-test using non-paired data.

The allopurinol-treated group consisted of 25 rabbits, and the control group consisted of 17 rabbits. Blood samples were obtained on a weekly basis.

FIGURE 13(a)

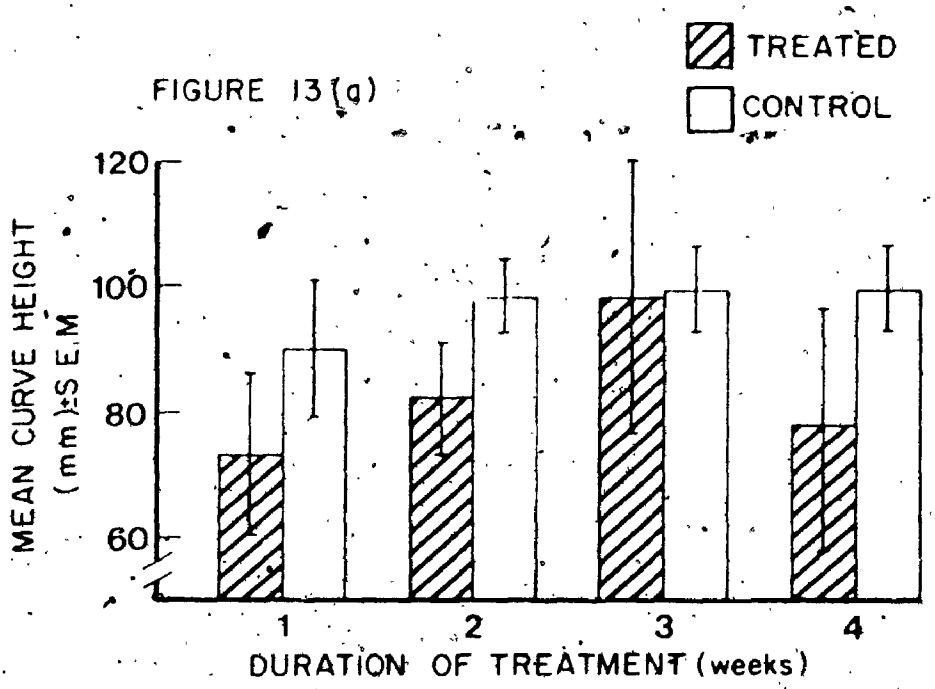
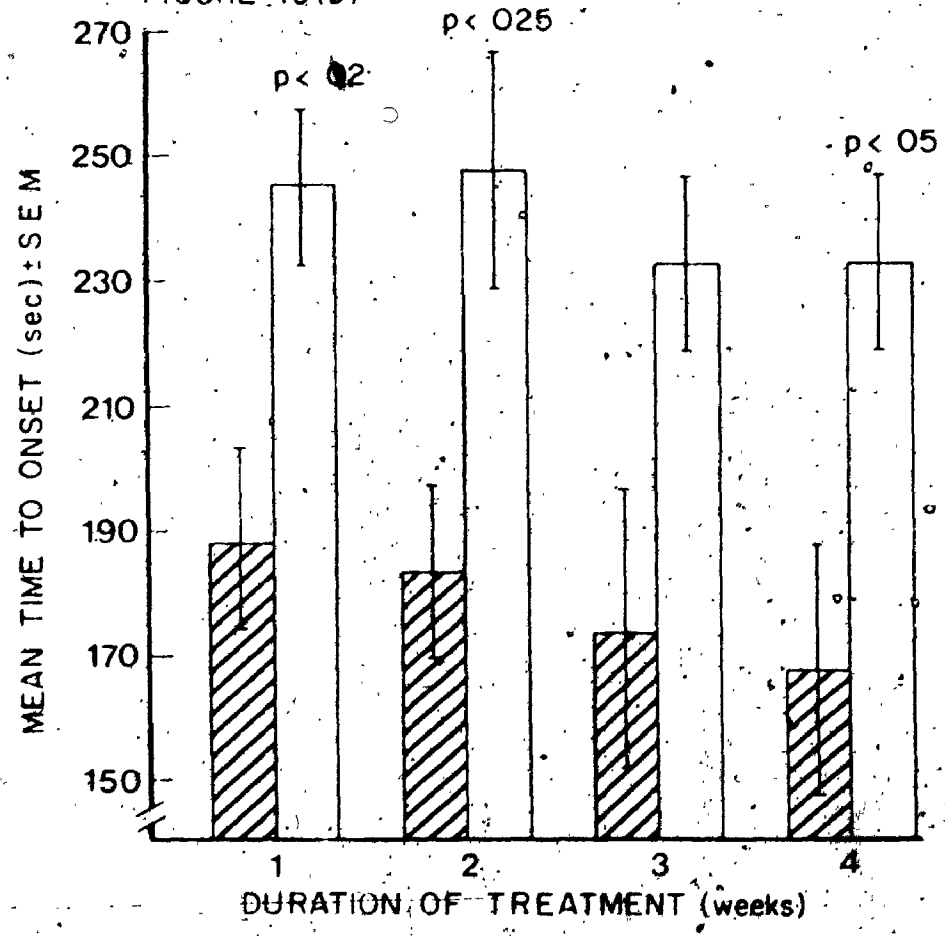


FIGURE 13(b)



control levels (Fig. 14). A control group of rabbits receiving lactose instead of allopurinol, did not show significantly elevated levels of total adenine nucleotides when analyzed as described above.

This elevation of total adenine nucleotides in platelets of allopurinol-treated rabbits was determined to be due largely to elevation of ADP levels in these platelets (Fig. 15). When one compares these data (Fig. 15) with those in Figure 14, one finds an identical pattern present. The ADP levels were relatively unchanged in the 1-7 day period, but prominently elevated in the 8-14, 15-21, and 22-28-day periods. Again, when the data for the 8-28 day period were analyzed, the elevation of ADP, above the initial pre-treatment values (Fig. 15), was statistically significant ($p < .05$). Again, the ADP levels of lactose-treated control rabbits' platelets were not significantly elevated.

The measurement of ATP levels in platelets of rabbits treated with allopurinol (40 mg./kg./day, orally) revealed only a slight elevation at the 8-14 day period of treatment, with lack of statistically significant changes at all levels.

Similarly, the ratios ATP/ADP were determined, but no statistically significant changes were noted.

Fig. 14.

Effect of Allopurinol on the Total Adenine Nucleotide Content of Rabbit Platelets.

Note that virtually no change in platelet total adenine nucleotide levels was evident in Days 1-7 in allopurinol (40 mg./kg./day, orally)-treated rabbits. The treatment periods 8-14, 15-21, and 21-28 days, however, were all elevated. When the data from the latter three treatment periods were analyzed, exclusive of the 1-7 day treatment period, a significantly elevated total adenine nucleotide level during this 8-28-day treatment period was noted. The p-value shown was obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 14

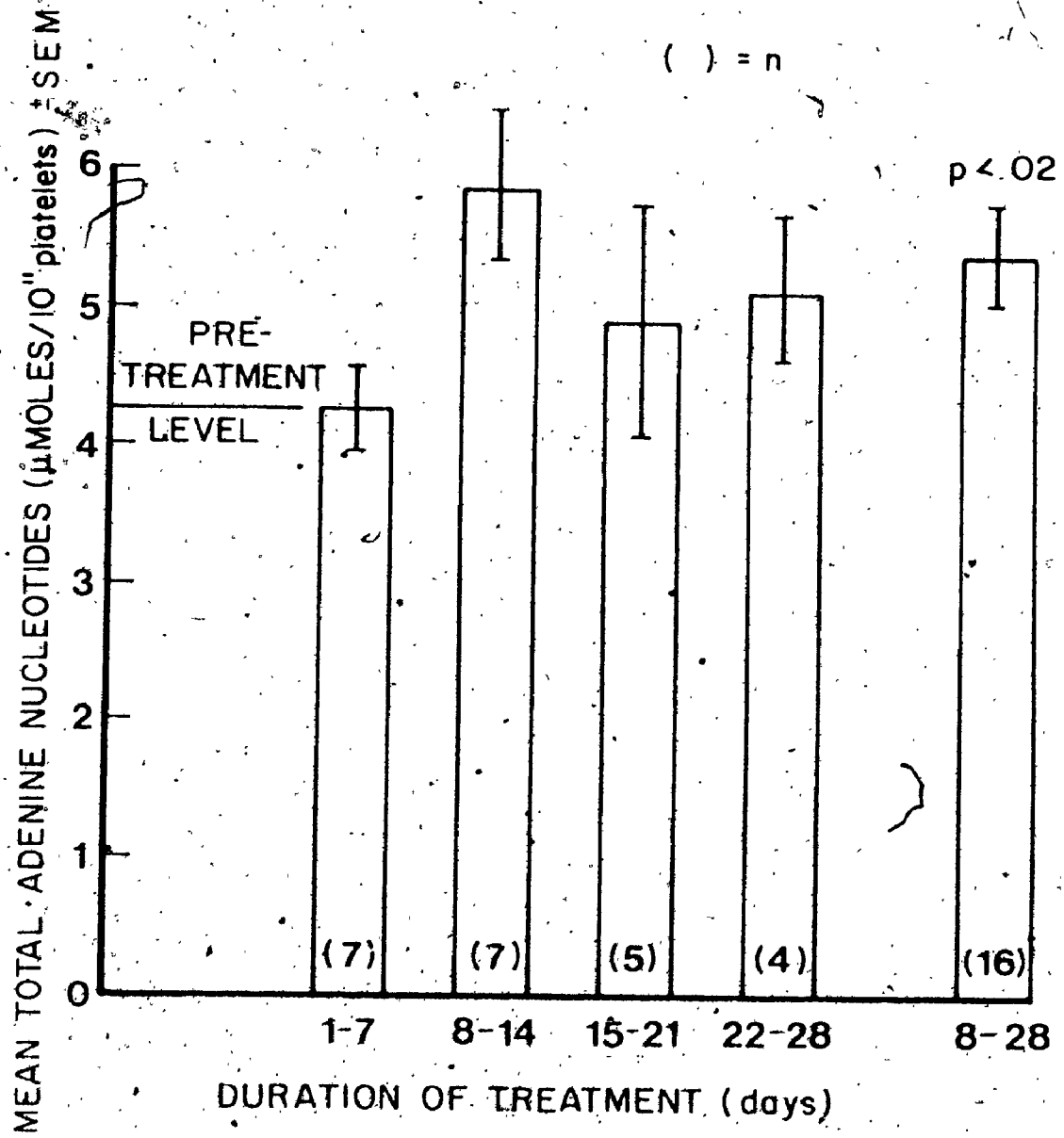

( ) = n
p < .02
MEAN TOTAL ADENINE NUCLEOTIDES (μMOLES/10¹¹ platelets) ± SEM
PRE-TREATMENT LEVEL
0
1
2
3
4
5
6
(7)
(7)
(5)
(4)
(16)
1-7
8-14
15-21
22-28
8-28
DURATION OF TREATMENT (days)

