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ADENINE NUCLEOTIDE METABOLISM

IN

HUMAN BLOOD PLATELETS

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree of

MASTER OF SCIENCE in CLINICAL BIOCHEMISTRY

at

MASSEY UNIVERSITY
NEW ZEALAND

BRUCE MATHEW FARNDALE

1985

At present, a skeletal muscle biopsy provides the most specific test for susceptibility to Malignant Hyperthermia (MH). This procedure is unsuitable for large scale screening of individuals and a simpler, less invasive test to distinguish MH-susceptible (MHS) people from those not possessing the genetic defect, is highly desirable.

Platelets contain a calcium-activated contractile system, a calcium-storing and releasing system, and an active ATP-generating system. It is thus logical to assume that the same processes occur in platelets as those in muscle during MH-induced accelerated metabolism. In this research, [8-^{14}C]adenine incorporation into blood platelet adenine nucleotides was investigated with a view to using differences between platelets from normal and MHS individuals as the basis for a clinical test.

It was assumed that under resting and/or halothane-stimulated conditions, nucleotide turnover in MHS platelets is significantly abnormal, and that the turnover abnormality is reflected in differences in adenine incorporation to platelet nucleotides via the salvage pathway.

MHS platelets took up less adenine and assimilated it into nucleotides at a slower rate than normal platelets. However, after two hours, 20% more labelled ATP was extracted from MHS platelets than normal, with a concomittant decrease in ADP levels. Halothane had little effect on normal platelets but caused a 10% decrease in incorporation into ATP in MHS platelets. AMP labelling was lower than normal in MHS platelets, indicating increased deamination of this

nucleotide.

Specific radioactivities of nucleotides were not measured since [^{14}C]adenine distributes evenly among metabolic ATP, ADP, and AMP; therefore, the total radioactivities were used as a measure for the levels of adenine nucleotides within the metabolic pool.

From the limited number of individuals screened, results suggest that MHS platelets have a higher basal ATP turnover rate than normal. When challenged with halothane the adenylate energy charge decreased, causing an increased nucleotide turnover rate which in turn led to a decreased ATP level due to the increased deamination of AMP. The appearance of more hypoxanthine and inosine than normal in the extraplatelet medium is consistent with the above sequence of events.

The platelet-halothane bioassay displays a limited ability to distinguish between normal and MHS individuals and may have the potential to become a less invasive equivalent to the "ATP-depletion test" in muscle.

I would like to thank my supervisor, Dr Bob Greenway, for sharing his time and wisdom with me for the duration of this research, and for tolerating the unavoidable encroachment of extra-university activities on my studies.

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AEC	- Adenylate Energy Charge
ADP	- Adenosine Diphosphate
AMP	- Adenosine Monophosphate
ATP	- Adenosine Triphosphate
BTB	- Bromthymol Blue
CCD	- Citrate-Citric Acid-Dextrose
CPD	- Citrate-Phosphate-Dextrose
CPK	- Creatine Phosphokinase
CPM	- Counts Per Minute
HPLC	- High-Performance Liquid Chromatography
HX	- Hypoxanthine
MH	- Malignant Hyperthermia
MHS	- Malignant Hyperthermia-Susceptible
PCA	- Perchloric Acid
PPO	- 2,5-diphenyloxazole
POPOP	- 1,4-bis-2-(5-phenyloxazolyl)benzene
mol	- mole(s)
mmol	- millimole(s)
M	- Molar
L	- Litre
ml	- millilitre
cm	- centimetre
min	- minute
μl	- microlitre(s)
mCi	- millicuries
^{o}C	- degrees Celcius

CHAPTER 1: INTRODUCTION.

1.1 MALIGNANT HYPERTHERMIA.

1.1.1 General Features.

The spectrum of diseases described by the phrase "inborn errors of metabolism" results from mutations of genes whose expression is non-lethal, but sufficiently non-trivial to show as pathological changes in body chemistry. In some cases, the fundamental molecular changes are well described (as in sickle-cell anaemia), while in others the biochemical lesion underlying the observed pathology is not well known. Included in the second group is a hereditary predisposition to severe complications during anaesthesia, termed either Malignant Hyperthermia or Malignant Hyperpyrexia. The syndrome is characterised by reactions to several kinds of drugs normally used during anaesthesia, and to extreme physical and emotional stress. Such drugs and stresses may trigger an enormous acceleration of muscle catabolism resulting in a metabolic crisis which presents to attending physicians signs including hypercapnoea, metabolic acidosis, muscle rigidity (in 75% of cases (Britt, 1979a)), and a progressive increase in body temperature at the rate of up to 12^{o}C/hour (Gronert, 1980) towards 43-45^{o}C at which death occurs if no treatment is instigated (Ellis, 1984).

The aetiology of Malignant Hyperthermia (MH) is directly related to the presence of a genetically inherited muscle abnormality, and has been intensively researched during the last twenty years.

Although symptoms corresponding to those of MH were reported as early as 1900 little attention appears to have been paid to the syndrome, and as recently as 1940, the prevailing attitude among specialists was that "The condition of hyperthermia during anaesthesia is of rare enough occurence scarcely to cause concern about its prevention" (Burford, 1940). It was not until awareness was raised by a brief report from Canberra by Denborough and Lovell (1960), concerning accelerated metabolism during anaesthesia, that the pathophysiology of MH began to be appreciated by anaesthetists at large. Previously, epidemiologists had been preoccupied with environmental causes of MH (Gronert, 1980).

The mortality rate of MH at the time of the announcement of Denborough and Lovell approached 90% (Aldrete, 1981), and in 1968, despite symptomatic therapy, it was still greater than 70% (Britt and Kalow, 1968). The higher the absolute maximum temperature reached during the crisis, and the longer the duration of the anaesthesia, the greater was the mortality rate (Britt and Kalow, 1970).

As a result of improved knowledge of the clinical and laboratory features of MH reactions, and of the management of the syndrome, the mortality rate had decreased to 64% by 1970 (Britt and Kalow, 1970), and in 1976, it was 28% (Britt, 1979d). However, MH still claimed the lives of up to 75% of those individuals whose syndrome was not treated promptly (Noble, 1980; Aldrete, 1981; Kolb et al., 1982).

Considering the intensity of the fulminant syndrome, and the high mortality rate at the time it was first described, it was appropriate

that the adjective "malignant" was used to describe the acute hyperthermic reaction triggered by drugs or stress.

The first documented case of MH in New Zealand occurred in 1968 when a 29-year-old male patient died under general anaesthesia at Palmerston North Hospital (Newson, 1978). Twelve further cases of MH have been recorded (Table 1.1), including one in which anaesthesia and surgery were aborted following an atypical response to succinylmethonium (SM) in a patient. SM is a commonly used muscle relaxant, but is also a potent MH-triggering agent. Of these 13 patients, seven survived (including the patient in whom anaesthesia was terminated). Four of the victims (including three of the fatalities) were members of a widely scattered, Otaki-based, Maori family.

Towards the end of the second decade of "MH-awareness" (the late 1970s) the first specific therapeutic agent for the clinical syndrome - Dantrolene Sodium - passed through its developmental and experimental stages to become available as a stable intravenous preparation (Hall, 1980). Dantrolene had been available in New Zealand since 1976 in lyophilised form and had been used preoperatively on at least 4 occassions, when survivors of previous MH-episodes, or relatives of individuals known to be MH-susceptible (MHS) presented for elective surgery (Newson, 1978). The introduction of Dantrolene Sodium ("Dantrium" in New Zealand) heralded the commencement of a new era in the treatment of MH as the drug had potential for application in both prophylactic and symptomatic therapy (Hall, 1980).

CASE #	SEX	AGE AT EPISODE	HOSPITAL	YEAR	OUTCOME
* 1	M	29	PALMERSTON NORTH	1968	FATAL
2	M	14	MIDDLEMORE	1970	SURVIVED
3	M	16	MIDDLEMORE	1970	FATAL
4	M	10	MIDDLEMORE	1971	FATAL
5	M	24	WAIKATO	1971	SURVIVED
* 6	F	19	AUCKLAND	1972	FATAL
7	M	14	MIDDLEMORE	1974	SURVIVED
8	M	26	PALMERSTON NORTH	1974	FATAL
9	M	18	HASTINGS	1975	SURVIVED
* 10	M	26	WELLINGTON	1976	FATAL
* 11	M	24	WELLINGTON	1976	SURVIVED
12	M	11	WAIKATO	1976	SURVIVED
13	F	6	AUCKLAND	1976	SURVIVED

TABLE 1.1

Documented cases of Malignant Hyperthermia occurring in New Zealand between 1968 and 1978 (* indicates closely related members of an Otaki-based family).

(From Newson, 1978).

While control of a MH-crisis is now possible, the best treatment remains accurate diagnosis coupled with awareness of the diagnosis, not only by the MHS individual but by all attending medical personnel (Britt, 1979a). At present, preanaesthetic diagnosis of MH-susceptibility depends most heavily on the results of isolated muscle contracture studies.

Muscle from MHS individuals shows increased sensitivity to caffeine and develops contractures in the presence of halothane (Moulds and Denborough, 1974; Anderson et al., 1980). While the number of cases reported in the medical literature has snowballed as awareness of MH has increased, improved preanaesthetic diagnosis should result in a true reduction in the incidence of MH during anaesthesia as techniques for identifying individuals at risk improve and become more widespread.

The frequency of incidence of susceptibility to MH is almost certainly higher than that calculated from the medical literature. Factors contributing to an under-estimation of the true incidence of MH include;

(i) the syndrome does not necessarily manifest itself during the first anaesthetic procedure. This is evidenced by the case of the individual in whom MH occurred during the thirteenth anaesthetic (Aldrete, 1981)

(ii) 50% of the patients in whom the syndrome develops have had prior anaesthesia without recognised MH occurring (Britt and Kalow, 1970; Halsall et al., 1979). Many patients with mild MH-susceptibility experience low, transient fevers during or after anaesthesia (Britt,

1979a).

These complications have led to reports estimating the occurrence of MH to be between 1:15 000 anaesthetic events in children (Britt and Kalow, 1970), and 1:50 000 to 1:150 000 in adults undergoing general anaesthesia (Ellis and Halsall, 1984), to an estimate of 1:200 000 for the general population (Zakarian, 1981). Therefore, insusceptibility to MH cannot be assumed from previous uneventful exposure to the known triggering agents, and all patients suspected of having inherited MH-susceptibility should be screened by *in vitro* muscle biopsy to assess their susceptibility.

With regard to the difference in reported frequency of anaesthetic-induced MH reactions in children and adults and to the details of those reactions that have occurred in New Zealand (Table 1.1), it is evident that MH is particularly prevalent in young males; especially those in adolescence and early adulthood. This observation was first made by Britt and Kalow (1970). The greater incidence of MH in males may be due to the greater muscle mass and strength of the male, or perhaps to some unknown hormonal influence (Britt, 1972). However, since MH has occurred in females, and as direct transmission occurs between father and son, the MH-susceptibility trait is sex-influenced but not X-linked (Britt, 1972). It has also been established that that half the offspring of affected parents are also afflicted (Britt, 1979a); therefore, the mode of inheritance must be autosomal dominant (Ellis, 1983).

Other genetic features of the MH trait include;

(i) reduced penetrance (transmission occurring from an apparently normal parent to his or her offspring), and

(ii) variable expressivity (the extent to which the trait is manifested by carrier individuals in a family).

(Relton et al., 1973; Aldrete, 1981);

MH is a true pharmacogenetic disease (Noble, 1980) as either or both triggering drugs and stresses are necessary for the development of a crisis in a geneticlly predisposed individual. MH does not necessarily manifest itself during an anaesthetic procedure and both the initial attack and reoccurrence have occurred during recovery from general anaesthesia (Mathieu et al.,1979). MH can also occur when the individual is fully conscious ("awake episodes"), for which the trigger is usually stress (Gronert, 1980), but the syndrome is most frequently associated with the administration of halogenated anaesthetic agents (e.g. halothane) and the depolarising muscle relaxant SM (Britt and Kalow, 1970). Table 1.2 is a fairly complete list of factors capable of precipitating an MH reaction.

Once MH has been diagnosed, an MHS-individual will always have to avoid triggering drugs and try to control physical and emotional stresses in order to prevent MH reactions (Britt, 1979a). However, even some of the "safe" anaesthetic agents and muscle relaxants have been incriminated as weak triggers in humans (Gronert,1981; Fitzgibbons, 1981).

MALIGNANT HYPERTHERMIA TRIGGERING AGENTS.

Within the operating theatre:

1. ALL Potent Inhalational Agents

Methoxyflurane
Halothane
Enflurane
Isoflurane
Trichloroethylene
Fluroxene
Diethyl Ether
Ethylene
Cyclopropane

2. MOST Skeletal Muscle Relaxants

Succinylmethonium
Decamethonium
Gallamine
d-Tubocurarine

3. ALL AMIDE Local Anaestheteics.

Lidocaine
Mepivacane
Bupivacane
Prilocaine

Outside the operating theatre:

Extreme emotional excitement
High environmental temperature
Mild infections
Muscle injury and/or infections

Aggravating factors:

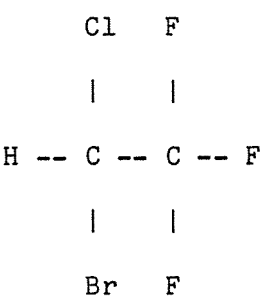
Sympathomimetics
Parasympatholytics
Cardiac Glycosides
Quinidine analogues
Calcium salts
Caffeine and Theophylline
Ethyl Alcohol

TABLE 1.2: Factors capable of precipitating a Malignant Hyperthermia reaction.

(From Britt, 1979a; Noble, 1980).

1.1.2 Halothane and its Action.

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is one of a series of fluorinated hydrocarbons synthesised in the early 1950s, and it possesses the following structure;



The anaesthetic was received enthusiastically by specialists in New Zealand (Simcock, 1959; Climie, 1959) and is probably the most commonly used inhalational anaesthetic in the world at present (Calvey and Williams, 1982). It is a volatile, colourless liquid which is unstable in the presence of light (Hall and Clarke, 1983).

Halothane is administered by means of a calibrated vaporiser; concentrations of 1.5-3.0% are required for induction and 0.5-1.5% for the maintenance of anaesthesia (Calvey and Williams, 1982). The blood:gas partition coefficient of halothane is reasonably small (2.3) so that both induction and recovery are relatively rapid, and the level of anaesthesia is easily controlled (Calvey and Williams, 1982).

The main site of action of both local and general anaesthetic is known to be the nerve synapse but conflicting hypotheses exist regarding the nature of the primary binding site for general

anaesthetics (Roth, 1980; Lechleiter and Gruener, 1984). It is generally accepted, however, that a direct or indirect interaction with Ca^{++}-dependent secretory or permeability mechanisms plays an important part in their action (Campbell, 1983).