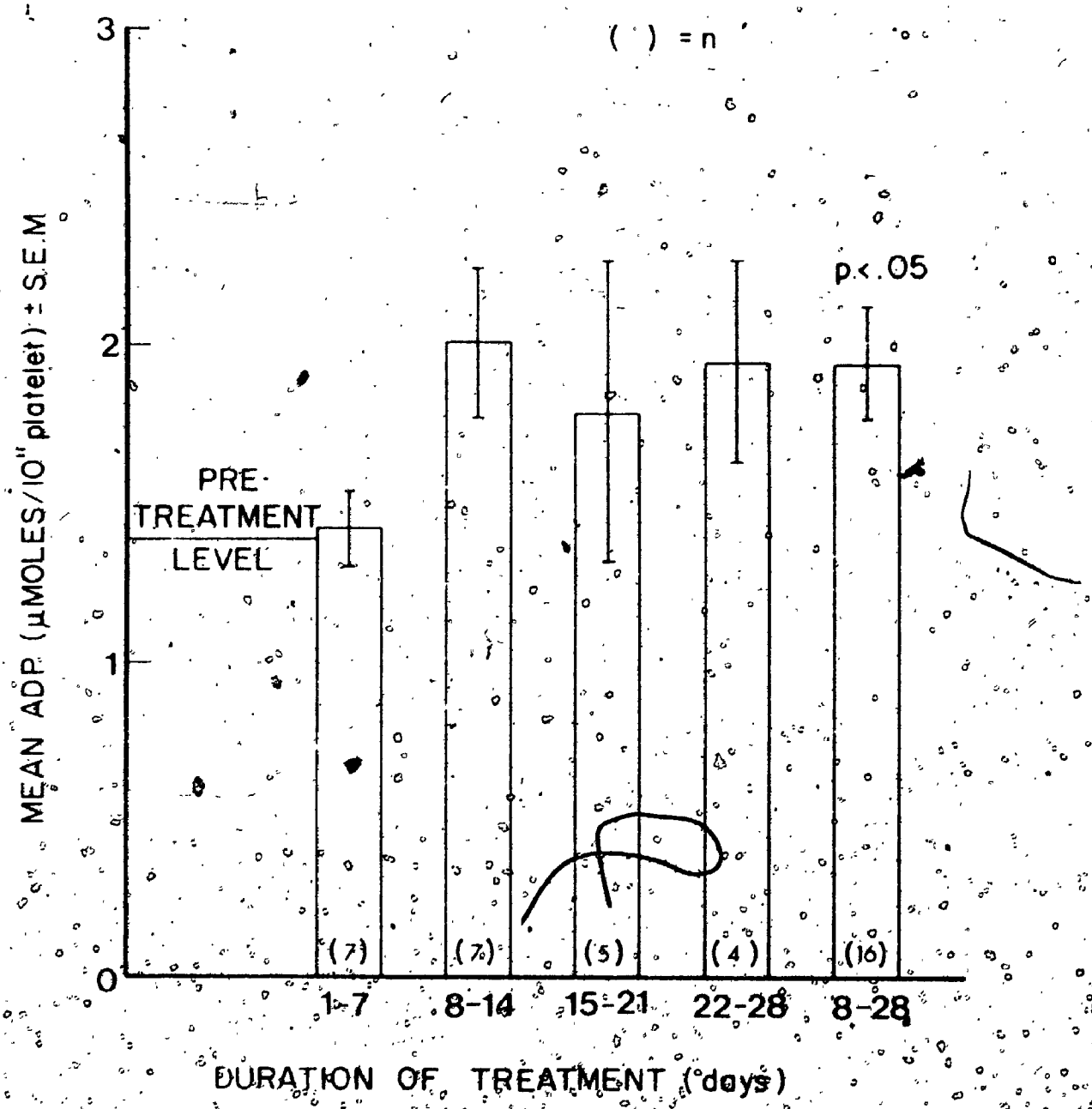
Fig. 15.

Effect of Allopurinol on the ADP Content of Rabbit Platelets.

Note that there was virtually no change in rabbit platelet ADP levels during 1-7 days of allopurinol (40 mg./kg./day, orally) treatment, but that the ADP levels were elevated thereafter. When the data from the 8-14, 15-21, and 22-28-day treatment periods were analyzed, exclusive of the 1-7 day period, a significant elevation of platelet ADP for this 8-28-day period was noted. The p-value shown was obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 15.


( ) = n
p<.05
PRE-TREATMENT LEVEL
MEAN ADP (μMOLES/10" platelet) ± S.E.M
0
1
2
3
(7)
(7)
(5)
(4)
(16)
1-7
8-14
15-21
22-28
8-28
DURATION OF TREATMENT (days)

Platelet aggregation studies carried out simultaneously with the adenine nucleotide determinations produced some interesting correlative data. ADP (3.3×10^{-6}M., final concentration)-induced platelet aggregation in CPRP of allopurinol (40 mg./kg./day, orally)-treated rabbits disclosed a gradually increasing Aggregation Index (Fig. 16) over the 1-7, 8-14, and 15-21 day treatment periods, approaching statistical significance at the 15-21 and 22-28-day periods. When the data for the 8-28-day treatment period were analyzed, however, the increased Aggregation Index was highly significant ($p < .01$). These findings were not observed in CPRP obtained from control rabbits receiving lactose treatment.

Use of a higher (1.25×10^{-5}M., final concentration) concentration of ADP, produced platelet aggregation effects similar to those of the lower concentration of ADP described above, with a statistically significant ($p < .02$) elevation (Fig. 17) of the Aggregation Index during the 8-28-day treatment period. A comparable control group of rabbits receiving lactose appeared to experience an increased level of platelet aggregation, but this increase was not characterized by statistical significance.

Fig. 16.

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP.

Note the gradually increasing Aggregation Index (A.I.) in ADP (3.3×10^{-6}M., final concentration)-induced platelet aggregation in CPRP of allopurinol (40 mg./kg./day, orally)-treated rabbits. Again, when the data from the 8-28 day treatment period were analyzed, a statistically significant increase in platelet aggregation could be demonstrated for this 8-28-day treatment period. The p-value shown was obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

Fig. 17.

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP, Using a Higher Concentration of ADP.

Use of a higher concentration of ADP (1.25×10^{-5}M., final concentration) to induce platelet aggregation in CPRP of allopurinol (40 mg./kg./day, orally)-treated rabbits produced results similar to those illustrated in Figure 16. With this higher concentration of ADP, elevated Aggregation Index (A.I.) values were noted, being statistically significant during the 8-28-day treatment period. The p-value shown was obtained by student's t-test, using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

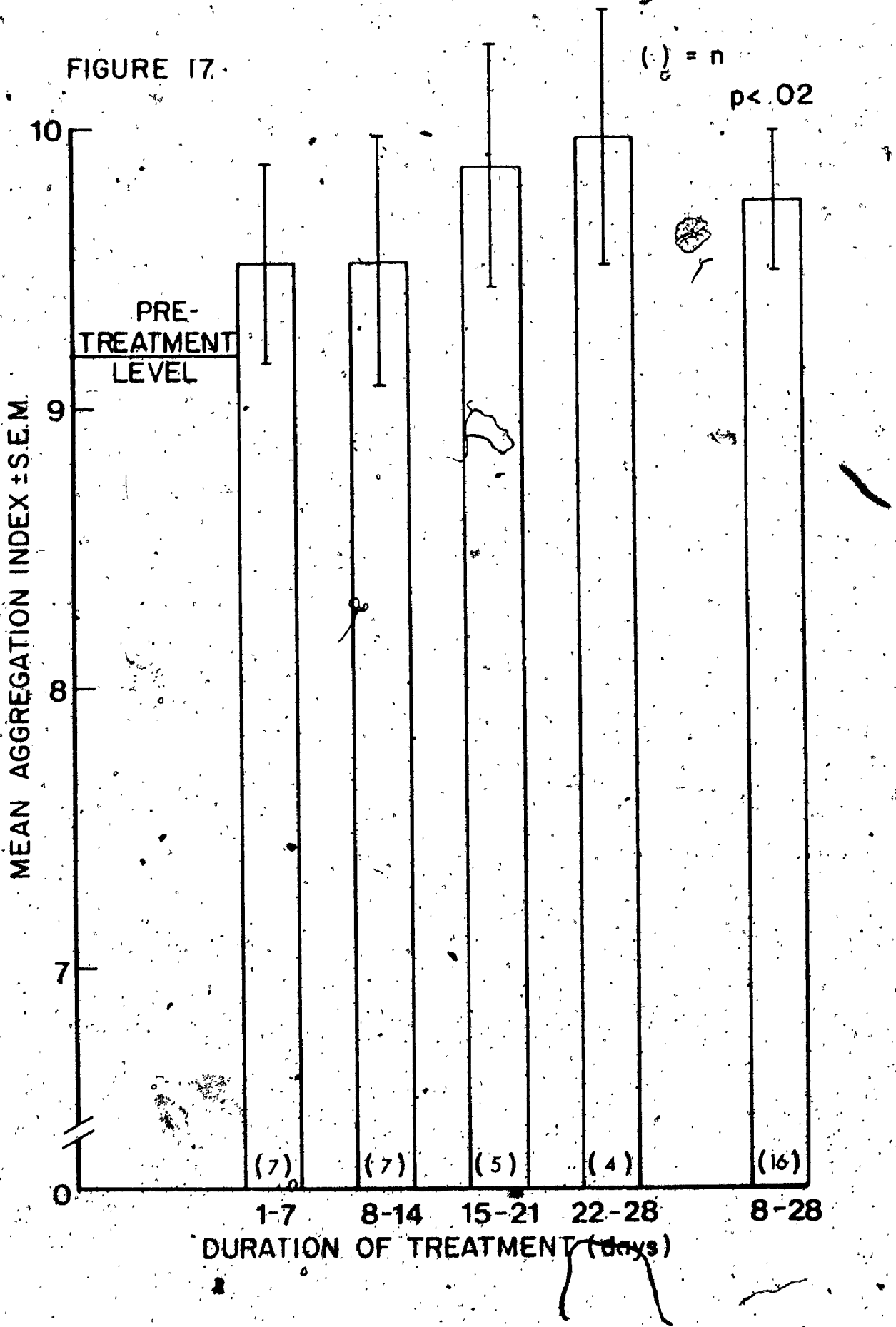
Fig. 17.

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Rabbit CPRP, Using a Higher Concentration of ADP.

Use of a higher concentration of ADP (1.25×10^{-5}M., final concentration) to induce platelet aggregation in CPRP of allopurinol (40 mg./kg./day, orally)-treated rabbits produced results similar to those illustrated in Figure 16. With this higher concentration of ADP, elevated Aggregation Index (A.I.) values were noted, being statistically significant during the 8-28-day treatment period. The p-value shown was obtained by student's t-test, using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 17


( ) = n
p< .02
10
9
8
7
0
PRE-TREATMENT LEVEL
MEAN AGGREGATION INDEX ± S.E.M.
(7)
(7)
(5)
(4)
(16)
1-7
8-14
15-21
22-28
8-28
DURATION OF TREATMENT (days)

Collagen-induced platelet aggregation in CPRP from allopurinol (40 mg./kg./day, orally)-treated rabbits resulted in significantly elevated ($p < .05$) mean collagen curve-heights throughout the 28-day treatment period (Fig. 18). No statistically significant changes in collagen curve height were observed in the control group.

The time to onset of collagen-induced platelet aggregation, was not significantly altered in either the allopurinol (40 mg./kg./day, orally)-treated or the control groups.

Several other parameters were measured in these experiments in conjunction with the previously described adenine nucleotide and platelet aggregation measurements. The whole-blood, platelet count in the allopurinol (40 mg./kg./day, orally)-treated rabbits was significantly reduced (Fig. 19) at the 8-14 ($p < .05$), and 22-28 ($p < .02$)-day treatment periods. Analyzing this data during 1-28 ($p < .01$) days of treatment (Fig. 19) was even more significant. Quite on the contrary, however, the control rabbits exhibited elevated whole-blood, platelet counts during the 1-28 ($p < .001$)-day period of the experiment (Fig. 20).

Fig. 18.

Effect of Allopurinol on Collagen-Induced Platelet Aggregation in Rabbit CPRP.

The mean curve height (mm.) of collagen-induced platelet aggregation in CPRP of rabbits treated with allopurinol (40 mg./kg./day, orally) was significantly elevated during 1-28 days of treatment. The p-value shown was obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 18.

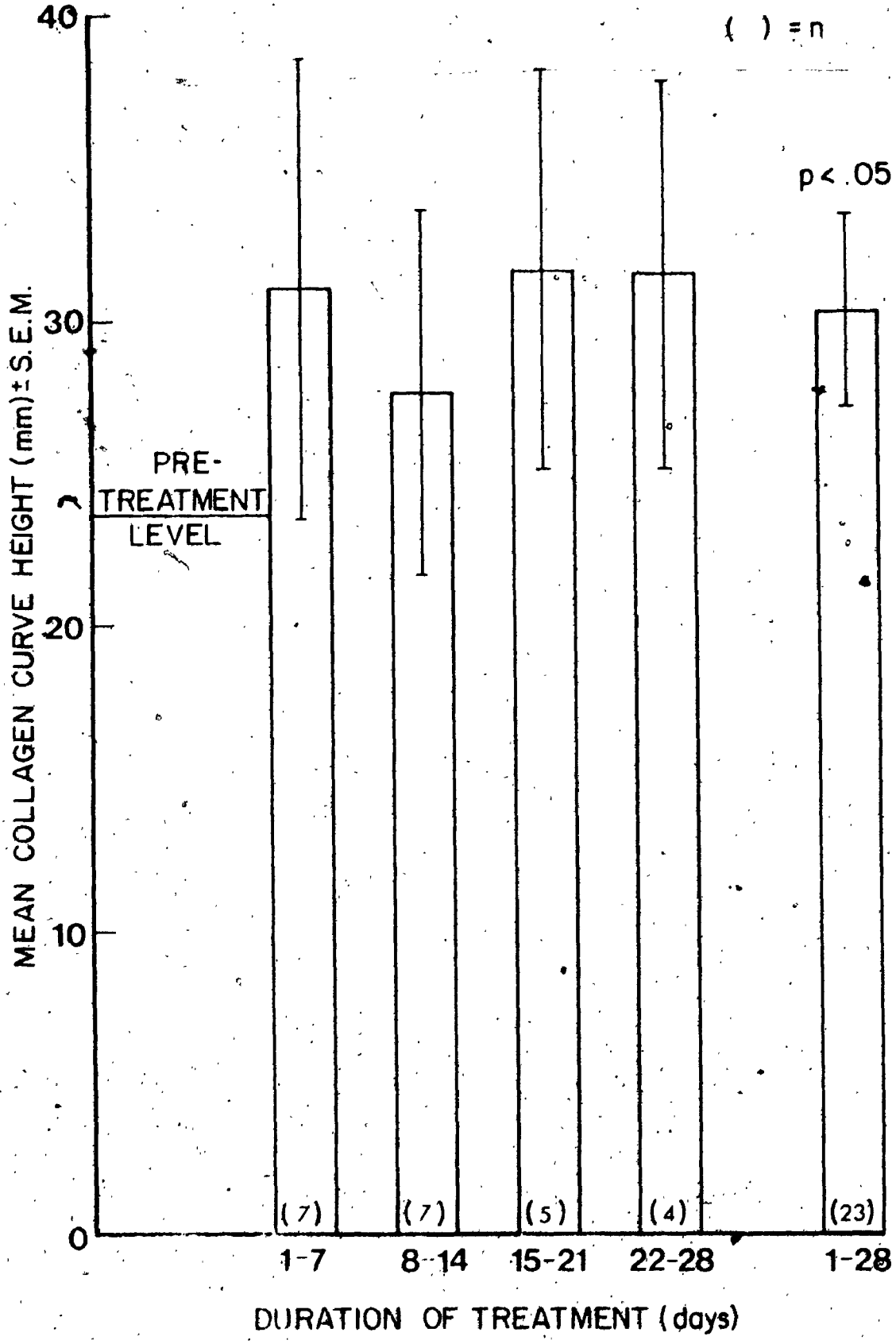

40
30
20
10
0
( ) = n
p < .05
PRE-
TREATMENT
LEVEL
MEAN COLLAGEN CURVE HEIGHT (mm) ± S.E.M.
(7)
(7)
(5)
(4)
(23)
1-7
8-14
15-21
22-28
1-28
DURATION OF TREATMENT (days)

Fig. 19.

Effect of Allopurinol on the Whole-Blood, Platelet Count in Rabbits.

The whole-blood, platelet count was significantly reduced in the 8-14 and 22-28-day periods of treatment of rabbits with allopurinol (40 mg./kg./day, orally). This reduced platelet count was even more evident when the whole 28-day (1-28) treatment period was considered. The p-values shown were obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 19.

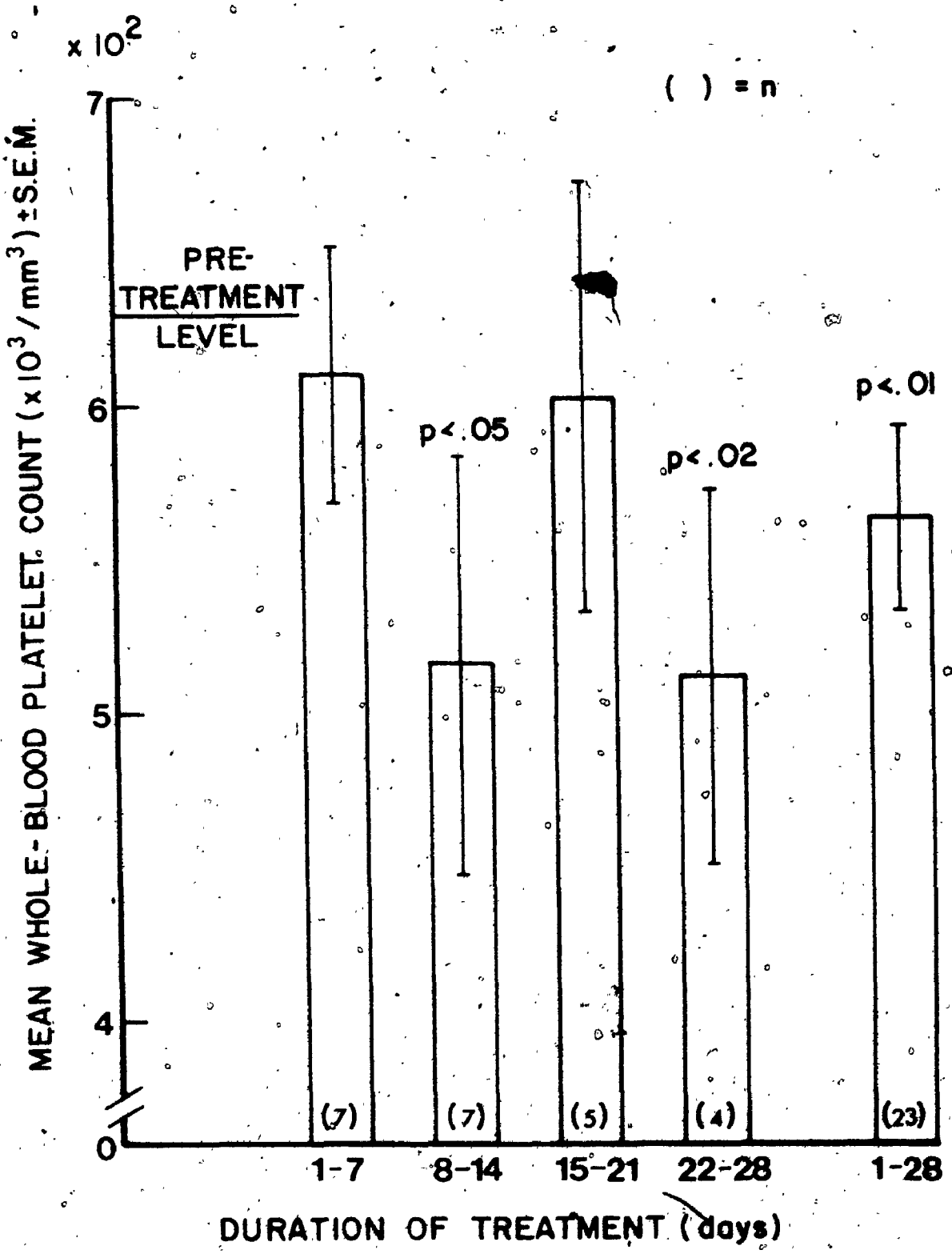

x 10²
( ) = n
MEAN WHOLE-BLOOD PLATELET COUNT (x 10³/mm³) ± S.E.M.
7
6
5
4
0
PRE-TREATMENT LEVEL
p<.05
p<.02
p<.01
(7)
(7)
(5)
(4)
(23)
1-7
8-14
15-21
22-28
1-28
DURATION OF TREATMENT (days)