Due to the difficulty of reproducing physiological conditions *in vitro*, investigations into the effect of halothane on membrane fluidity and permeability have caused much speculation and debate. A thorough study by Mastrangelo *et al.* (1978), which avoided many of the pitfalls of previous investigators, suggested that clinical concentrations of halothane increased the internal fluidity of lipid bilayers. However, this probably does not result in a non-specific increase in membrane permeability (Herbette *et al.*, 1982). In a separate study (Heffron and Gronert, 1979), the inhibitory effects of halothane on calcium sequestration were reduced by Ca^{++} and Adenosine Triphosphate (ATP). Therefore, halothane might interact with the ATP-binding site of the calcium-transport protein.

In several cases, halothane-induced MH was found to be associated with adenylate kinase deficiency (Sachsenheimer *et al.*,1977). They suggest that halothane binds only to the niche which has been identified as the binding site for the adenine moiety of Adenosine Monophosphate (AMP) on adenylate kinase. Inhibitory effects of halothane on adenylate kinase activity during anaesthesia could result in a degree of deregulation of the ratio of [ATP]:[ADP]:[AMP], which occurs before the observed sequence of metabolic derangements in MH (ADP = Adenosine Diphosphate).

The two proteins discussed above (calcium-dependent ATPase and adenylate kinase) and all other proteins for which specific interactions with halothane have been demonstrated possess functionally important binding sites for purine nucleotides (Sachsenheimer et al., 1977).

1.1.3 Biochemical Defect of Malignant Hyperthermia.

Confused by the heterogeneity of the triggering agents and confounded by the unpredictable occurrence of the syndrome, the exact cause which predisposes to the occurrence of MH remains elusive. This unpredictability has prevented the systematic collection of physiological and biochemical data (Hall et al., 1980), and the study of survivors and their relatives is limited to the analysis of blood samples and muscle biopsies. Therefore, Heffron (1984) contends that documentation of the 600 cases that had occurred world-wide by 1979 has contributed little to the elucidation of the etiology of the human MH syndrome. This file is held at the University of Toronto by Dr. Britt and is growing by about 70 cases each year (Winstanley, 1979).

Animal models of human disease have historically been crucial to our understanding of the mechanisms of many pathological processes. Fortunateley, such a model exists for MH. Anaesthetic-induced MH in stress-susceptible pigs was described by Hall et al. (1966) who noted the development of fatal hyperthermia with muscle rigidity following the administration of SM to several pigs. Subsequent extensive investigations using this model have provided important information

concerning the etiology of the syndrome (Gronert, 1980).

Most experimental findings suggest that the regulation of intracellular free calcium in skeletal muscle is defective (Heffron, 1984). Thus, it was logical to examine the properties of the cellular organelles known to be responsible for regulating the intracellular free calcium concentration; the sarcoplasmic reticulum (SR), mitochondria, and sarcolemma.

The observations that increased free calcium concentrations in muscle can increase oxygen consumption and that higher concentrations can induce contracture, have suggested that the MH syndrome can be initiated by a sustained increase in myoplasmic free calcium in the muscles of susceptible individuals (Aldrete, 1981). Data suggesting that calcium depletion blocks the halothane-induced abnormal contracture of muscle that occurs in <u>in vitro</u> MH supports this theory (Gronert, 1980). There have been several theories proposed to explain why calcium builds up in the myoplasm;

(i) altered accumulation of calcium in the SR

(ii) defective accumulation of calcium in the mitochondria

(iii) fragile sarcolemma with passive diffusion of calcium into the myoplasm

(iv) exaggeration of catecholamine release with multiple indirect effects on myoplasmic calcium uptake

(v) decreased sensitivity of the myofibrillar ATPase to calcium

(vi) adenylate kinase deficiency

(Aldrete, 1981).

The acceptance of one or more theories regarding the nature of the primary biochemical defect of MH-susceptibility does not necessarily exclude another theory from being correct, as MH-susceptibility may be a widespread membrane disease involving not only membranes of different cell types, but also different membranes within a given cell type (Britt, 1972).

1.1.4 Biochemical Events of Malignant Hyperthermia.

Small increases in the level of calcium in the myoplasm activate phosphorylase kinase (Ellis, 1983). Catecholamines activate adenylate cyclase, which in turn converts ATP to cyclic AMP, which stimulates the activity of phosphorylase kinase. The lactate produced by the subsequent breakdown of glycogen under the anaerobic conditions prevailing during a MH-crisis may be transported to the liver where it is converted to carbon dioxide and water, or to glucose. The increase in temperature of the livers of MHS pigs in the early stages of MH probably results from the heat production associated with glycogen metabolism.

Carbon dioxide and water are also produced from pyruvate transported into the mitochondria with the net result of oxygen consumption and further heat production (Britt, 1972).

The acceleration of anaerobic glycolysis and oxidative phosphorylation would both stimulate ATP production in the early stages of MH. A contribution to energy production is also made by the conversion of creatine phosphate and ADP to creatine and ATP (Britt, 1972).

Somewhat higher levels of calcium activate myosin ATPase which hydrolyses ATP to ADP in an exothermic reaction (Aldrete, 1981). Under normal conditions, the myoplasmic calcium returns to its usual level, but during a MH-crisis the sarcoplasmic reticulum is unable to resequestrate the calcium and muscle contraction is sustained (Britt, 1972). The anaesthetist may experience some difficulty in intubating the trachea due to muscle contraction after administration of SM, and this should warn him or her of an impending MH-crisis (Ellis and Halsall, 1984).

As myoplasmic calcium continues to rise, some of the excess gains entry into the mitochondria (Britt, 1972). The increased calcium ion concentration uncouples oxidative phosphorylation from electron transport, thereby decreasing ATP production and accelerating oxygen consumption and output of lactic acid, carbon dioxide, and heat (Aldrete, 1981).

The net effect of the above events is an increase in muscle

temperature as well as a decline in muscle ATP and creatine phosphate.

A fatal outcome of an MH-crisis becomes inevitable when ATP levels are too low to permit muscle relaxation. Failure of the sarcolemmal ATPases allows molecules to follow their natural concentration gradients, and consequently, potassium, magnesium, phosphate, enzymes, and myoglobin leak out. Simultaneously, calcium flows in and exacerbates an already calcium-dependent biochemical derangement (Aldrete, 1981).

1.2 PLATELETS.

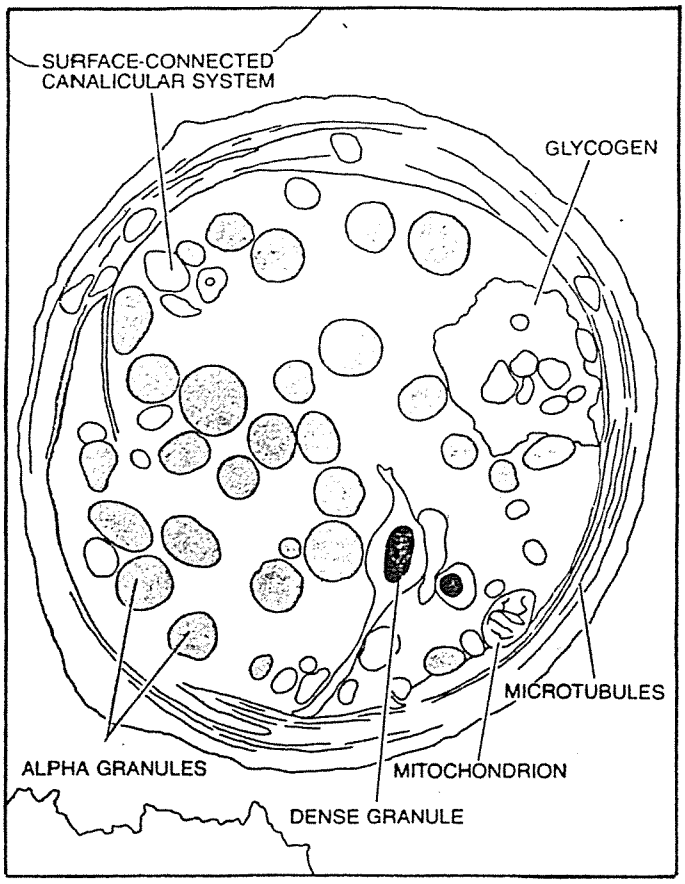
While the presence and some of the functions of the red and white cells was recognised in the early days of microscopy, the very existence of platelets was not firmly established until near the end of the nineteenth century (Zucker, 1980). It was soon clear that they were involved in haemostasis and blood clotting, but their fundamental role in these processes only came to be understood in the late 1950s. The general functional properties of platelets are now known to include adhesion, aggregation, procoagulant activity, clot consolidation, and 5-Hydroxytryptamine (serotonin) uptake (Frojmovic and Milton, 1982). Platelets, therefore, play their central role in haemostasis and thrombosis by orchestrating a complex set of events associated with the maintenance of blood vessel integrity.

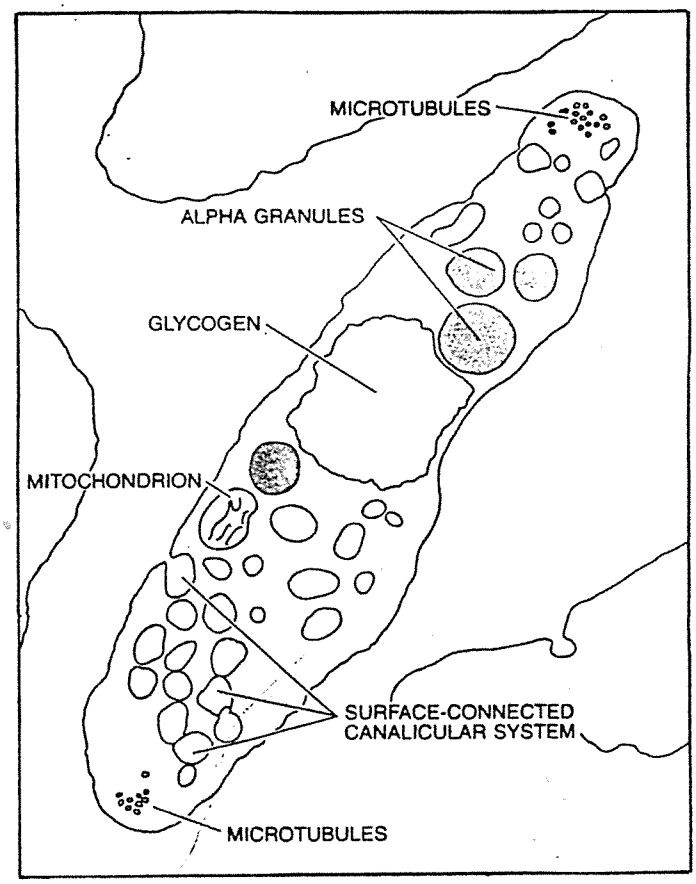
Platelets also play a part in inflammation, immunological reactions, and interactions with tumour cells, and have been proposed to serve as readily available models for neurosecretory cells (Frojmovic and Milton, 1982). They are also a factor in several pathological states e.g. transient ischaemic attack, stroke, vascular occlusive disorders of hypertension, and diabetes (Longenecker, 1980), and have been implicated in the pathogenesis of migraine attacks (Hannington et al., 1981).

Figure 1.1 shows two sections, in different planes, of a single platelet and indicates some of its major structural features. Platelets are merely cytoplasmic fragments as they are formed and released into the circulation from megakaryocytes (Triplett, 1982).

FIGURE 1.1

Two sections, in different planes, of a resting platelet. (From Zucker, 1980).


SURFACE-CONNECTED
CANALICULAR SYSTEM
GLYCOGEN
MICROTUBULES
ALPHA GRANULES
MITOCHONDRION
DENSE GRANULE


MICROTUBULES
ALPHA GRANULES
GLYCOGEN
MITOCHONDRION
SURFACE-CONNECTED
CANALICULAR SYSTEM
MICROTUBULES

Therefore, a platelet has no nucleus, and the endoplasmic reticulum and golgi complex are virtually absent.

Two types of granules have been identified within the cytoplasm of platelets. The majority (alpha-granules) are of moderate electron density and contain enzymes such as acid hydrolases that are characteristically associated with lysosomes. There are approximately 20-200 alpha-granules per platelet. Storage sites for ADP, ATP, serotonin, and calcium are found within dense granules - the second type of platelet granule. They are more electron-dense than the alpha-granules and number between two and ten per platelet (Triplett, 1982). Selective processes that release the contents of the granules have been extensively studied (Holmsen and Weiss, 1979).

The platelet membrane is invaginated at many points to form a network of tortuous channels that "burrow" through the platelet cytoplasm. This system of channels, which is called the "surface-connected canalicular system" (SCCS), enlarges the total surface-area of the platelet membrane and serves as a means for the uptake of substances and for the rapid extrusion of granule-bound secretory products during the release-reaction of the platelet.

Intimately associated with the SCCS is another system of tubules called the "dense-tubular system" (not shown in Figure 1.1). It has been proposed that the latter system may be derived from endoplasmic reticulum, and the relationship between the dense-tubular system and the SCCS in platelets may be similar to that of the sarcoplasmic reticulum and transverse tubule system in skeletal muscle (Triplett,

1982).

Platelets also contain a microtubular system, which is a circumferential band of hollow tubular structures similar to the microtubules found in many cells. The function of the microtubules in the platelet awaits definition but their primary role is probably to maintain a cytoskeletal structure for the platelet (Triplett, 1982).

Other structures within the platelet cytoplasm include mitochondria and glycogen granules. The latter are similar to those in muscle cells and indicate a large energy potential. In some instances inclusions of lipid droplets, bacteria, viruses, and carbon particles, have been identified in the ultrastructure of the platelet, indicating that platelets are phagocytic and may aid in the removal of foreign particles in vivo.

Platelet activation is a series of morphological and functional changes, and appears to be initiated by the "stimulus-induced translocation of intracellular calcium stores" (Feinstein, 1978). Calcium functions as the primary intracellular messenger or transmitter of information from the surface membrane, where stimulating agents react with their receptors, to the internal contractile apparatus (Ardlie, 1982).

The release of internal calcium sets in motion a complex integrated set of responses whereby pseudopod formation and centralisation of platelet granules due to the shortening of contractile proteins accompany the secretion of serotonin, ATP, ADP,

calcium, and the various metabolites of arachidonic acid (Frojmovic and Milton, 1982; Vargaftig et al., 1981). The primary stimulus for platelet activation appears to involve membrane glycoproteins, and receptors have been partially identified for the most extensively studied platelet-stimulating agent, thrombin (Vargaftig et al., 1981).

The role of platelets in haemostasis is illustrated in Figure 1.2. Contact of a platelet with collagen in the presence of von Willebrands factor initiates a pathway that stimulates the secretion of ADP from the dense-granules. The adhering platelet changes shape, spreads out along the collagen, and degranulates. Meanwhile, a number of steps involving tissue factor, calcium ions and other clotting factors convert prothrombin in the plasma into thrombin. Thrombin also stimulates secretion, and converts fibrinogen from both plasma and platelets into fibrin. Under the influence of collagen, ADP and thrombin, platelets aggregate and strands of fibrin reinforce the plug. The process may either stop at this stage or go on to form a larger thrombus with trapped red blood cells (Zucker, 1980).

FIGURE 1.2

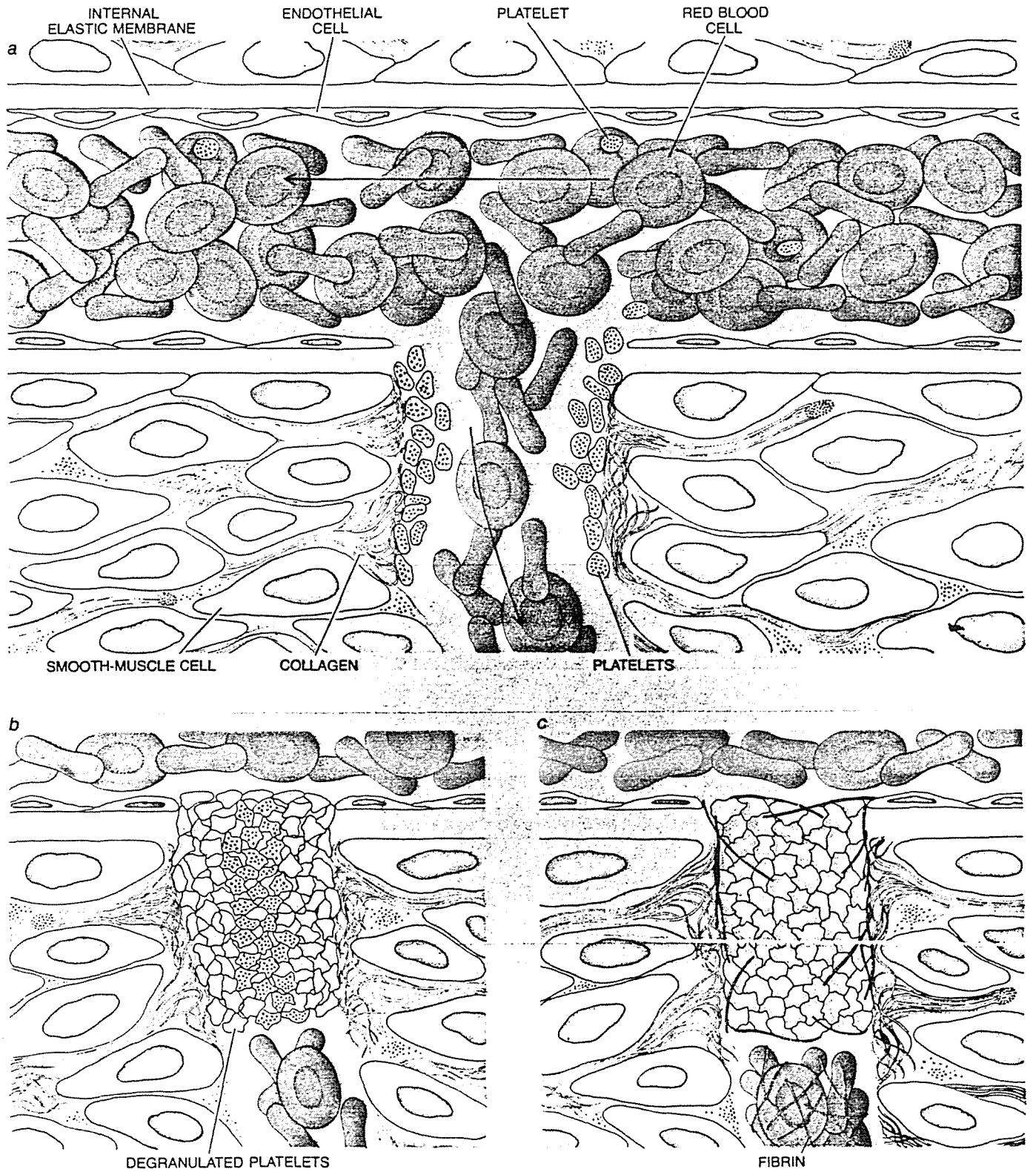
Role of platelets in haemostasis.

(a) As blood begins to flow through a cut in the vessel wall, platelets adhere to collagen in the wall.

(b) Platelets are thereby stimulated to secrete the contents of their granules, including ADP, and other passing platelets adhere to the first layer.

(c) Fibrin strands are formed, which reinforce the platelet plug.

(From Zucker, 1980).


a
INTERNAL ELASTIC MEMBRANE
ENDOTHELIAL CELL
PLATELET
RED BLOOD CELL
SMOOTH-MUSCLE CELL
COLLAGEN
PLATELETS
b
DEGRANULATED PLATELETS
c
FIBRIN

1.3 PLATELET ADENINE NUCLEOTIDES.

1.3.1 Introduction.

Fantl and Ward (1956) presented the first detailed analysis of the nucleotides of blood platelets and were astounded at the preponderance of adenine over the other bases. They were at a loss to explain the significance of this observation but noted that "The presence of ATP and ADP suggests a 'connexion' with energy metabolism." Having said this, however, Fantl and Ward were clearly worried by the earlier observation that platelets had a very low oxygen consumption (1/100 that of leucocytes). Nor could they confidently assign a major role to platelet adenine nucleotides in blood clotting and the subsequent contraction of the fibrin clot; "Their ATP content is far too low to be of significance in these processes." Fantl and Ward rather despairingly concluded their paper with the remark that "Perhaps the release of platelets from the megakaryocytes is a process which requires the energy liberated from ATP." An important contribution by Fantl and Ward, however, was to reiterate an earlier suggestion that aspects of platelet function can be compared with muscle contraction - a comparison that has been the object of the attention of many investigators during the past 30 years.

Platelets contain higher levels of nucleotides than any other mammalian cell type (Ugurbil and Holmsen, 1979). The proportions of the total platelet acid-soluble nucleosides, mono-, di-, and triphosphates is shown in Table 1.3.

	ADENOSINE	GUANOSINE	URIDINE	CYTIDINE
MONOPHOSPHATE	0.704	0.299	0.118	0.063
DIPHOSPHATE	4.420	0.747	0.187	0.169
TRIPHOSPHATE	5.479	0.697	0.436	0.112

TABLE 1.3

Distribution of nucleoside mon-, di- and triphosphates in human platelets.

The values represent μmol/10^{11} platelets.

With the exception of the relatively high ADP levels observed in human platelets, the nucleotide composition in platelets from other species is very similar.

The explosion of information relevant to platelet metabolism in the mid- to late- 1960s stemmed from a requirement for the demonstration of as many acid-soluble organophosphates as possible in small amounts of platelets in connection with work on the effect of collagen on platelets *in vitro* (Holmsen, 1965b). Earlier chemical methods of nucleotide analysis (Fantl and Ward, 1956) required several litres of blood, while the isotope methods developed at that time lacked resolving power. Therefore, Holmsen (1965a) developed a two-dimensional paper chromatographic method for the separation and identification of radioactive organophosphates from platelets labelled with ^{32}P *in vitro.*

As a result of his pioneering work virtually all of our present knowledge of platelet adenine nucleotide metabolism was contributed by Holmsen or by others inspired by his series of publications that began in the mid-1960s.

1.3.2 Adenine Nucleotide Formation in Blood Platelets.

Incubation of PRP with ^{32}P-orthophosphate failed to label platelet AMP, indicating very low, if any, *de novo* synthesis of adenine nucleotides in platelets (Holmsen, 1965a). This observation was supported by evidence that glycine was not incorporated into adenine or any other nucleotides (Holmsen and Rozenberg, 1968a).

Holmsen and Day (1971) reported that labelled adenine and adenosine were used in the synthesis of platelet adenine nucleotides, although it had previously been established that a small proportion of the radioactivity from [8-^{14}C]inosine could be found in adenine nucleotides after incubation with washed human platelets (Ireland and Mills, 1966).

The salvage of these preformed purine compounds can occur by either of two general mechanisms;

(i) Phosphoribosylation of the free purine bases by specific enzymes requiring phosphoribosyl pyrophosphate (PRPP) as the ribose-phosphate donor

(ii) Phosphorylation of purine nucleosides at their 5'-hydroxyl groups.

In human platelets, the first mechanism is quantitatively more important, and the reactions catalysed by the the two enzymes that perform this task are depicted in Figure 1.3. The existence of platelet adenosine-phosphoribosyl transferase was recognised by Holmsen and Rozenberg in 1968, whereas the presence of hypoxanthine-guanosine-phosphoribosyl transferase was not confirmed until 1974 when Rivard's group reported that 0.6% of the adenine and guanine nucleotides produced in platelets per hour were from hypoxanthine (HX). This corresponds to the proportion of adenine nucleotides catabolised to HX per hour in [8-^{14}C]adenine labelled PRP (Rivard et al., 1974).

FIGURE 1.3

Major pathways for the synthesis of purine nucleotides in platelets.

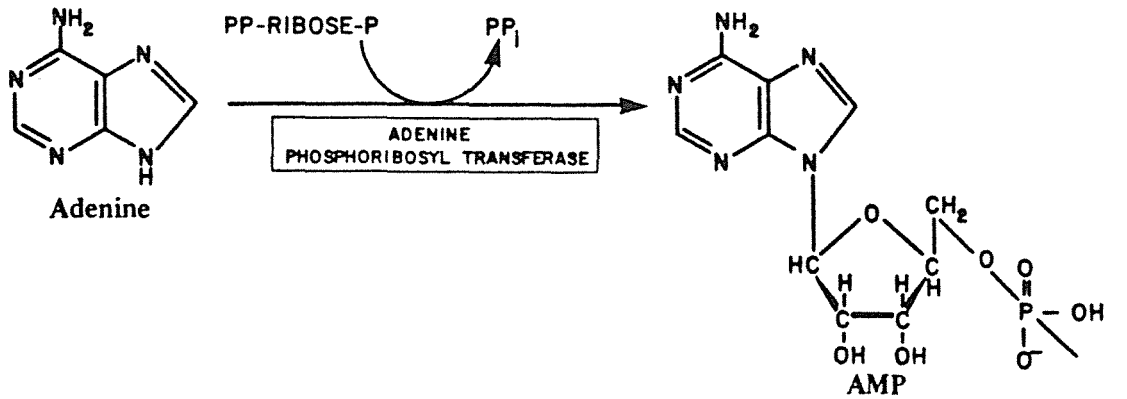
(i) Phosphoribosylation of adenine by adenine phoshoribosyl transferase, to form AMP

(ii) Phosphoribosylation of hypoxanthine by hypoxanthine-guanine phosphoribosyl transferase, to form IMP

(iii) Phospophorylation of adenosine to AMP by adenosine kinase

(From Martin, 1977).

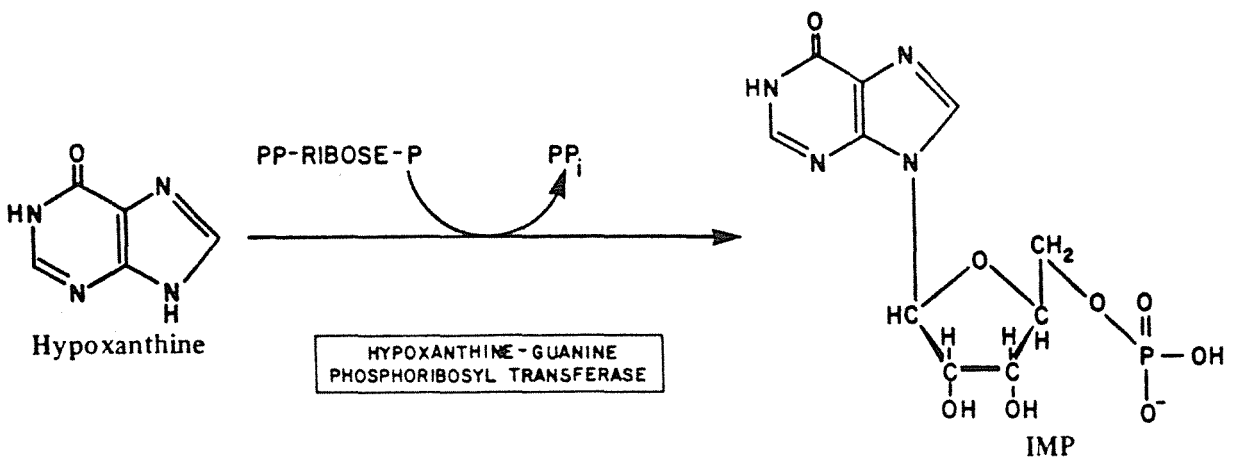
(i)



Adenine

AMP

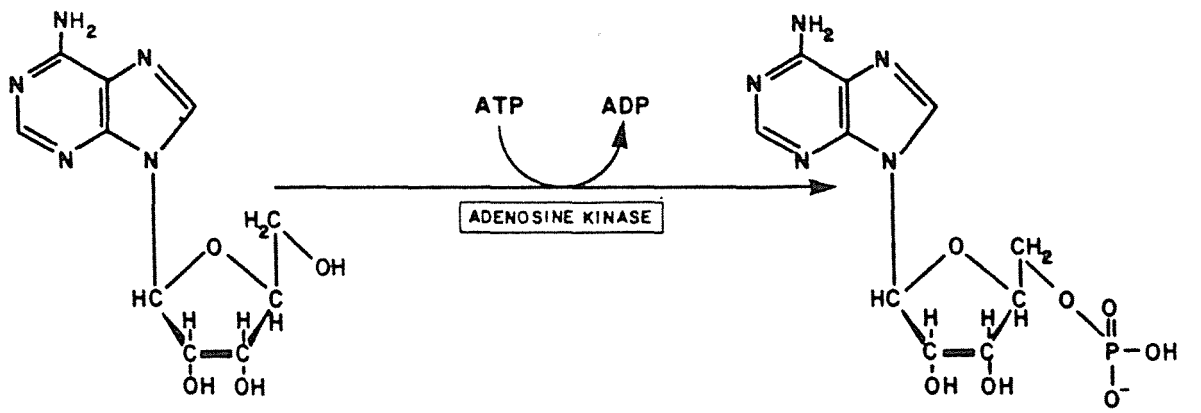
(ii)



Hypoxanthine

IMP

(iii)



The salvage of purine nucleosides to purine nucleotides in platelets is carried out by adenosine kinase (Figure 1.3), although inosine can be used to a small extent (Ireland and Mills, 1966).

The reactions leading to the formation of AMP in human platelets are given in Figure 1.4.

Interestingly, in the human organism as a whole, the consumption of PRPP by the salvage pathways is greater than the consumption of PRPP for the de novo synthesis of purine nucleotides (Martin, 1977).

The isotopic labelling techniques that facilitated the elucidation of the synthetic pathways for adenine nucleotides in human platelets have been equally useful in the investigation of the metabolism of adenine nucleotides. Figure 1.5 illustrates the isotopic labelling of adenine nucleotides in platelets and the theoretical movements of radioactive label. Platelets have high adeylate kinase activity, so labelled AMP resulting from the condensation of adenine with PRPP (or the phosphorylation of adenosine with platelet ATP) is rapidly distributed among the metabolically active platelet ADP and ATP pools (Holmsen and Day, 1971).