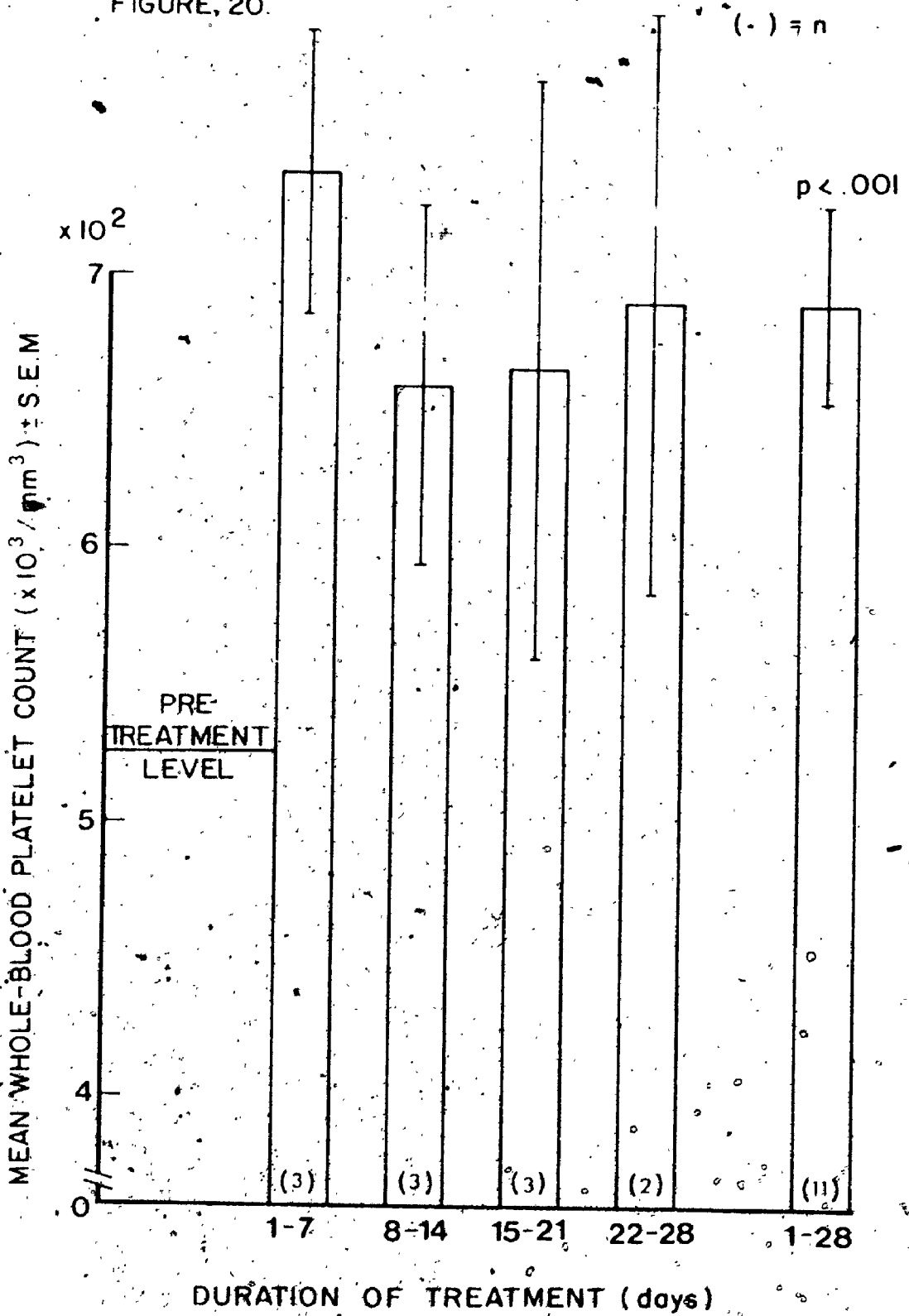
Fig. 20.

Elevation of Whole-Blood, Platelet Counts in Control Rabbits.

Whole-blood, platelet counts in the control group were significantly elevated during the 28-day (1-28) period of the experiment. The p-value shown was obtained by student's t-test using paired data.

In this experiment the control group consisted of three rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration indicated. For details, see Appendix III.

FIGURE, 20.


(-) = n
p < .001
x 10 2
7
6
5
4
0
MEAN WHOLE-BLOOD PLATELET COUNT (x 10 3 / mm 3) ± S.E.M
PRE-TREATMENT LEVEL
(3)
(3)
(3)
(2)
(11)
1-7
8-14
15-21
22-28
1-28
DURATION OF TREATMENT (days)

Even though the same CPRP dilution techniques were used for the allopurinol (40 mg./kg./day, orally)-treated or the control groups throughout the 28-day experiment, examination of the "diluted" CPRP counts, however, revealed a reduced diluted CPRP count in the allopurinol-treated group which was significant at the 22-28 ($p < .005$)-day treatment period (Fig. 21). Conversely, the diluted CPRP count was elevated in the control group during the comparable period, this elevation being significant at the 8-14 ($p < .05$) day level (Fig. 22). This control group data indicated statistical significance as well throughout the 1-28 ($p < .001$)-day period (Fig. 22).

Simultaneous monitoring of the packed cell volumes (PCV) during these adenine nucleotide experiments did not reveal any significant difference in either the allopurinol (40 mg./kg./day, orally)-treated or the control groups.

Measurement of rabbit weights throughout these experiments indicated in both groups, only the expected weight gains consistent with normal growth during the 28 days of the experiment.

Fig. 21.

Lowered Platelet Counts in CPRP of Allopurinol-Treated Rabbits.

The CPRP platelet counts, determined after the described CPRP dilution procedure, were significantly reduced in the allopurinol (40 mg./kg./day, orally)-treated rabbits in the 22-28-day period of treatment. The p-value shown was obtained by student's t-test using paired data.

In this experiment, the allopurinol-treated group consisted of seven rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration of treatment indicated. For details, see Appendix III.

FIGURE 21

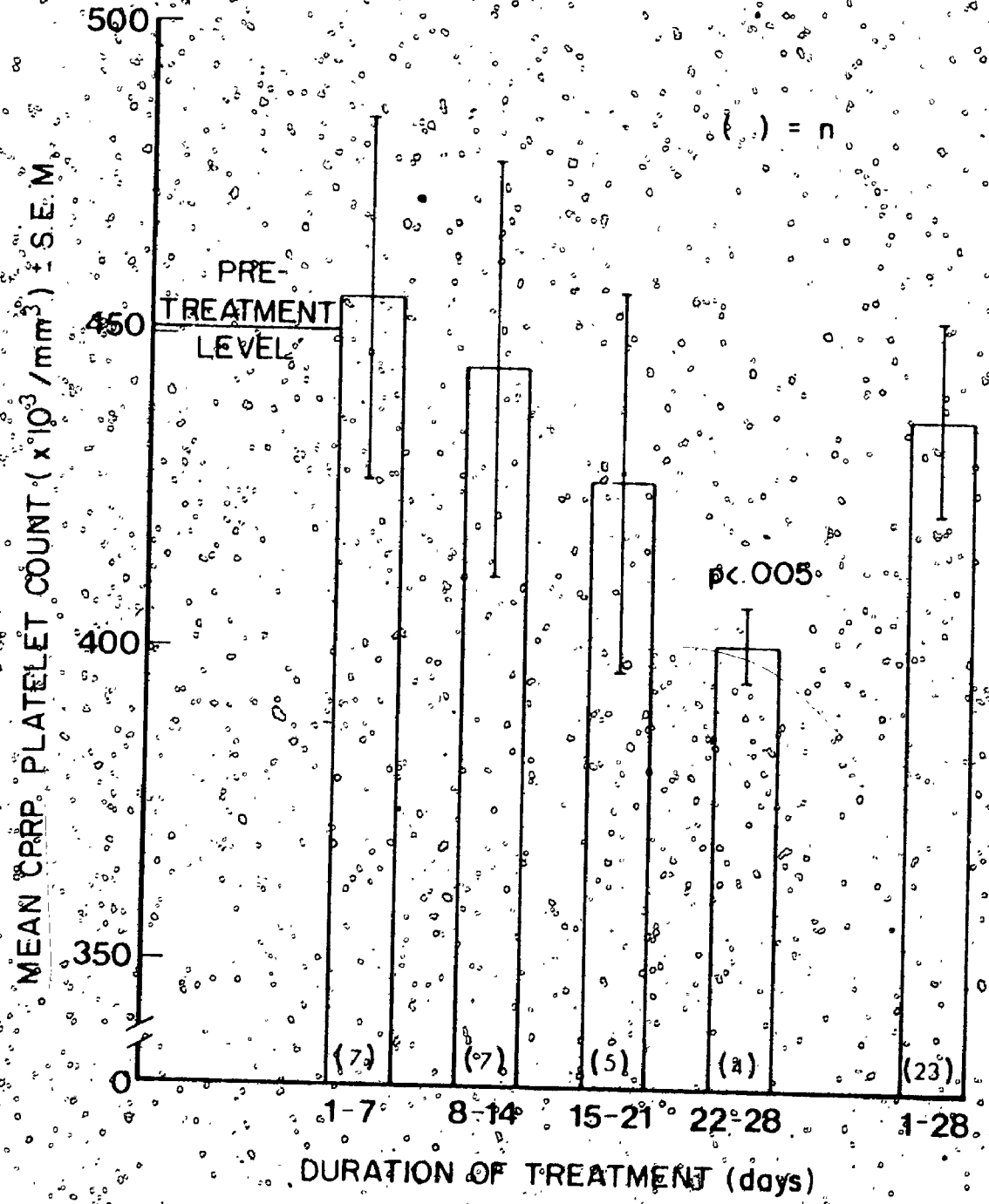

500
450
400
350
0
PRE-TREATMENT LEVEL
( ) = n
p<.005
(7)
(7)
(5)
(4)
(23)
1-7
8-14
15-21
22-28
1-28
DURATION OF TREATMENT (days)
MEAN CRP PLATELET COUNT (x10³ /mm³) ± S.E.M.