(i) ADENOSINE + ATP -----> AMP + ADP

(Adenosine Kinase)

(ii) (a) ADENOSINE + P_i -----> ADENINE + RIBOSE-1-PHOSPHATE

(Purine Nucleoside Phosphorylase)

(b) RIBOSE-1-PHOSPHATE -----> RIBOSE-5-PHOSPHATE

(c) RIBOSE-5-PHOSPHATE + ATP ---> 5-PHOSPHO-RIBOSE-PYROPHOSPHATE + AMP

(Ribose Phosphate Pyrophosphokinase)

(d) ADENINE + 5-PHOSPHO-RIBOSE-PYROPHOSPHATE -----> AMP + PYROPHOSPHATE

(Adenine Phosphoribosyl Transferase)

FIGURE 1.4

The major pathways of AMP synthesis from adenosine in platelets.

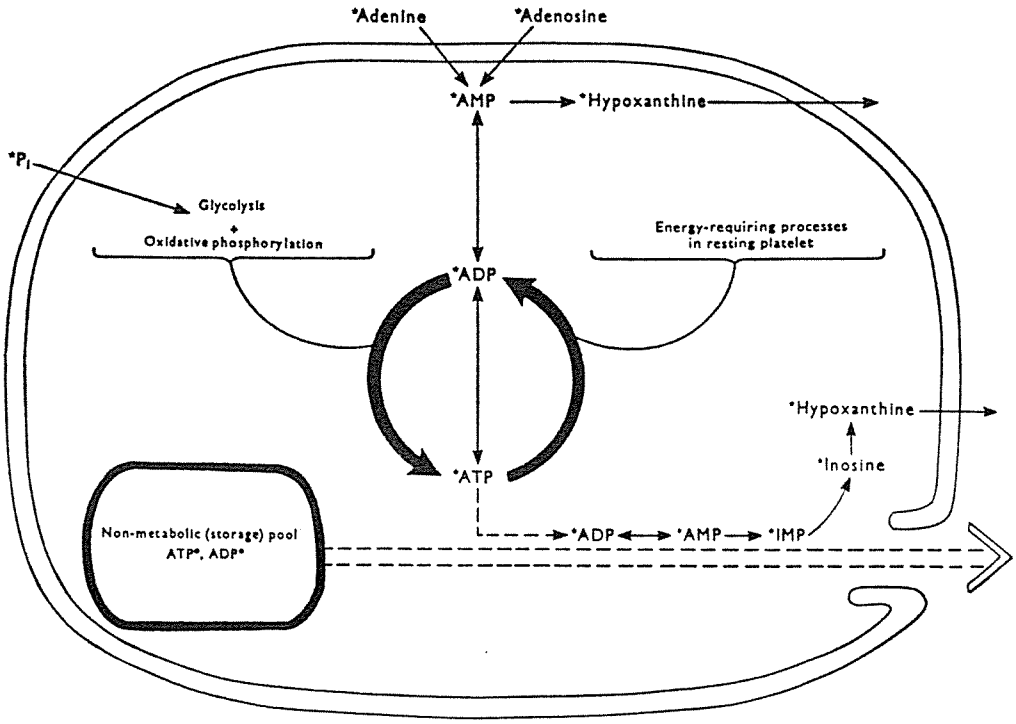


FIGURE 1.5

Schematic representation of adenine nucleotide metabolism in resting and release-stimulated platelets.

Radioactive precursors (*P_i, *adenine, and *adenosine) can enter the platelet and label the metabolic pool of platelet nucleotides; all members of this pool are designated by *. The fairly good balance between ATP-producing and ATP-utilising processes keeps the concentrations of metabolic ATP, ADP, and AMP in PRP reasonably constant during the course of an experiment. However, a small amount of the metabolic nucleotides in the resting platelets is catabolised to HX, which diffuses into the medium (upper right). In human platelets about two-thirds of the adenine nucleotides from the non-metabolic pool (^{o}), are stored in granules and do not participate in metabolism. Upon release-stimulation by thrombin and collagen this pool is extruded through channels to the surrounding medium (broken, double-lined arrow). Concomitant with this extrusion a certain amount of metabolic ATP is rapidly degraded to HX, which diffuses out of the platelet.

(From Akkerman et al., 1983).

1.3.3 Adenine Nucleotide Pools in Blood Platelets.

A full understanding of the role of purine nucleotides in cells is complicated by the presence of inter- and intracellular nucleotide compartments. Three main types of nucleotide compartment have been described (Daniel et al., 1980);

(i) contains nucleotides that are tightly bound to proteins (e.g. actin, transport ATPases)

(ii) involves the partition of nucleotides between the cytoplasm and intracellular organelles

(iii) is found between cells in organised tissues.

Holmsen (1967) found that ATP and ADP released from platelets that had been incubated with ^{32}P in vitro, had a markedly lower specific radioactivity than the nucleotides remaining in the platelets. This result suggested that platelets have different pools of adenine nucleotides, and can be assigned to the second type of compartmental arrangement described above.

Subsequent investigations revealed at least two such pools (Figure 1.5). One nucleotide pool participates in cellular metabolism, (as revealed by its ability to be isotopically labelled), and is retained by the platelets during the release reaction. Another pool, which is released, does not participate in metabolism since it is not isotopically labelled. The metabolically active pool ("cytoplasmic

pool") is present in platelet cytosol, mitochondria, and membranes and contains about two-thirds of the total adenine nucleotides of human platelets. This pool can be subdivided into 2 functionally different pools;

(i) the "basic metabolic pool", which participates in glycoloysis and oxidative phosphorylation and supplies ATP for contraction, membrane pumps, and biosynthesis

(ii) the "release energy pool", consisting of ATP which breaks down intracellularly during the release reaction (Holmsen, Day, and Storm, 1969). It shows an apparently different time-course of isotope incorporation from the nucleotides remaining in the platelets (the basic metabolic pool) in that it is slowly, but strongly labelled (Holmsen and Rozenberg, 1965b; Holmsen, 1967).

The remaining one-third of the adenine nucleotides belong to the metabolically inactive pool and are stored together with serotonin in specific, dense granules ("granular pool"). Equilibrium between the cytoplasmic and granular pools is complete by 18 hours (Reimers et al., 1975).

The investigation of metabolic processes in platelets often requires the incorporation of radioactive isotopes into adenine nucleotides. Therefore, platelets must be extracted to facilitate the determination of the pattern of incorporation of radioactive label into the separated components.

When resting platelets containing radiolabelled cytoplasmic nucleotides and unlabelled granule nucleotides are extracted, the nucleotides from the two pools mix. Consequently, as noted above, isotope dilution occurs and the specific radioactivity of the nucleotides in the extract is lower than that of cytoplasmic nucleotides. The specific radioactivity of ATP in the extract is three times higher than that of ADP (since cytoplasmic ATP constitutes about 80% of total platelet ATP, while cytoplasmic ADP constitutes only 50% of total platelet ADP). Thus, isotope dilution by the unlabelled granular adenine nucleotides is greater for ADP than for ATP.

1.3.4 Metabolism of Platelet Cytoplasmic Nucleotides.

Most of the normal functions of blood platelets, e.g. aggregation, adhesion, clot retraction, serotonin uptake and storage, the release reaction and synthetic and transport functions, depend on the presence of intact glycolytic or respiratory processes for their operation (Solomons and Handrich, 1975; Akkerman et al., 1978). This implies a direct or indirect dependence on intracellular ATP availability.

In performing its function of supplying energy to the platelet, ATP is degraded by adenyate kinase to ADP and AMP, from which ATP can be regenerated by glycolysis and oxidative phosphorylation (Holmsen et al., 1969a). AMP is also further degraded to hypoxanthine (HX) (Figure 1.5).

In resting platelets adenylate kinase appears to work

unidirectionally, since AMP deaminase removes AMP from the equilibrium (Holmsen and Day, 1971). Holmsen and Rozenberg (1968b) were first to show the presence of AMP-deaminase in platelets and suggested a possible route of ATP-HX conversion in which the two energy-rich phosphoryl groups of ATP are consumed during an energy-requiring reaction, with the participation of an ATPase and adenylate kinase. The end product (AMP) is then deaminated to Inosine Monophosphate (IMP) which is dephosphorylated by the membrane bound 5'-nucleotidase (Day et al., 1969). The inosine formed is rapidly phosphorylated to HX and Ribose-1-Phosphate; the HX probably leaves the platelet by simple diffusion whereas the ribose phosphate probably remains in the platelet. Shielding of AMP-deaminase from the adenine nucleotides by localisation in subcellular structures has been proposed (Holmsen and Rozenberg, 1968b), but the extreme lability of this enzyme has prevented determination of its exact location (Holmsen et al., 1969b).

The ATP-HX conversion rate is limited through the deamination of AMP to IMP and NH_3 by AMP deaminase (Ashby and Holmsen, 1983a and 1983b). However, both "release energy ATP" and ATP having a function intracellularly during "special energy requiring processes", are converted to IMP with a time-course indicating a close relationship between ATP metabolism and the ATP requirement of the cell (Holmsen and Day, 1971). The products - IMP, inosine, and HX - cannot be utilised for ATP resynthesis; thus, the ATP-IMP conversion represents a net loss of metabolically active ATP (Solomons and Handrich, 1975). The depletion of adenine nucleotides is balanced by the formation of HX Ugurbil and Holmsen, 1979).

The irreversible deamination of AMP to IMP and the subsequent degradation of IMP to HX, causes a shift in nucleotide composition within platelets (Solomons and Handrich, 1975), and the profile can therefore be used as an indicator of metabolic disturbances during altered metabolism.

1.3.5 Protein-Bound Nucleotides in Blood Platelets.

The method employed in the extraction of platelets has a large effect on the cytoplasmic nucleotides determined. In extracts prepared by perchloric acid, the [^{14}C]ATP/[^{14}C]ADP ratios are distinctly lower than those obtained from ethanol extracts (Ugurbil and Holmsen, 1979). The difference between the two extraction methods results from the failure of ethanol to extract 30-40% of the isotopically labelled ADP (Holmsen, 1972), which is equivalent to 5-25% of the total platelet ADP (Holmsen and Day, 1971). Therefore, referring to the cytoplasmic pool as the metabolic pool is not strictly correct as part of the ADP which can be isotopically labelled is protein-bound and is not accessible to metabolic enzymes.

The most likely candidate for the ADP-binding protein is F-actin, which can be precipitated by ethanol (Daniel et al., 1979). Intact, biologically active F-actin (polymerised F-actin) contains one molecule of ADP bound per actin monomer and this ADP can be extracted from F-actin by perchloric acid (Ugurbil and Holmsen, 1979). The amount of ethanol-insoluble ADP is roughly equivalent to half the total amount of actin monomers in the platelets (Ugurbil and Holmsen, 1979).

Myosin, ATP-utilising enzymes, and other proteins which are known to bind ADP, are present in much smaller amounts than the ethanol-insoluble ADP complex.

1.3.6 Adenylate Energy Charge.

The ratio nominated by Atkinson (1968) to be the primary regulator of important metabolic sequences was termed the Adenylate Energy Charge (AEC);

$$\frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]}$$

Purich (1977) walks the line between opponents and supporters of the AEC when he states that the ratio may be one of many indices of energy status, but it may not necessarily be the central regulator. He questions the justification for isolating the adenylate control from other substrate/product/effect interactions. Indeed Holian et al. (1977) found that control of respiration in isolated mitochondria depended upon $[ATP]/[ADP][P_i]$ and not $[ATP]/[ADP]$, illustrating that the energy charge expression (through omisson of P_i) may not account for regulation of the primary rate of ATP resynthesis in aerobic cells. Shargool (1977), however, was sympathetic to the idea of the energy charge hypothesis being a simple preliminary explanation of how certain enzymes react to the presence of certain important metabolites.

A metabolic response which also provided a basis for the impression that the AEC was important in metabolic control was reported by Dietzler et al. (1974). Not only can the high value of the energy

charge be maintained during haemostasis, but also during metabolic stress. They demonstrated that in response to metabolic stress, a cell can lose up to 67% of the normal complement of adenine nucleotides while totally maintaining the initial high value of the AEC.

Similarly, Holmsen and Robkin (1977) found that platelets were able to function normally *in vitro* under conditions of hydrogen peroxide-induced lowering of ATP levels, provided that the AEC did not fall by more than 5%; at a greater AEC reduction platelet functions were inhibited. These results indicated that cell functions depend more on the AEC than on the ATP concentration.

This supported the earlier proposal by Mills (1973) that a sudden large increase in the rate of energy utilisation by a cell would be expected to cause a reduction of the AEC. Small changes in AEC correspond to large alterations in the metabolic activity of the cell, i.e. increased ATP turnover.

1.4 PREANAESTHETIC DIAGNOSIS OF MALIGNANT HYPERTHERMIA.

1.4.1 Introduction.

Identification prior to anaesthesia of individuals carrying the MH-susceptibility trait is of major therapeutic importance, since this step alone can reduce mortality from MH reactions to nearly zero (Britt, 1979a). However, no single test that is sufficiently inexpensive and noninvasive has yet been devised to permit application to the general population. A small battery of physiological, biochemical, and pharmacalogical tests is curently available to the clinician to assist in the diagnosis of MH-susceptibility.

1.4.2 Non-invasive Studies.

Musculo-skeletal

Most MHS individuals and their relatives lead active, normal lives, and their susceptibility is detectable only by specific testing. However, MHS individuals tend to be emotionally tense and hyperactive (Britt, 1979a) and most have obvious musculoskeletal abnormalities (Gronert, 1980).

The latter are present in 67% of all MHS patients and in about 36% of their first degree relatives (Britt, 1979a). A physical examination of the MHS may reveal some of the following typical characteristics;

short, stocky stature

crossed eyes (squint or strabismus)

droopy eyelid (ptosis)

sway back (lumbar lordosis)

curvature of the spine (kyphoscoliosis)

club foot

various hernias

double joints (joint hypermobility)

easy dislocation of the joints

skeletal muscle cramps

difficulty with fine motor coordination (e.g. dyslexia)

(Britt, 1979a).

The last characteristic occurs despite the fact that muscle bulk and strength are excessive (Britt, 1979b). At the onset of exercise, muscles tend to be strong but with sustained exercise the muscles fatigue more rapidly than normal.

More than one-third of confirmed MHS patients have abnormal ECGs and about one-half have myocardial perfusion defects (Britt, 1979b).

Blood.

High serum creatine phosophokinase (CPK) concentrations in MHS patients and their relatives were first described by Denborough et al. (1970). Somewhat conflicting CPK studies of MHS families have since been reported by other workers in attempts to use CPK as a marker of MH (Britt, 1979b). False lows occurred if the sample was exposed to light or if it was not properly frozen in dry ice or liquid nitrogen. Normal

subjects may have had elevated serum CPK levels if they have exercised within a week or two of sampling. Excessive tourniquet pressure, forcible suction of blood through the needle, or squirting of blood against the wall of the syringe also lead to results that were higher than expected.

Many other muscle damaging conditions elevate the serum CPK e.g. a recent myocardial infarction, muscular dystrophy and other myopathies, paranoid schizophrenia, various neurological disorders, hypothyroidism, acute and chronic alcoholism, a recent injection, or muscle trauma.

In spite of the problems encountered in its application to the diagnosis of MH, elevation of serum CPK occurs in nearly 80% of MHS-individuals and in some of their relatives. In the remaining families, the CPKs are elevated in some individuals but the elevation tends to be small and not always reproducible. CPK measurement cannot, therefore, be used as the sole diagnostic criterion. There is, however, a significant positive correlation in members of MHS families between a high serum CPK and clinical muscle abnormalities (Britt, 1979b). Thus, the combined presence of both a high serum CPK and a muscle abnormality in the same individual is a much better marker of the MH trait than is the presence of either alone.

In normal people, little relationship between age and serum CPK is observed, whereas in MHS patients values tend to be higher in early and middle adulthood than in childhood or old age.

1.4.3 Invasive Studies.

The studies described thus far, while useful in screening MHS families in order to identify branches most likely to be affected, are not sufficiently accurate to be pathognomonic in any one individual. For a definitive diagnosis, a skeletal muscle biopsy is needed.

Electrical and mechanical properties of human skeletal muscle are difficult to examine because of the unavailability of viable, intact (tendon to tendon) fibre preparations. Fragment biopsies of human muscle provide a feasible alternative, and their contracture responses were proposed by Kalow et al. (1970) as a means of differentiating between normal individuals and those susceptible to MH.

At the Second International Workshop on MH (Rosenberg, 1980a) diagnostic testing for MH using strips of muscle obtained at biopsy, was discussed at great length. It was clear that each laboratory had its own established controls and criteria for diagnosing MH since agreement among laboratories on the best diagnostic test was often lacking. The tests used to differentiate normals from MHS individuals are;

(i) Contracture to caffeine alone

Caffeine accelerates the release of calcium into the myoplasm causing contraction of muscle. Greater contracture is observed in muscle afflicted with MH than in normal muscle.

(ii) Contracture to halothane alone

Halothane causes contracture in severely afflicted MHS-muscle but never has this effect on normal muscle.

(iii) Caffeine contraction in the presence of halothane

Muscle only mildly afflicted with MH requires the simultaneous addition of both halothane and caffeine in order to induce contracture greater than that seen in normal muscle tissue under similar circumstances.

(iv) ATP-depletion test

This measures the ratio of ATP concentrations between two muscle strips in different baths. Oxygen is bubbled through both baths, and halothane is added to one. After half an hour, the concentration of ATP is measured in both muscle strips and the ratio decreases for MHS-muscle, while a ratio of one or more is normal.

Despite attempts to standardise caffeine contracture testing at previous workshops, it seems that information cannot be easily compared among laboratories perhaps because of variation in fibre size, bath size, bath temperature, and other similar factors.

Gronert (1979) carefully compared several methods for diagnosing susceptibility to MH using two groups of pigs defined as genetically susceptible or normal. He found that caffeine-induced skeletal muscle

contractures on muscle fragments were valid in determining susceptibility to MH in pigs; contractures induced by the combination of halothane and caffeine, by halothane alone, or by measurements of ATP depletion, were less reliable.

1.4.4 Platelets in Malignant Hyperthermia.

There is some evidence that the defect of MH is a generalised one involving membranes other than those of the skeletal or cardiac muscle.

Platelets removed from some (Zsigmond et al., 1978), but not all (Rosenberg et al., 1980b, 1981) MHS individuals exhibit abnormal aggregation in the presence of epinephrine and collagen.

While it could be that the haemolysis which occurs in severe cases of MH is secondary to the fever itself (Gronert, 1980), it may be due to a primary defect in the membrane of either red blood cells or platelets (Britt, 1972).

Britt (1972) notes that the platelet contractile mechanism is similar to that of the muscle cell; an observation made by pioneers of research on platelet biochemistry (Fantl and Ward et al., 1956) and by more recent investigators (Solomons and Masson, 1984b). However, it was Solomons and coworkers who first used platelets in diagnostic tests for MH which fulfilled a prediction made by Mustard (1971) that "Studies with platelets may provide clues to understanding some of the general aspects of cell function that are very difficult to explore in other systems".

Because of the painful and debilitating nature of the muscle biopsy operation, its replacement with a simple blood test would revolutionise the preanaesthetic diagnosis of MH.

Except for two reports (Giger and Kaplan, 1983; 1984), the only published investigations into the appropriateness of such a test has been a series of papers by Solomons and coworkers (Solomons et al., 1978; Solomons et al., 1980; Solomons and Masson, 1982a, 1982b, 1984a, 1984b) The ancestor of this series appears to be a report describing the development of a sensitive in vitro method for studying the energy metabolism of platelets under stress, which appeared in a volume of collected papers that had the rather unlikely title "Biomedical Applications of Polymers."

Solomons saw his procedure as a useful tool in helping to determine the biocompatibility of synthetic prostheses using the platelets of the recipient of the implant. He saw potential for the application of the platelet bioassay to the study of a wide variety of metabolic disorders, and published a paper in 1978 concerning platelet metabolism in MH.

Solomons performed a series of investigations on normal individuals and obtained a typical nucleotide pattern when platelets were incubated with [^{14}C]adenine. The ratio R;

$$\frac{[^{14}C]ATP + [^{14}C]ADP}{[^{14}C]AMP}$$

was 9.6±2.0, and this value decreased to 8.0±2.1 following exposure to halothane. In patients with a previously recorded episode of MH and positive muscle biopsy, R was significantly reduced to 1.5±1.5. Since the reductions were due to decreases in ATP, the platelet bioassay is analogous to the ATP depletion test in muscle strips.

Solomons adapted the thin-layer chromatographic method he had previously employed, to one involving HPLC and modified the ratio R to account for changes in HX;

$$\frac{[^{14}C]ATP + [^{14}C]ADP}{[^{14}C]AMP + [^{14}C]HX}$$

The value of R was reduced by 74.9±2.7% by halothane in clinically diagnosed MH patients compared with a reduction of 23.3±3.0 for normal patients. The reproducibility and specificity of this platelet-halothane biassay encouraged Solomons to incorporate it into routine clinical use at the University of Colorado.

However, Giger and Kaplan (1983) could not reproduce Solomons' results and concluded that, although ATP in platelets exposed to halothane was depleted significantly over a period of 30 minutes, there was no difference between platelets from MHS patients and those from non-susceptible controls. A discussion concerning methodology is now in progress between the opposing groups (Solomons and Masson, 1984a; Giger and Kaplan, 1984).

Apparently, guidelines for the standardisation of the

platelet-halothane bioassay will have to be decided upon as was attempted for the pharmacological testing of muscle strips. Therefore, the need for other investigators to provide evidence which may lead to the establishment or rejection of the platelet-halothane bioassay as an aid in the preanaesthetic diagnosis of MH is apparent.

THE PRESENT RESEARCH

The present research,started prior to the methodological controversy referred to above, is an attempt to develop a simple clinical test for MH-susceptibility based on the assumptions that;

(i) the muscle defect in MHS-subjects is also reflected in the platelet contractile system

(ii) under resting and/or halothane-stressed conditions, the nucleotide turnover in MHS-platelets is significantly abnormal

(iii) the turnover abnormality is reflected in differences in adenine incorporation to platelet nucleotides via the salvage pathway.

CHAPTER 2: MATERIALS AND METHODS.

MATERIALS.

2.1 CHEMICALS.

2.1.1 Radioactive Chemicals.

[8-^{14}C]adenine, code CFA.348, 54 mCi/mmol, was obtained from the Radiochemical Centre, Amersham.

2.1.2 Solvents.

"AnalR" grade solvents (BDH) were used wherever possible; otherwise, all other solvents were redistilled prior to use.

Deionised, distilled water was used when required.

2.1.3 Other Chemicals.

All other chemicals were obtained from the Chemical Store, Department of Chemistry and Biochemistry, Massey University. Only chemicals were manufactured by Sigma Chemical Company were used, with the exception of the following items;

Halothane (Fluothane)	- ICI
Glucose	- Glaxo
Sodium Phosphate	- Reidel-De Haen

Ammonium Oxalate	- E. Merch Darmstadt
Sodium Azide	- Reidel-De Haen
Potassium Carbonate	- May and Baker
Sodium Chloride	- BDH
Charcoal	- Norvit
Pyridine	- Sinar
Potassium Hydroxide	- May and Baker
Triton X-100	- Rohm and Haas
Acetonitrile	- Reidel-De Haen
Potassium Phosphate	- Baker
Tetrabutylammonium Hydroxide	- BDH
Dimethyldichlorosilane	- BDH

2.2 SUBJECTS FOR STUDY.

2.2.1 Normal subjects.

Whole blood was drawn in this Department from volunteer donors who were all members of the staff or graduate students. Only subjects who were not currently taking any form of medication were used. Donors who were not aware of any unexplained sudden death of close family members were considered to be Malignant Hyperthermia (MH) negative.

2.2.2 Malignant Hyperthermia-Susceptible Subjects.

The donors EC and SC (uncle of EC) had previously been ascertained to be MH-positive on the basis of muscle contracture tests performed by Dr. I.L. Anderson of the Department of Veterinary Clinical Science at Massey University (Anderson *et al.*, 1980). They were invited to Massey University where whole blood samples were drawn by the same person on each occassion.

A blood sample was also obtained from a suspected MH-positive patient prior to surgery at Palmerston North Hospital.

The donor, PU, was a young adult male who had survived a previous MH episode and was about to undergo muscle biopsy surgery. This patient was subsequently classified MH-positive on the basis of a muscle contracture test.

Blood was drawn from PU in the anaethesia induction room at Palmerston North Hospital while the patient was being prepared for muscle biopsy surgery. The sample was brought back to this Department where platelet-rich-plasma (PRP) was prepared and experiments begun within two hours of the blood being drawn.

The spectrum of MH susceptibility existing in humans (Nelson and Flewellen, 1983) is reflected in the response of muscle specimens from susceptible individuals to muscle contracture testing (Rosenberg, 1982). In the tests performed by Dr. Anderson, muscle samples from PU and SC displayed roughly three times the contracture response of LC.

2.3 CHROMATOGRAPHY.

Whatman No. 1 (qualitative) filter paper (46 cm x 57 cm) was used for all paper chromatography.

METHODS.

Most platelet investigators recommend that all glassware likely to come into contact with platelets is siliconised. However, it is not certain whether there is any advantage in using siliconised or plastic surfaces when preparing and storing PRP. Platelets adhere to siliconised surfaces as readily as they do to glass (Day et al, 1975). Despite this observation, every effort was made in the course of this research to allow platelets to come into contact only with plastic surfaces. When it was necessary for platelets to contact glass surfaces the latter were always siliconised; either commercially (vacutainer tubes) or in the laboratory using dimethyldichlorosilane in 2% 1,1,1-trichloroethane solution.

2.4 COLLECTION OF BLOOD.

Table 2.1 is a list of the anticoagulants and methods employed in the collection of blood and preparation of PRP by several major groups investigating platelet adenine nucleotides. The list indicates the lack of standardisation in the procedure for the preparation of platelets for metabolic studies. Since platelet behaviour is known to depend on the method of preparation of platelet suspensions (Day, 1979), different methods may lead to different results between these studies.

	ANTICOAGULANT	PREPARATION OF PLATELET-RICH PLASMA
1.	3.8% Sodium Citrate	170 x g for 10 minutes
2.	129 mM Trisodium Citrate Dihydrate	1000 xg for 15 minutes at 4°C
3.	0.13 M Trisodium Citrate or 0.027 M EDTA + 0.12 M NaCl	200 x g for 15 minutes 900 x g for 2 minutes (remove red cells) 2000 x g for 20 minutes (all at 5°C)
4.	3.13 mg/ml Trisodium Citrate	180 x g for 10 minutes
5.	0.11 M Citrate	180 x g for 12 minutes
6.	0.11 M Citrate	500 x g for 12 minutes
7.	3.8% Trisodium Citrate	320 x g for 10 minutes
8.	3.8% Trisodium Citrate	200 x g for 10 minutes
9.	Acid-Citrate-Dextrose Dextrose	1200 rpm for 18 minutes (remove red cells) 2200 rpm for 28 minutes (all at 4°C)
10.	12 mM Trisodium Citrate Dihydrate	1000 x g for 15 minutes (at 4°C)

TABLE 2.1

Preparation of platelet-rich plasma for metabolic studies by some major research groups.

Collection of blood into acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) solutions prevents the pH from rising as high as when sodium citrate is used as the anticoagulant (Day et al, 1975). While some metabolic processes of platelets, e.g. the release reaction, are impaired below pH 7.6-7.7, a pH of 6.5 or less is necessary to prevent platelet aggregation (Day et al., 1975).

In the course of this research either of the two following methods of blood collection was used.

2.4.1 Method 1.

In the initial experiments, 9 ml samples of blood were drawn by venipuncture into commercial vacutainer tubes (donated by Palmerston North Hospital) each containing 1 ml CPD solution of the following composition:

COMPONENT	mmol/L
trisodium citrate	0.102
citric acid	0.0170
glucose	0.142
monobasic sodium phosphate	0.0185
-pH 5.6-5.8	

Unfortunately, commercial production of CPD vacutainer tubes was discontinued early in this research and further supplies could not be

obtained. This necessitated the preparation of CPD vacutainer tubes in this laboratory.

2.4.2 Method 2.

The initial procedure was to add 1 ml CPD solution to plain, sterilised, siliconised vacutainer tubes, replace the stoppers, and re-evacuate the tubes with a vacuum pump. However difficulty was experienced in manufacturing tubes with a constant vacuum, which led to variable volumes of blood being drawn. The best alternative method involved drawing 10 ml blood samples into plain vacutainer tubes and transferring 9 ml from each sample into separate 15 ml plastic centrifuge tubes containing 1 ml each of CPD solution.

To minimise activation of the clotting process blood was mixed with anticoagulant almost immediately and never remained in the vacutainer tubes for more than 30 seconds.

In later experiments the anticoagulant used was citrate-citric acid-dextrose (CCD) of the following composition:

COMPONENT	mmol/L
trisodium citrate	0.100
citric acid	0.007
glucose	0.140
-pH 6.5.	

Both function and metabolism of platelets depend greatly on the pH of the suspending medium (Akkerman et al., 1978). Therefore, care was exercised to ensure that each solution of anticoagulant prepared was of the exact pH required.

In accordance with advice given by the Department of Pathology at Palmerston North Hospital no blood or fraction of blood was refrigerated before or during the preparation of PRP or during experiments until the platelet extract had been prepared.

Storage of citrated PRP at room temperature has a less injurious effect on the platelet than storage at $4^{o}C$. Platelets stored under the latter conditions have an increased tendency to aggregate because of the inactivation of temperature-dependent oxidative phosphorylation (Day, 1979). This results in a decreased synthesis of ATP and an increased permeability for ADP, which causes platelet aggregation (Day, 1979).

2.5 PREPARATION OF PLATELETS.

2.5.1 Introduction.

The various isolation procedures have different effects on platelet functionality, morphology, and metabolism (Lages et al., 1974). Therefore, the method employed must be suitable for the particular investigation being carried out.