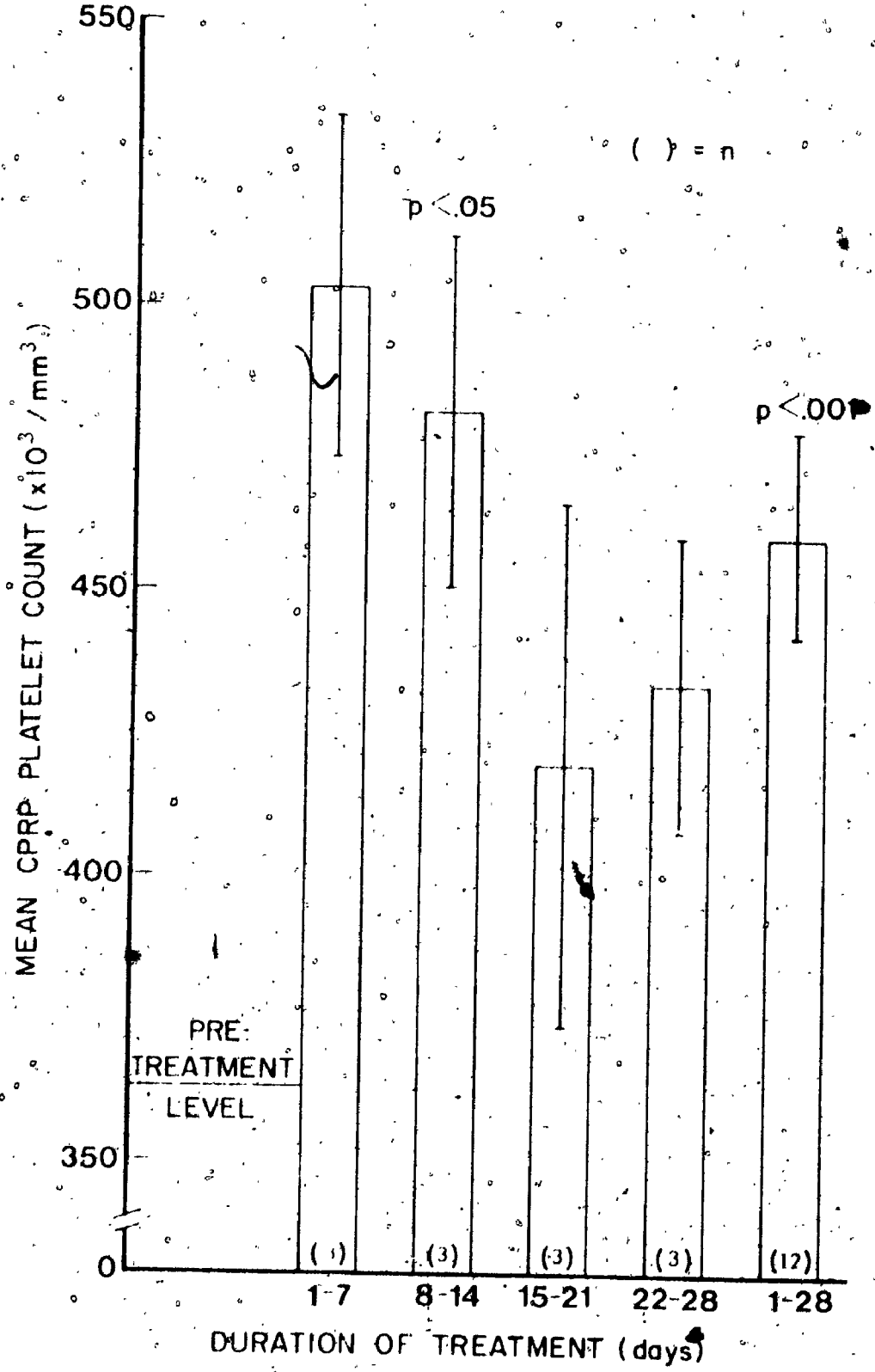
Fig. 22.

Elevated Platelet Counts in CPRP of Control Rabbits.

The CPRP platelet counts, determined after the described CPRP dilution procedure, were significantly elevated in the control group of rabbits in the 8-14-day period, as well as through the 1-28-day period. The p-values shown were obtained by student's t-test using paired data.

In this experiment the control group consisted of three rabbits, a blood sample being obtained from each rabbit, every seven days. Since not all rabbits survived the full 28 days of the experiment, the n values shown reflect the total number of blood samples obtained, for the duration indicated. For details, see Appendix III.

FIGURE 22


550
500
450
400
350
0
MEAN CPRP PLATELET COUNT (x10³ / mm³)
( ) = n
p < .05
p < .00
PRE-TREATMENT LEVEL
(1)
(3)
(3)
(3)
(12)
1-7
8-14
15-21
22-28
1-28
DURATION OF TREATMENT (days)

D. Effect of Clinical Use of Oral Allopurinol on Adhesiveness and Aggregation of Human Platelets

Throughout the period of allopurinol treatment, platelet adhesiveness was unchanged, from the initial pre-treatment levels, in the patients studied.

Again, as in the experiments with rats and rabbits, there was a trend for ADP (4.1×10^{-6}M., final concentration)-induced platelet aggregation to be increased during allopurinol treatment (Fig. 23a), with a significantly increased ($p < .01$) mean curve height in the first month of treatment (Fig. 23b).

A highly significant correlation ($r=0.66$, $p < .005$) was demonstrated (Fig. 24) between decreasing serum uric acid levels, as measured by the fall (mg.-%) from the initial value, and increasing platelet aggregation (Aggregation Index) in the patients studied. Patients whose serum uric acid levels fell by 6-9 mg.-%, showed an increase of 2-4 Aggregation Index units. Those who showed little change in uric acid levels, also showed little change in platelet aggregation.

Fig. 23.

Effect of Allopurinol on ADP-Induced Platelet Aggregation in Human CPRP.

(a) ADP(4.1×10^{-6}M., final concentration)-induced platelet aggregation (as illustrated by mean rate of rise) in CPRP of patients receiving allopurinol was slightly elevated during the treatment period, each patient's monthly treatment data being compared to the pre-treatment control data for that patient.

(b) Similarly, the mean curve height was elevated during allopurinol therapy, being significantly so in the first month of treatment. The p-value shown was obtained by student's t-test using paired data.

Nine patients were included in the study, but since not all patients completed the six-month duration, the n-values shown reflect the number of patients participating at the duration of treatment shown.