Techniques for preparing concentrated platelet suspensions include:

(i) Separation of platelets from plasma.

- differential centrifugation
- albumin density gradient centrifugation
- isolation by gel filtration

(ii) Platelet suspensions in plasma.

-Most methods for preparing platelet-rich plasma (PRP) involve the centrifugation of whole blood collected into an anticoagulant. The yield of platelets by this procedure is no better than 75-85% (Karpatkin, 1979). Anticoagulant used, and time of centrifugation is variable. (A selection of methods is shown in Table 2.1).

Disadvantages of methods to produce plasma-free platelets include the necessity for high g-forces in differential centrifugation and the presence of a variable, high concentration of albumin in the final preparation of platelets from density gradient separation. Although gel filtration produces morphologically and functionally well preserved platelets which have not compacted, the platelet count is only about half of that in the PRP applied to the column (Day et al., 1975). Higher numbers of platelets are recovered in PRP.

Lages et al. (1974) analysed the total adenine nucleotide content

and the pattern of ^{14}C incorporation into the metabolic adenine nucleotide pool in platelets isolated by gel filtration and in PRP. They found no significant changes in the ATP and ADP contents or in the adenylate energy charge (AEC).

For the current research, PRP was prepared because of the higher yields of platelets obtained and the less complicated procedure involved.

2.5.2 Platelet-Rich Plasma.

Day (1979) considered the ideal PRP to contain the maximum yield of platelets in the maximum recovery of plasma, with a minimum number of red and white blood cells. He suggested that, utilising present techniques, the closest one can approach this ideal is a recovery of 37-42% plasma, a platelet count of $3 \times 10^5/\mu l$, and 2-4 red blood cells per microscopic field (at 400X magnification).

Day (1979) also summarised a study by others of the factors influencing the preparation of PRP, which suggested that centrifugation of citrated whole blood at an average of 193.6 x g for 10 minutes gave a satisfactory harvest of PRP.

In practice, however, some investigators use high g-forces for short times while others use low g-forces for longer times. There is an ideal moment when virtually all the platelets are still present in the plasma and all of the RBC have been sedimented. If centrifugation is prolonged, platelets will sediment with RBC (Day et al., 1975).

The volume of blood centrifuged and the geometry of the centrifuge tube affect the yield. PRP prepared by centrifuging a small volume of blood in a large tube has a lower platelet count than PRP prepared from a large volume centrifuged in the same size tube at the same g-force for the same time because the average g-force in the small volume is higher than in the larger volume.

Blood contains populations of platelets with different densities (Day et al., 1975): the heaviest platelets will be removed first during prolonged centrifugation and the resulting PRP will contain a greater proportion of smaller, lighter platelets than the original blood. Loss of the larger, heavier platelets can lead to erroneous results. Thus, the concentration of adenine nucleotides per platelet was less in platelets obtained from a small volume of blood than in platelets from a large volume.

Centrifugation of blood at room temperature ($22^{o}C$) is best for maintaining platelet function. Shape change and increased platelet "stickiness" occur at $5^{o}C$.(Mustard et al., 1972).

Either of two methods was used in this work:

2.5.2.1 Method 1.

This method is used at Palmerston North Hospital for platelet function testing and has the advantage of a high yield but the disadvantage of being time-consuming.

10 ml anticoagulated blood was centrifuged at 1000 x g for 3 minutes at room temperature. This force can be achieved using a Gallenkamp bench top centrifuge at setting 3 (slightly higher than 1000 x g) with a rheostat to adjust the voltage supplied to the appropriate level. After removal of plasma from the red blood cells, the former was re-centrifuged at 1000 x g for 15 minutes and left to stand for one hour. All but 1 ml of the supernatant plasma was discarded and the platelet button allowed to redisperse for one hour (many investigators use EDTA to resuspend the platelets (Day et al.,1975)).

The best yield obtained using this method was 1860 x 10^9 platelets/L of PRP, and routine yields were 1000 x 10^9 platelets/L.

Figure 2.1 is a flow-chart of method 1, indicating the percentage recovery of platelets during a typical preparation.

2.5.2.2 Method 2.

This method was preferred when a large amount of PRP was required eg. for time-course experiments and platelet concentration was secondary to total yield.

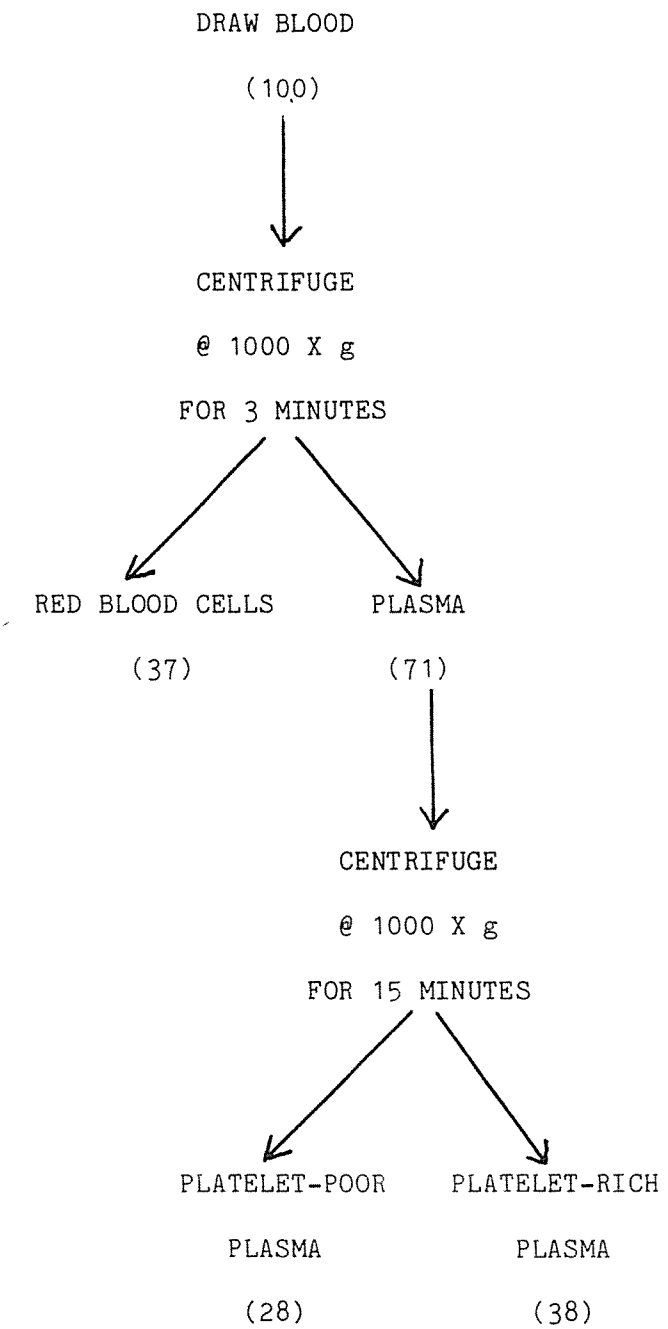


FIGURE 2.1

Preparation of platelet-rich plasma.

Numbers in parentheses indicate the recovery of platelets as a percentage of the number of platelets in whole blood.

Anticoagulated blood was collected as before, and centrifuged at 188 x g for 10 minutes at room temperature (setting No. 1 on a Gallenkamp benchtop centrifuge). The PRP was then removed from the sedimented red blood cells.

This method routinely produced PRP which had platelet counts of around 300 x 10^9 platelets/L PRP, which is consistent with the yield reported by Day (1979) for a similar g-force over the same length of time.

PRP made from blood that had been standing for several hours at room temperature was observed to contain platelets of abnormal shape: they became more pleiomorphic. Therefore, PRP was made as soon as possible after the blood was drawn.

Microhaemolysis may occur during withdrawal and centrifugation of blood, and ADP released from cells may lead to platelet aggregation (Haslam and Mills, 1967). Even the slow centrifugation usually used to prepare PRP (100-300 x g) may result in exposure of platelets to ADP, probably from platelets themselves (Day et al., 1975). Therefore, consistency in methods of blood withdrawal and centrifugation is important.

2.6 PLATELET COUNTING.

2.6.1 Introduction.

As with the preparation of platelet suspensions, many methods have

been developed for the enumeration of human blood platelets. A recent review of platelet counting methods (Wertz, 1979) classifies these methods into three general groups-

(i) Hemocytometer platelet counts.

(ii) Automated and semi-automated platelet counters

(iii) Electronic particle counting

The technique used for platelet counting in this study was one used at Palmerston North Hospital which was based on phase contrast microscopy of platelet suspensions mounted on a haemocytometer (Brecher and Cronkite, 1950).

2.6.2 Procedure.

To allow visualisation of platelets in a microscopic field, contaminating RBC must be lysed. For this purpose a diluent solution was prepared:

ammonium oxalate	10 g/L
sodium azide	1.0 g/L

Reported problems of turbidity developing in this diluent were not observed when it was made up in 100 ml batches.

Small amounts of diluent were filtered through a 0.45 micron Millipore filter as required and 380 µl of filtrate was added to a 20 µl aliquot of the platelet-containing sample in a plastic tube. After standing for 5 minutes a small amount of the diluted platelet

suspension was loaded onto an "Improved Neubauer" counting chamber which was then placed in a covered petrie dish containing a circle of moist blotting paper to prevent the edges of the loaded sample from contracting due to evaporation. The platelet count was performed after a further 20 minutes using phase contrast microscopy at 400X magnification. Five squares on the grid were counted which normally contained a contamination of 4-5 white blood cells/300 platelets.

2.6.3 Results and Discussion.

Evidence suggests that ammonium oxalate may lead to falsely low counts due to thrombolysis (Wertz, 1979). Wertz proposed a saline dilution-sedimentation scheme for use with phase contrast microscopy and describes a formalin-acetone diluent which "further clarifies the image of the platelets". He also suggests that insufficient settling time in all haemocytometer methods may be a major source of error, and a sedimentation time of 40 minutes may be necessary.

Using the method of Brecher and Cronkite (1950) the normal value for a human platelet count is 248 with a standard deviation of 55 x 10^9 platelets/L with a precision of 9-17%. Note that in Figure 2.1 the percentage of platelets in the plasma plus red-blood-cell fraction does not equal 100. This is not surprising in view of the reported precision of the counting method.

Whole blood samples from normal individuals who participated in this research had platelet counts from 250 to 300 x 10^9 platelets/L. The MHS blood samples had the following platelet counts;

LC	186×10^9 platelets/L
SC	198×10^9 "
PU	320×10^9 "

All determinations were performed in duplicate and, while the first two MHS patients had markedly lower platelet counts than normal, the third patient had a platelet count higher than the normal range.

2.7 PREPARATION OF PLATELET EXTRACTS.

2.7.1 Introduction.

Three methods of following the incorporation were used in the course of this work. In all methods [8-14C]adenine (with a specific activity of 54 mCi/mmol) was added to a 1 ml sample of PRP and incubated in the presence or absence of liquid halothane in a water-bath at 37°C. Nucleotides were then extracted from the platelets into perchloric acid (PCA) which was neutralised and the extract chromotographed.

Aliquots of the PRP, supernatants, and extracts were taken at various stages during the above procedure for liquid scintillation counting.

2.7.2 Procedure.

2.7.2.1 Holmsen and Rozenberg (1968a)

Blood was drawn into CPD tubes (initially commercial and later using those prepared in this Department) and PRP was made by Method 1. Each 1 ml aliquot of PRP was preheated to 37°C for five minutes before the addition of 10 µl of [8-^{14}C]adenine. In paired experiments where one aliquot of PRP was to be incubated in the presence of halothane, 10 µl of the anaesthetic was added at this stage and 10 µl of 0.9% NaCl was added to the control tube. All tubes were tightly capped and incubated at 37°C in a shaking water bath.

Aliquots of 200 µl were removed from the PRP at various times during incubation and each was added to 20 µl of ice-cold 6.6 M PCA in 1.5 ml polypropylene centrifuge tubes. After vigorous mixing each sample was centrifuged at 12 000 x g for 2 minutes at room temperature to precipitate platelet debris and protein.

The nucleotide extract was removed from the precipitate and 10 µl of ice-cold bromthymol blue indicator solution was added. The extract was then titrated to the indicator colour change (orange to blue at pH 6.0-7.6) at 0°C with 2 M K_2CO_3 delivered from a micrometer syringe.

Typically, 30-50 µl of K_2CO_3 was required to neutralise each extract. The sample was then centrifuged at 3000 x g for 15 minutes (using a bench top centrifuge at 4°C). The extract was removed from the $KClO_4$ precipitate and stored at -20°C until required.

2.7.2.2 Solomons et al. (1978)

Blood was drawn into plain vacutainer tubes and added to plastic centrifuge tubes containing CCD. To 1 ml samples of PRP (prepared by Method 1) was added 10 µl of [8-14C]adenine with 10 µl of either halothane or 0.9% NaCl saline.

At the end of incubation at 37°C in a shaking water bath, each aliquot of PRP was centrifuged at 3000 x g for 3 minutes to produce a platelet pellet and a "supernatant fraction" (plasma) in which no platelets remained (determined by phase contrast microscopy of non-radioactive sample). The supernatant was removed and placed in a fresh 15 ml plastic centrifuge tube.

To the platelet pellet was added 1 ml ice-cold 0.6 M PCA and 1 ml ice-cold 1.2 M PCA was added to the supernatant. All tubes were shaken for 15 minutes at 4°C and then centrifuged at 3000 x g for 5 minutes.

The nucleotide extracts were adjusted to pH 7.0-8.0 with 1 M KOH using a micrometer syringe. In this case, pH was measured using the microelectrodes of a blood gas-analyser with slight modifications. Very accurate and consistent titrations were possible using this instrument.

A 14% solution of acid washed charcoal in water was prepared and 100 µl of this solution was added to each extract. The tubes were then shaken for 20 minutes at 4°C and centrifuged at 3000 x g for 5 minutes

at the same temperature. The charcoal precipitate was washed with 2 ml water and again centrifuged at 3000 x g for 5 minutes at 4°C. 100 µl of ice-cold 15% pyridine was added to each tube and, after 30 minutes shaking at 4°C, the charcoal was centrifuged down and the extract removed and chromatographed immediately.

2.7.2.3 Rao *et al.* (1981).

Blood was drawn into plain vacutainer tubes and added to plastic centrifuge tubes containing CCD. 1 ml aliquots of PRP (prepared by Method 2) were placed in 15 ml plastic centrifuge tubes containing 0.5 ml CCD. These tubes were prewarmed and [8-14C] adenine, plus halothane or 0.9% NaCl added as in the previous two methods. The tubes were tightly capped, returned to the water-bath, and shaken at a constant gentle rate.

At the end of incubation the tubes were centrifuged at 12 000 x g for 1.5 minutes at 4°C and the supernatant plasma was removed, frozen in liquid air, and stored at -20°C until required for chromatography. To the platelet button was added 100 µl of ice-cold 6.6 M perchloric acid and the button was completely redispersed manually using a fine stirring-rod. After vigourous mixing followed by sonication the tubes were centrifuged for 1.5 minutes at 12 000 x g at 4°C.

The extracts were carefully removed, placed in 1.5 ml polypropylene tubes, and neutralised with 5 M KOH to a pH of 5.5-8.0 using a micrometer syringe. The pH was measured by transferring the smallest amount of extract possible on the tip of a pasteur pipette to

a piece of indicator paper. This method was more cumbersome than that used in Section 2.7.2.2 but the blood-gas analyser was not available for these experiments.

A freeze-thaw cycle was then performed. Liquid air was used to freeze the extracts which were then thawed at room temperature. Three repetitions of this cycle were performed (3 freezes and 3 thaws). The extracts were then centrifuged at 12 000 x g for 1.5 minutes at $4^{o}C$, separated from the $KClO_4$ precipitate, and chromatographed immediately or stored at $-20^{o}C$ until required.

2.8 pH ADJUSTMENT OF PERCHLORIC ACID EXTRACTS.

2.8.1 Introduction.

The end point for titration of the acid extracts was very sensitive and often, near the end point, the addition of less than 1 µl of K_2CO_3 was enough to turn the Bromthymol Blue (BTB) from orange to blue.

The care necessary during titrations made them very tedious and time consuming to perform and probably inaccurate since a consistent end point (blue colour) was difficult to obtain.

When the blood-gas analyser became available (Section 2.7.2.2) the titration curves could be quantitated and four examples of these curves are shown in Figure 2.2. The addition of 1 µl of KOH increased the pH of each extract from less than 1 to a final pH of 8-10. It proved

impossible to obtain a final pH of 6.0-7.6 (which is the range over which BTB changes colour).

It was, therefore, necessary to investigate the effect of end point pH on recovery of nucleotides from the platelets. However, this was not possible until a reliable method for nucleotide extraction (Rao et al., 1981) was developed.

2.8.2 Procedure..

Three indicator solutions were prepared to cover most of the pH range between 5 and 11 (CRC Handbook of Chemistry and Physics, 1980-81);

Indicator	Approx. pH Range	Colour Change	Preparation
1. Methyl Red	4.8-6.0	Red --> Yellow	0.02%
2. Bromothymol Blue	6.0-7.6	Yellow --> Blue	0.04%
3. Thymolphthalein	9.4-10.6	Colourless --> Blue	0.04%

10 µl of [8-14C]adenine was added to each of three 1 ml aliquots of PRP which were then incubated at 37° C for 30 minutes in a shaking water bath. The platelets were extracted with Rao's procedure but,

prior to neutralising the extracts, 10 µl of one of the above indicators was added to each extract. Titration was then carried out until the end-point was reached. and the radioactivity in each extract (after centrifugation to remove the $KClO_4$ precipitate) was determined.

When the extract was titrated to pH 10, 20% of added radioactivity was recovered, whereas at a pH of between five and eight only 10-12% of the radioactivity was recovered.

2.8.3 Discussion.

The pH to which the platelet extracts were titrated obviously had a large influence on the subsequent recovery of radioactivity from the extract. While only three pH ranges were considered in this experiment, it appeared that akalinising the extracts was appropriate. (It was later noted that Giger and Kaplan (1982) also recommended making platelet extracts alkaline prior to adenine nucleotide determination). Therefore, extracts were subsequently made alkaline to pH 9-11.

Determining pH with indicator paper was naturally less accurate than with the blood-gas analyser but titration curves could still be produced consistently and examples are shown in Figure 2.3.

These results raised questions about the stability of nucleotides stored at low to neutral pH. Only one report was available concerning storage of nucleotides (Brown, 1971) No data were given but the author concluded that nucleotides were more stable when stored under alkaline

conditions.

In order to check the stability of nucleotides in solution, ATP solutions of known concentration were prepared in phosphate buffer (pH 4.3) and stored at various pH's for six weeks at -20°C. The experiment, detailed in Section 3.4, showed that ATP was most stable when stored at pH 9-10 (see Figure 3.7).

If this conclusion for the long term storage of ATP solutions can be applied to the storage of platelet extracts at -20°C then the findings of Brown (1971) are supported and it is indeed desirable to store nucleotides at an alkaline pH.

2.9 DETECTION OF NUCLEOTIDES.

2.9.1 Introduction.

The most widely used method of measuring nucleotides in platelet extracts is the Firefly Luciferase Assay (Holmsen et al., 1972). A recycled NAD-linked photometric assay was used by Mills and Thomas (1969) and simple absorbance at 260nm (Murer, 1969) has also been employed. Determination of an adenine-chloroacetaldehyde complex (Gordon and Drummond, 1974), column chromatography (Goetz et al., 1971), and HPLC (Brown, 1970) are other methods.

FIGURE 2.2

Titration of platelet extracts using a blood-gas analyser to determine the pH.

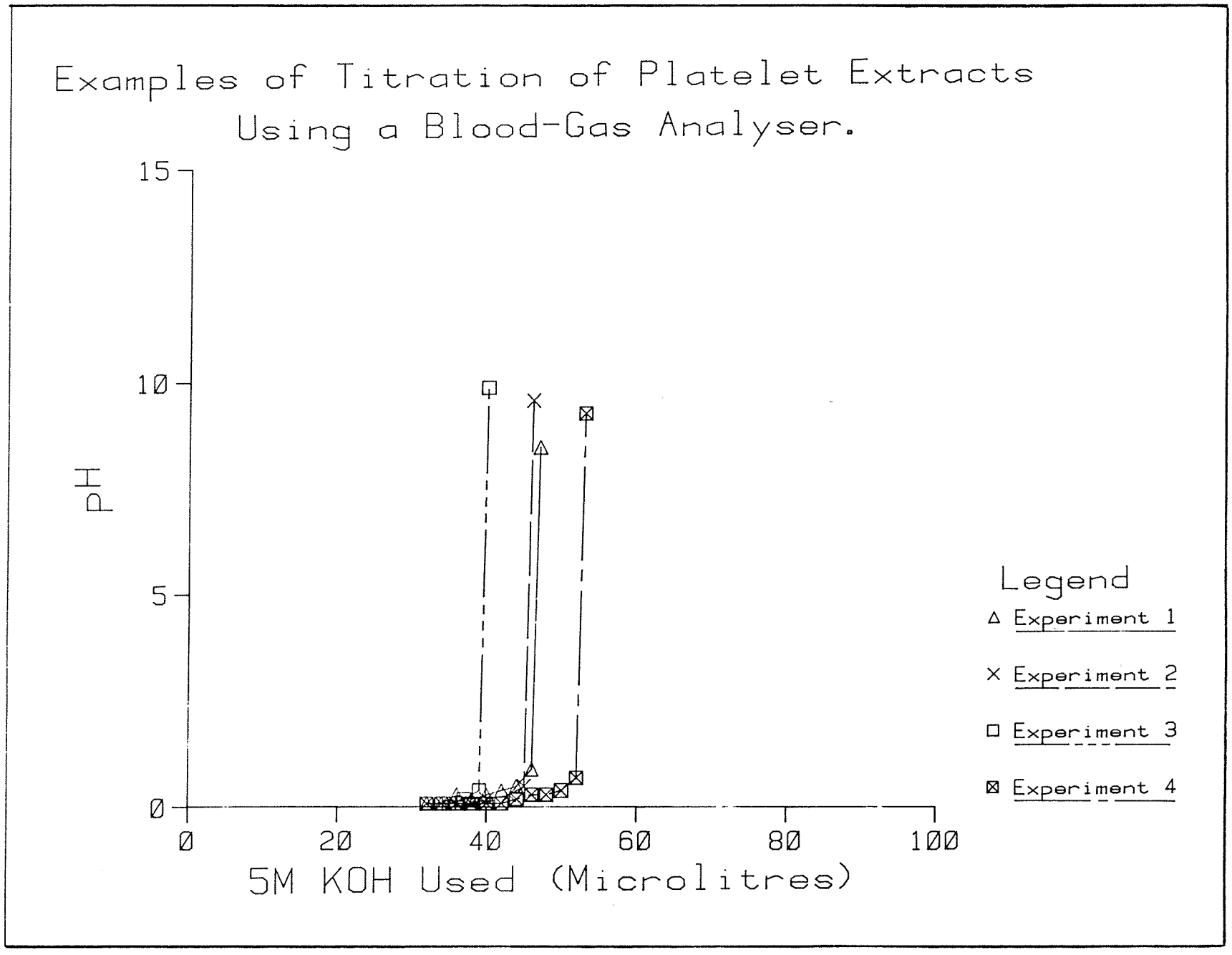
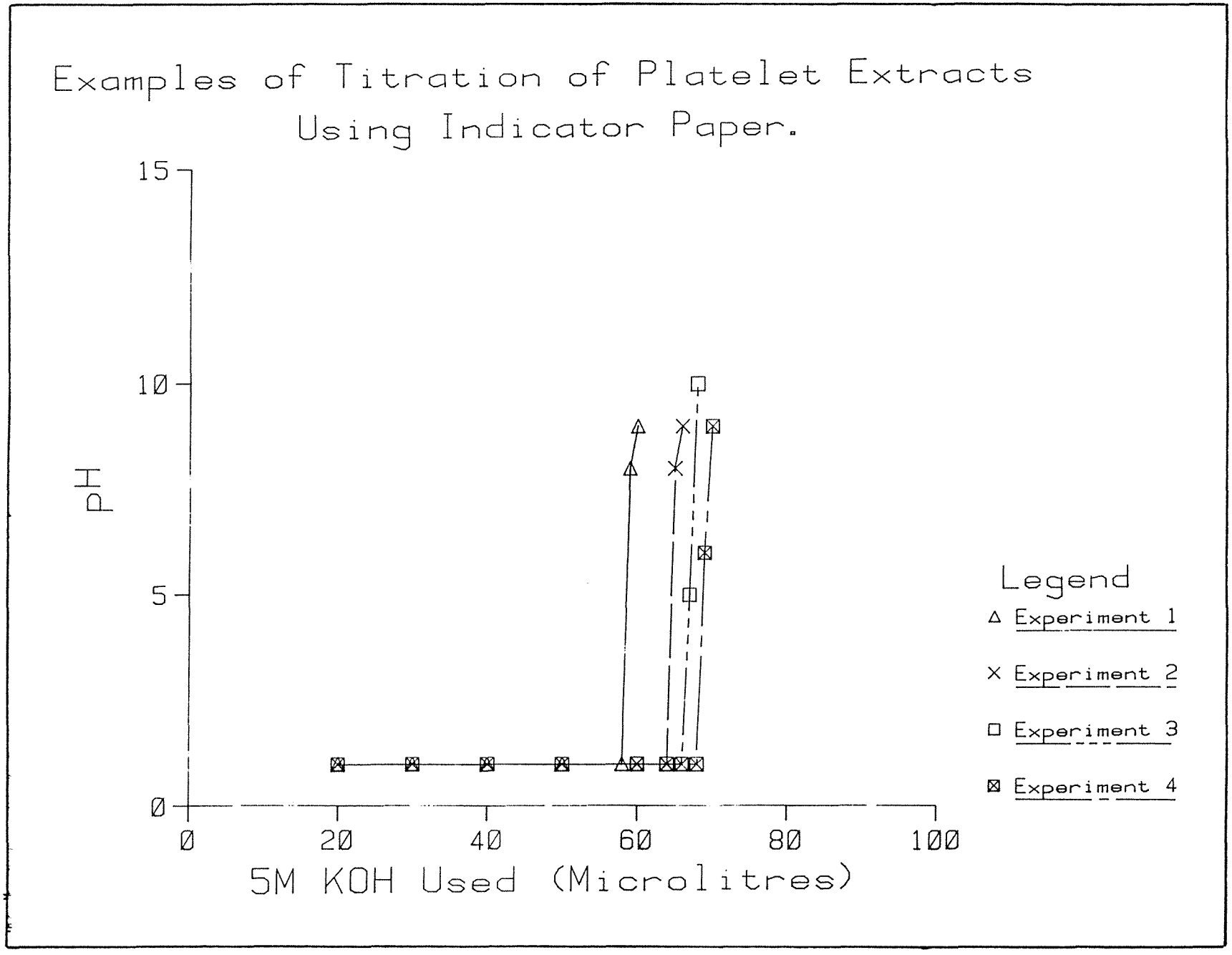

Examples of Titration of Platelet Extracts
Using a Blood-Gas Analyser.
15
10
5
0
pH
0
20
40
60
80
100
5M KOH Used (Microlitres)
Legend
Experiment 1
Experiment 2
Experiment 3
Experiment 4

FIGURE 2.3

Titration of platelet extracts using indicator paper to determine pH.


Examples of Titration of Platelet Extracts
Using Indicator Paper.
15
10
5
0
pH
0
20
40
60
80
100
5M KOH Used (Microlitres)
Legend
Experiment 1
Experiment 2
Experiment 3
Experiment 4

2.9.2 Paper Chromatography.

A line was drawn 10 cm from the top of a 46 cm x 57 cm sheet of Whatman no.1 filter paper. The page was divided into 3 cm or 4 cm wide columns, and 50 µl of a nucleotide extract was streaked across each strip (leaving a 1 cm wide border at both sides of the sample lane). The streak was dried with a stream of cold air. Normally markers were applied on top of this streak, and consisted of 2 µl each of 1 per cent solutions of some or all of ATP, ADP, AMP, IMP, cyclic-AMP, HX, inosine, and adenine. A 1 cm streak of red dye (from a marker pen) was applied close to the left and right margins of the chromatogram. This dye ran parallel to the HX marker making it a useful guide to the progress of the chromatogram as HX had the second highest R_f of markers used. The bottom edge of the chromatogram was cut to form a jagged edge to facilitate an even flow of solvent. The chromatogram was developed by descending chromatography for at least 24 hours to produce an adequate separation of the ATP and ADP markers.