FIGURE 23(a)
RATE OF RISE

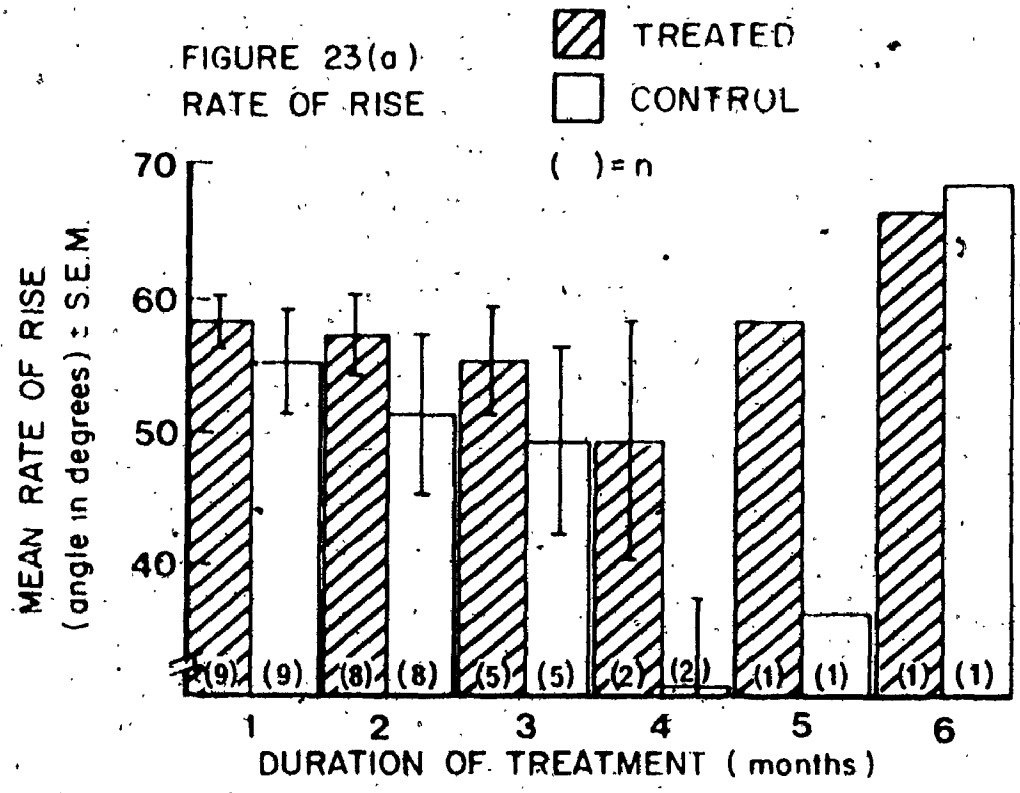


FIGURE 23 (b)
CURVE HEIGHT
$p < .01$

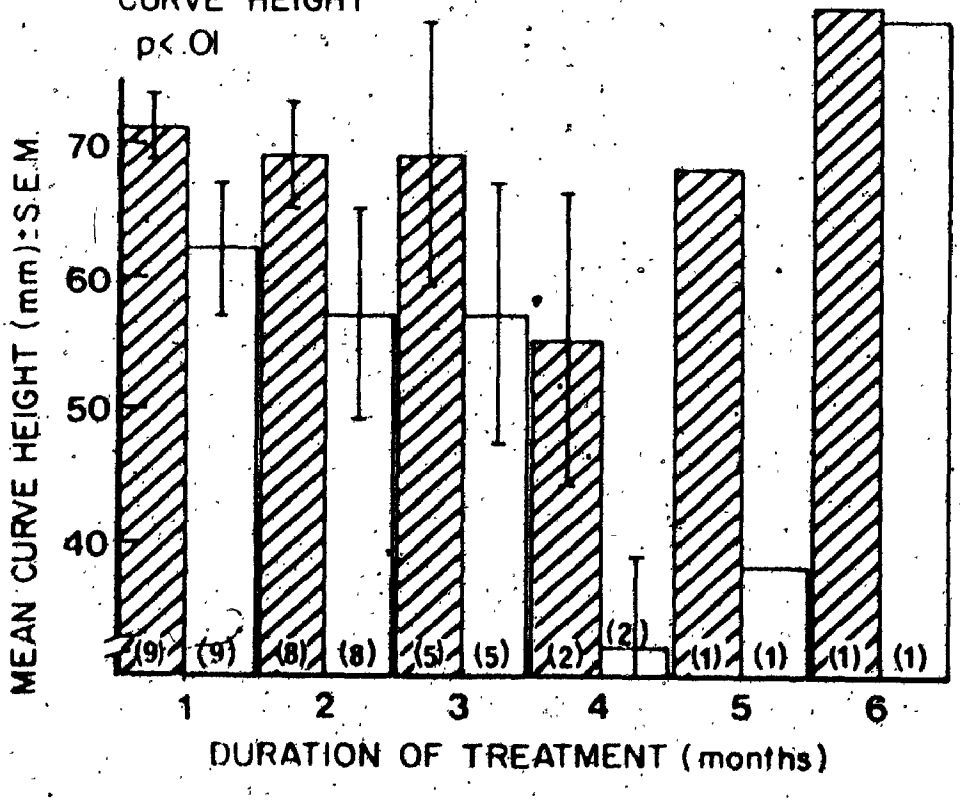
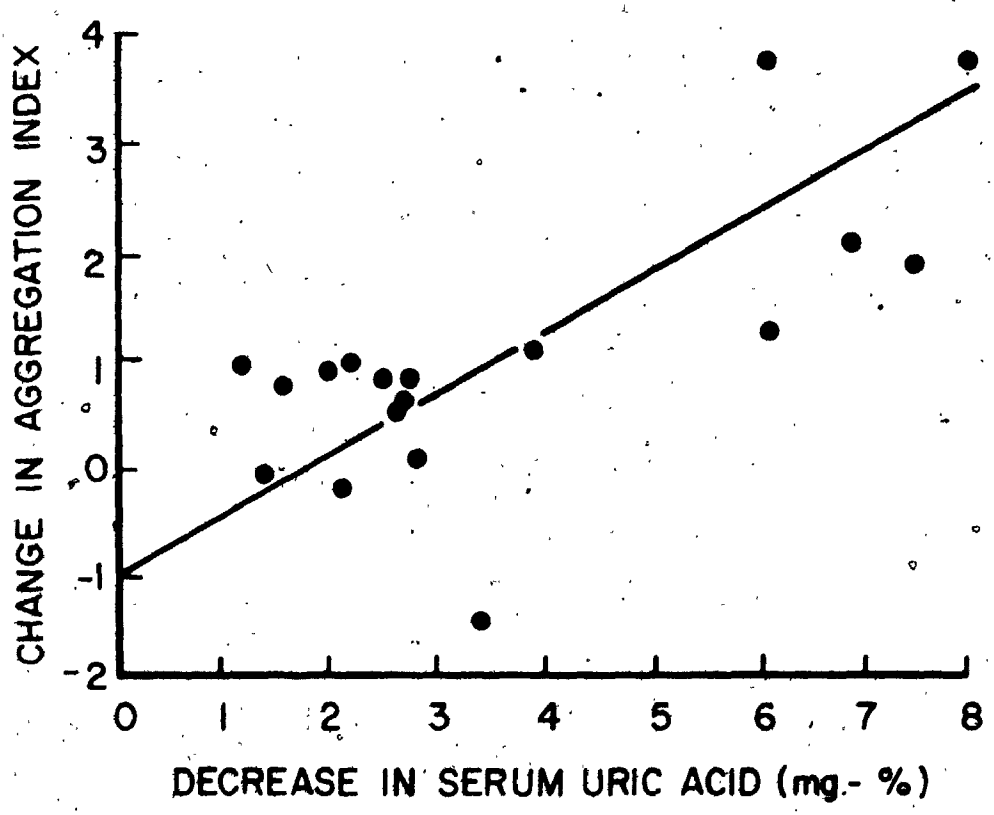


Fig. 24.

Relationship Between Changes in Platelet Aggregation and Decreases in Serum Uric Acid in Patients.

Correlation of the platelet aggregation of the patients studied, with the uric acid levels, revealed a highly significant correlation ($r=0.66$, $p < .005$). A drop of serum uric acid between 6-9 mg.-% was accompanied by an increase in aggregation index of 2-4 aggregation index units.

FIGURE 24.


CHANGE IN AGGREGATION INDEX
4
3
2
1
0
-1
-2
0
1
2
3
4
5
6
7
8
DECREASE IN SERUM URIC ACID (mg.- %)

DISCUSSION

Patients with untreated cancer, particularly hematological disorders, have an accompanying hyperuricemia due to rapid cell turnover (Krakoff, 1967). Treatment of cancer with X-rays or antineoplastic agents also results in hyperuricemia (Rundles, Wyngaarden, Hitchings, and Elion, 1969) due to increased tissue destruction and nucleoprotein turnover. Davis, Theologides, and Kennedy (1969) indicated that thrombosis has been associated with cancer for more than 100 years. It is not too surprising, therefore, that an association between thrombosis and hyperuricemia would be suspected. Such an association was reported by Pearce and Aziz (1969), in that of 60 acute stroke patients studied, 25% had hyperuricemia which was not transitory, leading these authors to suggest that hyperuricemia may be a factor in the pathogenesis of atheroma. Subsequent work (Pearce and Aziz, 1970) indicated that hyperlipidemia was also associated with cerebrovascular disease but that hyperuricemia was not sufficiently well-correlated ($p < .10$ with $> .05$) with hyperlipidemia to be a useful diagnostic predictor of cerebrovascular disease. Gertler, et al. (1964) had previously studied 490 men, 28-83 years of age, and had concluded that the "coronary-prone" individual could be characterized as "older, shorter, and with elevated cholesterol, uric acid, and phospholipid levels". Bluhm

and Riddle (1973) also found a significant correlation between serum uric acid levels and myocardial infarction. Newland (1968), using intravenous ADP to induce pulmonary thrombosis in rats, reported finding increased thrombi formation in rats pre-treated with intravenous urate. He postulated that the elevated urate levels possibly slowed the degradation of the infused ADP, resulting in an increased amount of ADP being available to induce pulmonary thrombosis. It may have been, however, merely phagocytosis of uric acid crystals (infused as part of the urate infusion, or precipitated in the blood stream immediately after infusion) by the platelets, which has been shown (Glynn, Mustard, Smythe, and Movat, 1966) to occur in vitro, leading to platelet aggregation. Furthermore, Palmer, Piper, and Vane (1970) have reported finding vaso-active substances released from isolated lungs by injection of particulate matter into the perfusate. Uric acid crystals, acting like particulate matter, might release substances from the lungs, which in turn, could affect platelet aggregation, suggesting that Newland's (1968) results might be due to "uric acid crystal-particulate matter". Uric acid, solubilized by litnium carbonate, and added to 5% glucose, has been infused intravenously into human subjects (Yu, Berger, and Gutman, 1962, and others) as part of urate excretion studies. This infusion raised the mean plasma urate level from 5.4 mg.-% before the infusion to 7.3 mg.-% after the infusion. These investigators did not monitor the effects, on platelets, of this urate infusion. Emmerson (1975) has recently discussed the potential hazards and risks involved in treating, or not treating, hyperuricemia. He identifies obesity,

type IV hyperlipoproteinemia, hypertension, diet, alcohol (especially beer), thyroid dysfunction, and parathyroid dysfunction, as factors (not necessarily causative factors) associated with hyperuricemia. Dosman, Crawhall, and Klassen (1975) studied seven male patients immediately following myocardial infarction, and found that their urate turnover was twice normal, and their urate pool size was 3-6 times normal. They concluded that hyperuricemia in the post-myocardial infarction state is clearly evident, but that a causative relationsnip between hyperuricemia and myocardial infarction has not yet been proven.

In the results reported here with acute gout patients receiving allopurinol orally for the first time, a significant correlation was found between decreasing serum urate and increasing platelet aggregation. Since it was not feasible to verify these data by further studies in human subjects, an hyperuricemic animal model was developed. The hyperuricemia of the animal model could thus be lowered with allopurinol and any similar correlation with platelet aggregation be observed as in the studies of patients with gout. Desiring to avoid the probable difficulties associated with urate infusions and uric acid crystals, the techniques of Johnson, et al. (1969) and Stavric, et al. (1973) in rats were used, developing a rabbit hyperuricemic model. Bluestone, Klinenberg, Waisman, and Goldberg (1974) have also described a rat hyperuricemic model, based on the techniques of Johnson, et. al. (1969). Using intravenous potassium oxonate, a uricase inhibitor, and a previous loading dose of intraperitoneal sodium urate, an hyperuricemia was

produced which gradually increased with time. This hyperuricemia in rabbits was found not to be associated with any changes in platelet aggregation. When this urate level was lowered with intravenous sodium allopurinol, however, a decreased platelet aggregation was observed, quite the contrary to the previous observations in gout patients. To determine whether this decreased platelet aggregation was due to the lowering of the urate level, or due to the intravenous allopurinol, the intravenous allopurinol experiments were repeated with normouricemic rabbits. Again, a similar decrease in platelet aggregation was observed. This suggested that intravenous allopurinol in rabbits, and not a change from hyperuricemia to normouricemia in rabbits, was responsible for the observed changes in platelet aggregation. It should be noted that even normouricemic control group rabbits experienced a slight hyperuricemia, which may have been due to the anesthetic and/or experimental procedures. This situation was accounted for by providing a control group in these experiments. These data suggest that allopurinol increases platelet aggregation during prolonged oral use (as in the gout patients), and decreases platelet aggregation as a result of intravenous, short-term contact with circulating platelets.

It has been known for some time that acetylsalicylic acid (Morris, 1967; Zucker and Peterson, 1968; and others) inhibits platelet aggregation and prevents the second phase release of ADP (and other platelet constituents) from platelets. O'Brien (1975) and Flower (1975) have recently summarized this subject, including discussion of the inhibiting effects of acetylsalicylic acid on the

platelet production of prostaglandins. Prostaglandins stimulate adenyl cyclase resulting in elevated levels of cyclic AMP(cAMP) which is associated with (not a causative relationship (McDonald and Stuart, 1974) decreased (or inhibited) platelet aggregation. More recently, McElroy and Philp (1975), working with dipyridamole and related agents, concluded that platelet function (including aggregation and the release reaction) is modulated by alterations in the relative concentrations of cAMP and cyclic GMP(cGMP), and not by one or the other agent in isolation. Because of allopurinol's structural similarity to xanthine, and xanthine's relationship to the methylxanthines (inhibitors of platelet phosphodiesterase, which results in elevated cAMP), it is possible that allopurinol may have a similar action. In the data presented, this might be most easily recognized by in vitro allopurinol's inhibition of second phase aggregation, and intravenous allopurinol's reduced platelet aggregation.

Packham and Mustard (1969) found that non-steroidal anti-inflammatory agents such as phenylbutazone inhibited platelet aggregation and thrombus formation. The possible pharmacological similarity of allopurinol to non-steroidal anti-flammatory agents becomes credible when one considers the report (Riesterer and Jaques, 1969) that allopurinol possesses anti-flammatory properties. This similarity is further substantiated by Jaques and Helfer (1971) who reported finding antinociceptive and anti-exudative effects of allopurinol and of xanthine.

These results, indicating that *in vitro* allopurinol inhibits collagen-induced platelet aggregation in rabbit CPRP, are consistent with the evidence suggesting that allopurinol is an anti-flammatory agent. Furthermore, CPRP from rabbits receiving oral allopurinol for 1-28 days revealed a significant *in vitro* allopurinol inhibition of ADP-induced platelet aggregation, whereas CPRP from control rabbits receiving lactose for 1-28 days was not significantly affected by *in vitro* allopurinol. This suggests that perhaps the platelets of orally-dosed allopurinol-treated rabbits were "conditioned" by previous exposure to allopurinol resulting in a pronounced *in vitro* allopurinol effect on these platelets. The evidence of *in vitro* allopurinol-inhibition of the second phase release reaction in human CPRP also is supportive of the hypothesis that allopurinol possesses a direct effect on platelets. This is further supported by the evidence of inhibition of ADP-induced and collagen-induced aggregation of platelets from rabbits having received intravenous allopurinol. The use of intravenous allopurinol suggests that the sudden presence of allopurinol in the blood stream of rabbits results in a direct effect on platelets which was not found when allopurinol was given orally.

The interference of allopurinol with purine metabolism (Pomales, *et al.*, 1963; Kelley and Wyngaarden, 1970a; Kelley and Wyngaarden, 1970b) was previously discussed in some detail. Fox, Wyngaarden, and Kelly (1970) reported that allopurinol was found to deplete the 5-phosphoribosyl-1-pyrophosphate (PRPP) in human erythrocytes. Although such an action of allopurinol would reduce or prevent purine synthesis *de novo*, it is also possible that

allopurinol, by preventing conversion of hypoxanthine to xanthine, could reduce the catabolism of substances such as inosine, adenosine, and thus other adenine nucleotides. Predominance of the latter effect would be expected to be accompanied by an accumulation of substances such as AMP, ADP, and ATP. Rivard, McLaren, and Brunst (1975) found that C^{14}-hypoxanthine was incorporated predominantly into ADP and ATP, in normal washed human platelets. Increased platelet levels of total nucleotides (determined to consist largely of ADP), as described earlier, would lend support to the possibility of hypoxanthine incorporation into platelets. This elevated ADP level also provides an explanation for the increased platelet aggregation observed in rats, rabbits, and man, all receiving allopurinol orally.

The near total absence of this effect during the first seven days of the 28-day experiment with rabbits suggests that oral allopurinol does not significantly alter nucleotide levels in existing circulating platelets, but rather, may produce elevated ADP levels primarily in developing platelets in the megakaryocytes. A platelet population higher than normal in ADP content, may result in a "stickier" platelet population, which might explain the observed thrombocytopenic effect of oral allopurinol in rabbits.

The foregoing discussion suggests that allopurinol possesses two distinct effects with respect to platelets:

1. A direct effect, preventing second phase release of platelet constituents, with decreased platelet aggregation. These effects were observable when sodium allopurinol was used *in vitro*, or *in vivo*

as an intravenous injection.

2. An indirect effect, which may involve the megakaryocytes, is manifested by elevated platelet levels of ADP, increased platelet aggregation, and an apparent thrombocytopenia which may be due to increased "stickiness" of the platelet population.

These distinctive effects are schematically illustrated in Fig. 25.

Fig. 25.

Schematic Representation of the Possible Mechanisms of action of Allopurinol on Blood Platelets.

(a) Sodium Allopurinol, in vitro, or given intravenously, may exert its platelet aggregation-inhibiting effects at any one or more of (i), (ii), or (iii) aspects of the overall mechanism. Allopurinol given orally for an extended time, results in elevated levels of platelet ADP (iv).

(b) In this simplified diagram of purine metabolism, it will be noted that allopurinol inhibits xanthine oxidase at (v) and (vi), and by so doing, results in elevated levels of hypoxanthine and xanthine, the former being re-utilized for the synthesis of IMP, AMP, and ADP, respectively.

FIG. 25 (a)

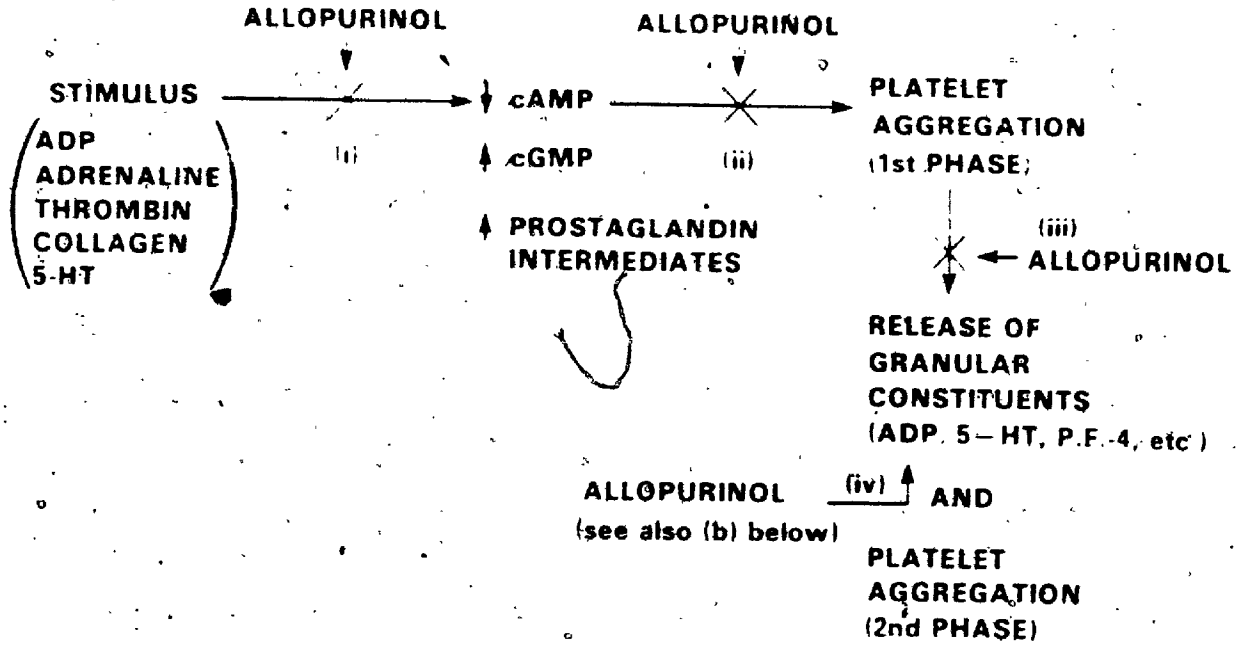
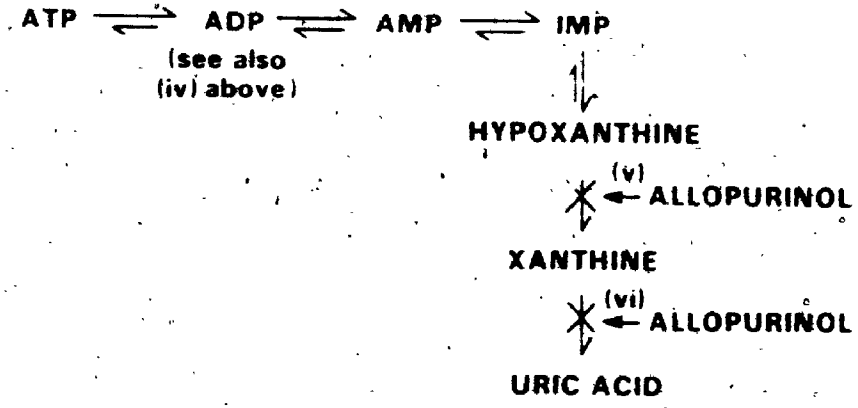


FIG. 25 (b)



SUMMARY AND CONCLUSIONS

The experimental results obtained may be summarized as follows:

1. Allopurinol, independent of the absolute levels of plasma urate or of changes therein, was found to inhibit platelet aggregation.

2. When added to rabbit CPRP, allopurinol *in vitro* resulted in a significant reduction in ADP-induced platelet aggregation, and complete inhibition of collagen-induced platelet aggregation.

   In human CPRP, *in vitro* allopurinol caused a complete inhibition of the second phase (release reaction) platelet aggregation.

3. Allopurinol fed orally to rats, and administered orally to rabbits resulted in increased ADP- and collagen-induced platelet aggregation. In addition, platelet levels of ADP (in rabbits) were elevated with allopurinol treatment. A significant reduction in platelet count was also noted in these allopurinol-treated rabbits.

4. Allopurinol administered orally to hyperuricemic and/or gout patients, as part of their normal therapy, resulted in increased ADP-induced aggregation of these patients' platelets.

Evidence has been presented which supports the hypothesis that allopurinol has a double action on platelets:

1. A short-term direct effect, manifested by decreased platelet aggregation, and prevention of release of platelet constituents.

2. A long-term effect, manifested by elevated levels of platelet ADP, resulting in increased platelet aggregation and apparent thrombocytopenia.

An animal model for hyperuricemia was developed, and has been presented in this manuscript. This model permitted successive serum urate determinations, over several hours, on the same animal.

Consistent evidence was obtained, and was presented here, with respect to the effect of allopurinol on the platelets of three species, rat, rabbit, and man.

The clinical significance of this work is recognized when one considers the ever-increasing concern with which the routine use of allopurinol in patients is being viewed. Although allopurinol has been used clinically for many years, and a few side-effects have been reported, it is only recently that heretofore unknown, but profound, unexpected effects are being reported. Fox, Royse-Smith, and O'Sullivan (1970) reported interference, by allopurinol, with pyrimidine nucleotide synthesis. The interference of allopurinol with purine synthesis has been discussed previously. Vesell, Passananti, and Greene (1970) reported that allopurinol, in normal subjects, had decreased the liver microsomal enzyme activity, suggesting possible toxic effects of concomitantly administered drugs which rely upon the liver microsomal enzymes for their catabolism.

One should not overlook the possible beneficial effects of intravenous allopurinol. If intravenous allopurinol prevents, in man, second phase release of platelet constituents and reduces platelet aggregation, as was determined in these experiments with rabbits, there may be a clinical role for intravenous allopurinol in the short-term prevention of thromboembolism.

Appendix I

Standards, Enzymes, and Solutions Used in the Assay of Adenine Nucleotides

(1) ATP Standard (100 ug./ml.)

Adenosine Triphosphate, Disodium Salt (M.W. 632.2) (Calbiochem)

Preparation: 100 ug./ml. in 0.15 M. NaCl

Storage: 2 ml. quantities, frozen at -20°C.

Stability: 2 months

(2) ADP Standard (100 ug./ml.)

Adenosine Diphosphate, Monosodium (M.W. 494.3) (Calbiochem)

Preparation: 100 ug./ml. in 0.15 M. NaCl

Storage: 2 ml. quantities, frozen at -20°C.

Stability: 4 months

(3) PEP-PK(A)

5 ul. Pyruvate Kinase (PK) suspension (Sigma), from rabbit skeletal muscle, TYPE II, crystalline suspension in $(NH_4)_2SO_4$; Stored at +4°C.; stable for 2 months

100 ul. Phosphoenolpyruvate (PEP) (Sigma) solution* Tricyclohexylamine salt (M.W. 465.6), crystalline.

q.s. 25 ml. with Distilled Water

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*PEP solution

0.04566 gm. PEP

0.96920 gm. KCl

0.98600 gm. $MgSO_4.7H_2O$

q.s. 10 ml. with Distilled Water

Storage: +4°C.

Stability: 1 month

(4) PEP-$PK_{(I)}$

Similar to PEP-$PK_{(A)}$, with the exception that PEP-$PK_{(I)}$ has been heated at 100°C. for 15 minutes.

Storage: not stored; prepared freshly for use each day.

(5) FLE

Firefly Lantern Extract (Worthington Biochemical Corporation)

(Firefly Luciferase)

-lyophilized extract of firefly lanterns

-each vial is equivalent to 50 mg. dry lanterns and when reconstituted (by the addition of 5 ml. of distilled water) contains 0.05 M. KH_2AsO_4 and 0.02 M. $MgSO_4$, pH 7.4

Reconstitution: 5.0 ml. Distilled Water added 2 hours before use

Storage: not stored when re-constituted; stored frozen when not re-constituted

(6) Suspending Fluid No. 1 (S.F. No. 1)

NaCl Solution, 0.154 M.	90%
Tris(hydroxymethyl) aminomethane -HCl Buffer* (pH 7.4)	8%
Disodium EDTA Solution, 0.077 M.	2%

Storage: +4°C.

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*Tris 0.2 M.

HCl 0.1 N.

and titrate with HCl to pH 7.4

(7) Suspending Fluid No. 2 (S.F. No. 2)

NaCl Solution, 0.154 M.	90%
Tris(hydroxymethyl) aminomethane ·HCl Buffer (pH 7.4)	10%

Storage: +4°C.

Appendix II

Aggregation Index, Method of Calculation

The Aggregation Index (A. I.) is a means of quantifying and integrating the rate and extent of the ADP-induced aggregation response of blood platelets. The A. I. is directly proportional to the rate and/or extent of platelet aggregation. The formula for the determination of the A. I. may be summarized as follows:

$$\text{A. I.} = 13 - \left(\text{mean of } \frac{\text{D. U.}}{\text{1/time}}, \text{ at time units of 3, 5, 7, 8 and 10.}\right)$$

where:

$$\text{D. U. ("Density Units")} = \log \frac{96 \text{ mm.}}{\text{(measured vertical curve height) (mm.)}}$$

Time is measured in "Time Units" (1 "Time Unit" = 15 sec. at chart speed of 1 in./min. using a Bausch & Lomb VOM5 Recorder)

and where:

96 mm. = the established maximum curve height for all aggregation curves (instrumental limitation)

* See also the attached aggregation curve, sample calculation and sample worksheet form.

AGGREGATION INDEX WORK SHEET

(Revised November 19, 1969)

R. A. Wildeman
Pharmacology Dept.
U.W.O.

(Exp. No.) (Patient No.) (Rabbit No.) (Date)

NOTE: Aggregation Index (A.I.) = 13 - mean of $\frac{D.U.}{1/time}$, at time units of 3, 5, 7, 8 & 10

where: D.U. = "Density Units" = log $\frac{96 \text{ mm.}}{\text{measured vertical curve height (mm.)}}$

Time = "Time Units", where 1 time unit = 15 sec. (chart speed = 1 inch/min.)

and where: 96 mm = the established max. curve height of all aggregation curves.

	mm.	D.U.	Time (Units)	1/Time	D.U. 1/Time	A.I.
			3	0.33		
Sample No.			5	0.20		
Curve No.			7	0.14		
ADP used ___ ml.			8	0.13		----
			10	0.10		

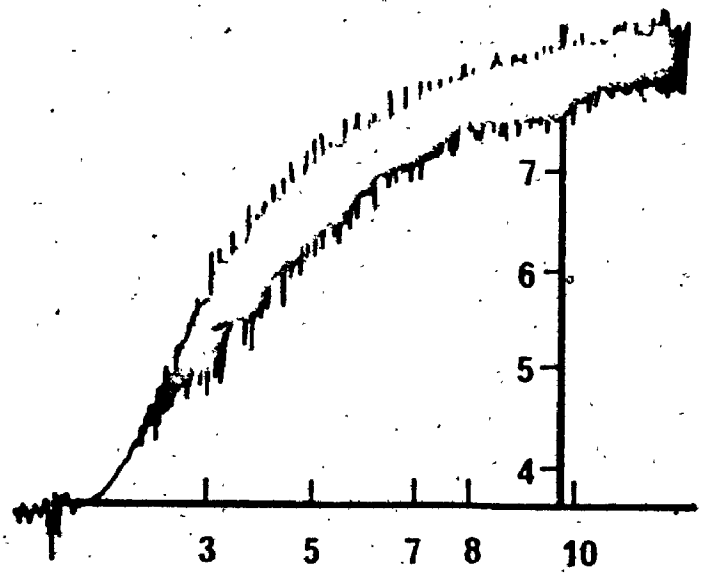
	mm.	D.U.	Time (Units)	1/Time	D.U. 1/Time	A.I.
			3	0.33		
Sample No.			5	0.20		
Curve No.			7	0.14		
ADP used ___ ml.			8	0.13		----
			10	0.10		

	mm.	D.U.	Time (Units)	1/Time	D.U. 1/Time	A.I.
			3	0.33		
Sample No.			5	0.20		
Curve No.			7	0.14		
ADP used ___ ml.			8	0.13		----
			10	0.10		

	mm.	D.U.	Time (Units)	1/Time	D.U. 1/Time	A.I.
			3	0.33		
Sample No.			5	0.20		
Curve No.			7	0.14		
ADP used ___ ml.			8	0.13		----
			10	0.10		

AGGREGATION INDEX -- SAMPLE CALCULATION

SAMPLE CURVE TRACING



TIME UNITS

SAMPLE CALCULATION

mm.	D.U.	Time (Units)	1/Time	D.U. 1/Time	A.I.
18	0.727	3	0.33	2.20	
36	0.427	5	0.20	2.14	
47	0.312	7	0.14	2.23	
51	0.274	8	0.13	2.11	
56	0.236	10	0.10	2.36	
			Average =	2.21	10.79

Appendix III Treatment Regimen of Seven Rabbits Receiving Allopurinol for 1-28 days

Rabbit No.	Treatment Summary: Lactose Treatment	Allopurinol Treatment	Weekly Cardiac Puncture No. 0	1	2	3	4
1*	Days 1-29		Day 1 (0)**	Day 8 (0)	Day 15 (0)	Day 22 (0)	Day 29 (0)
2		Days 1-28	1 (0)	8 (7)	15 (14)	22 (21)	29 (28)
3	Day 2	Days 3-29	2 (0)	9 (6)	16 (13)	23 (20)	30 (27)
4	Days 2 & 3	Days 4-29	2 (0)	9 (5)	16 (12)	23 (19)	30 (26)
5	Days 3-31		3 (0)	10 (0)	17 (0)	24 (0)	31 (0)
6***	Days 24, 25 & 26	Days 27-40****	24 (0)	31 (4)	38 (11)	*****	
7	Days 4,5,6,&7	Days 8-32	4 (0)	11 (3)	18 (10)	25 (17)	
8	Days 4,5,6,7,& 8	Days 9-32	4 (0)	11 (2)	18 (9)		
9	Days 5,6,7,8,9 & 10	Days 11-33	5 (0)	12 (1)	19 (8)	26 (15)	33 (22)
10R	Days 14-40		19 (0)	26 (0)	33 (0)	40 (0)	

* Control rabbits are No. 1, 5 and 10R.

** () = No. of days on allopurinol treatment

*** R indicates a replacement rabbit

**** Experiment was stopped on Day 40

***** Some Rabbits did not complete the series of five cardiac punctures

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