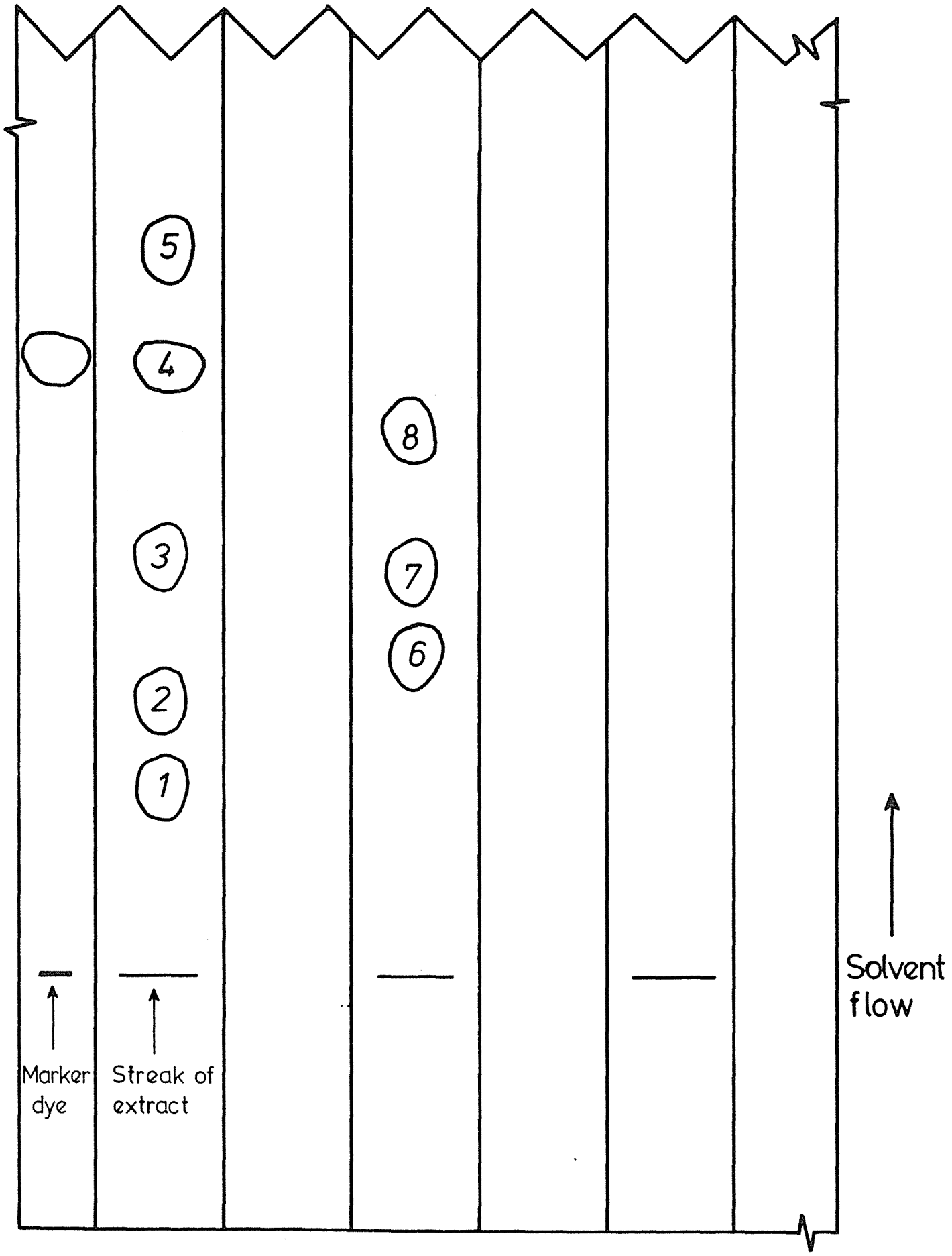
Figure 2.4 is a diagram of a chromatogram showing the positions of the marker nucleotides after developing the chromatogram. The combination of markers shown in the lanes indicated was not necessarily those used. The adenine marker (which was the farthest travelling marker) took about 40 hours to reach the bottom of the chromatogram.

FIGURE 2.4

Diagram of part of a typical paper chromatogram indicating the R_f of separated components.

(Scale 1:2)

1 = ATP
2 = ADP
3 = AMP
4 = HYPOXANTHINE
5 = ADENINE
6 = IMP
7 = CYCLIC AMP
8 = INOSINE


5
4
8
3
7
6
2
1
Marker dye
Streak of extract
Solvent flow

The solvent system was;

n-butanol	acetone	acetic acid	concentrated ammonia	water
45	15	10	2	28

(Holmsen and Rozenberg, 1968a).

Remaining solvent was returned to the container and reused. The developed chromatogram was dried in a fume hood overnight and the markers visualised next day in a UV "viewing-box". The chromatogram was cut into strips (sometimes for analysis on a radiochromatogram scanner) which were then cut into regions corresponding to the nucleotide markers and the radioactivity of each region was determined by liquid scintillation counting.

2.10 RADIOCHROMATOGRAM SCANNING.

This instrument was used at frequent intervals to verify that the markers were in fact running on the chromatogram at the same rate as the radioactive nucleotides. The strips were taped together end-to-end and loaded onto the scanner after being marked at regular intervals, in non-radioactive areas, with small spots of radioactive ink.

The instrument used was a Packard Model 7201 Scanner System and usual run conditions were:

Meter Range	: 1000
Slit Width	: 5 cm
Gas Flow	: 250 cm/minute
Chart Speed	: 0.5 cm/minute
Time Constant	: 3

Due to the large amount of noise at the sensitivity used, no attempt was made to use this instument for quantitative results. It was used solely to indicate the exact position of any radioactivity. A typical scanner output is reproduced in figure 2.5. Figure 2.6 shows the distribution of radioactivity on the same strip as determined by liquid scintillation counting of 1 cm sections and illustrates a problem encountered. In spite of the fact that the markers on the chromatogram separated discretely, ATP and ADP radioactivities were incompletely resolved on both the scan and counts of chromatogram segments. This emphasises the need for care in running the chromatograms and accuracy in cutting out the radioactive regions for counting.

FIGURE 2.5

Reproduction of a typical radiochromatogram scan.

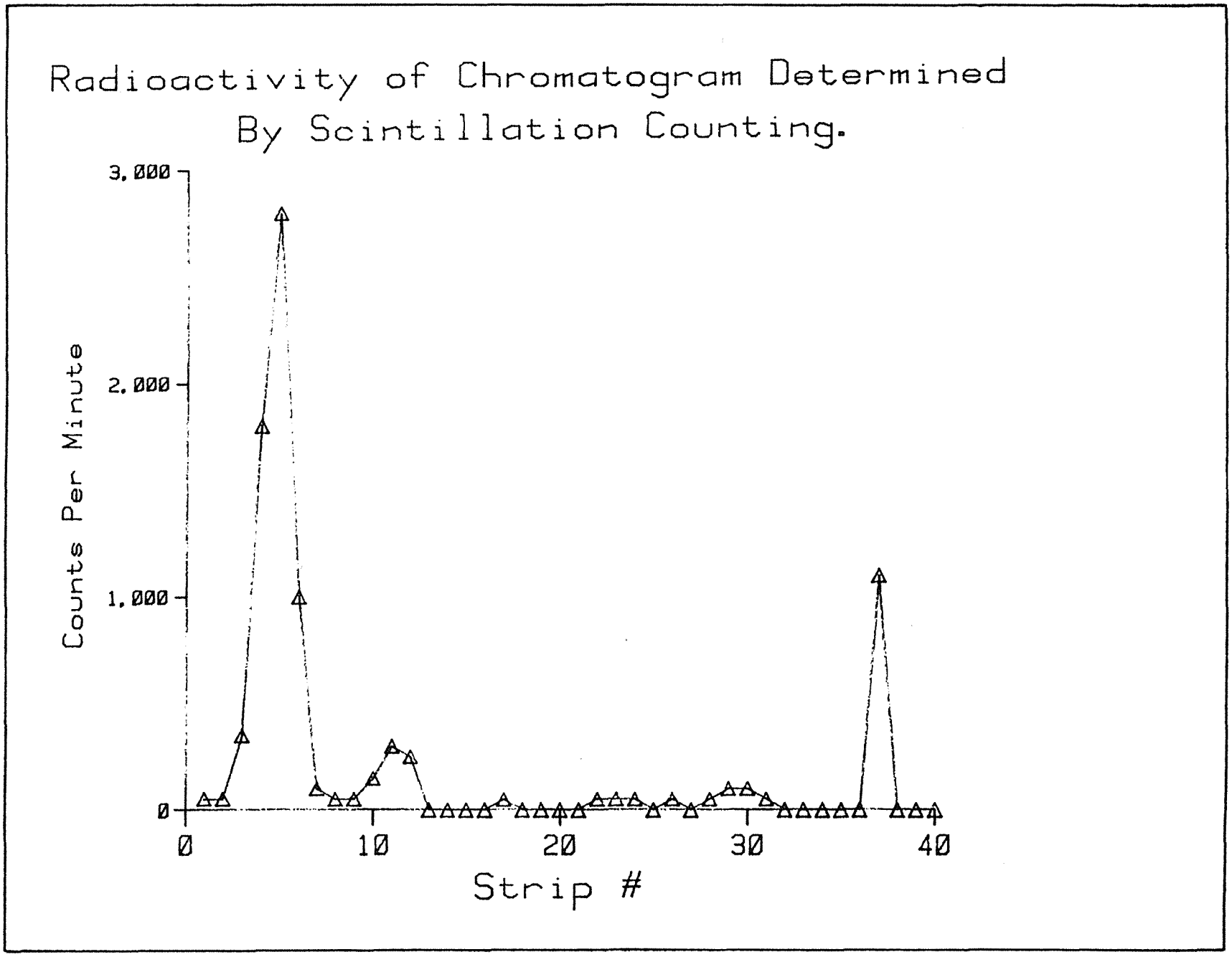
(Conditions detailed in text).

ATP
ADP
AMP
HYPOXANTHINE
ADENINE
Intek
Intek
Intek
Intek
0 10 20 30 40 50 60 70 80 90 100
EHR1687/1017 100-260

FIGURE 2.6

Determination of radioactivity by scintillation counting.

The same chromatogram as in Figure 2.5 was cut into 1 cm-wide strips and the radioactivity of each strip was counted.


Radioactivity of Chromatogram Determined
By Scintillation Counting.
3,000
2,000
1,000
0
Counts Per Minute
0
10
20
30
40
Strip #

2.11 SCINTILLATION COUNTING.

The composition of the scintillation fluid was;

PPO	12	g
POPOP	0.3	g
Triton x-100	1	L
Toluene	2	L.

Aqueous samples or paper squares carrying dried samples were added to 10 ml of scintillant in glass vials and counted in a Beckman LS7000 or LS8000 scintillation counter. All radioactive counting pertaining to a particular experiment was performed in one only of the above instruments due to variations in observed counts-per-minute (cpm) between them for a particular sample.

Vials were usually counted for one minute each, and recounted overnight for ten minutes each (data from the ten minute counts were used for calculations). An automatic quench compensation (AQC) facility was incorporated into the counting program, and because of this counting efficiency never deviated from the range 76-78% (for either one or ten minute counts).

An investigation into the effect of the volume of scintillation fluid used revealed that volume had no effect on counting efficiency

over the range 5-15 ml scintillant (when AQC was used). Increasing the volume of scintillant from 5 ml to 10 ml resulted in a 0.5% increase in corrected counts while the addition of a further 5 ml increased the corrected counts by 2-3 per cent. In the interests of conservation of solvent an increase of this magnitude did not justify using 15 ml of scintillant per vial. However, to ensure rapid and complete solubilisation of samples, 10 ml was used in preferance to 5 ml. Equal amounts of radioisotope sample in aqueous solution or in plasma produced identical results.

Initially, it was thought that the use of solvents to improve the miscibility of the sample in the scintillant would be useful and Tritosol, the scintillation fluid introduced by Fricke in 1975, was tried. The composition of this "emulsion scintillator" was;

PPO	3 g
Triton X-100	257 ml
Ethylene Glycol	37 ml
Ethanol	106 ml
Xylene	to make 1000 ml.

Experiments using this solvent revealed that, at an optimum volume of 10 ml, corrected cpm were only 6% higher than those obtained when an equal volume of triton-toluene was the scintillation fluid. An increase of this magnitude did not justify the regular use of Tritosol

which was more expensive and time-consuming to prepare.

The addition of various amounts of paper to vials had little effect on the counting efficiency (when AQC was used). For this investigation, aliquots of [^{14}C]hexadecane were applied to pieces of Whatman No.1 chromatography paper varying in size between 15 and 75 cm^2 (disintegrations-per-minute were determined by weighing the paper before and after the addition of the labelled compound to find the weight of [^{14}C]hexadecane). The counting efficiency varied between 72 and 75%, compared with an efficiency of 79% for [^{14}C]hexadecane added directly to scintillant. Correction was made for any quenching due to the amount of paper added to the vials.

CHAPTER 3: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.

3.1 INRODUCTION.

The first report concerning the determination of nucleotide profiles of cell extracts by High Performance Liquid Chromatography (HPLC) was published by Brown in 1970, who developed a gradient elution system which enabled her to detect naturally occurring mono-, di-, and triphosphate nucleotides qualitatively and quantitatively within 70 minutes. The enormous amount of research directed at simplifying this procedure and reducing the time required to determine a nucleotide profile (recently reviewed by Pogolotti, 1982; Brown, 1971), improved the technique to the extent that it is now possible to effect an isocratic elution of less than one nanomole of nucleotides using reverse-phase HPLC in about 10 minutes (Solomons and Masson, 1984b; Giger and Kaplan, 1983). These modifications have brought the technique within the scope of routine diagnostic procedures in the hospital laboratory. Indeed, the platelet bioassay first reported by Solomons et al. (1980) as being able to reliably detect MHS individuals is a HPLC analysis routinely used by his group to screen patients at risk prior to anaesthesia. Although Solomons and Masson's method has not been adopted generally, and discussion concerning the validity of his findings is continuing (Giger and Kaplan, 1983; Solomons and Masson, 1984b), the potential for HPLC methods in application to such diagnostic procedures is apparent.

The short investigation outlined here was an attempt to reduce the time required in determining the distribution of radioactivity in the

adenine nucleotides of platelet extracts and to improve the chromatographic separation of the components of each extract. Although non-radioactive markers applied to paper chromatograms were completely separated, radiochromatogram scans indicated that there was some carry-over of radioactivity from ATP to ADP. Unequivocal separation of measured components is of great importance if differences between normal and abnormal samples are to be used as the basis for a clinical test.

3.2 CHROMATOGRAPHIC CONDITIONS.

3.2.1 Rao et al. (1981).

3.2.1.1 System A.

For the initial work, similar apparatus to that used by Rao's group was employed;

Waters Association Model 204 HPLC consisting of;

- Model 450 Variable Wavelength Detector
- M6000 Solvent Delivery System
- Model 660 Solvent Programmer
- UK6 Universal Injector
- Radial-Pak C18 Column with RCM-100 radial compression module.

Gradient elution profiles (at room temperature) showed that the optimum composition of the mobile phase for isocratic elution of

adenine nucleotides was 70% of a 90% solution of acetonitrile in deionised, distilled water (pump A) plus 30% of a 10% solution of acetonitrile in water (pump B). Partly due to the required use of a guard column in series with the main column (packed with identical solid phase material) a flow rate of two ml/minute was the maximum attainable without the build-up of excessive back-pressure.

The paired-ion-chromatography (PIC) reagent was prepared by titrating an amount of orthophosphoric acid to pH 7.5 with tetrabutylammonium hydroxide to produce tetrabutylammonium phosphate at a final concentration of 0.005 mol/L of stock solvent.

3.2.1.2 System B

For later work, a different HPLC system was used;

- Model 450 Variable Wavelength Detector
- Model 721 Programmable System Controller
- Model 730 Data Module
- Model M-45 Solvent Delivery System
- Model 710B WISP (Waters Intelligent Sample Processor).

The solvent system employed was identical with that in Sytem A.

3.2.2 Solomons and Masson (1984b)

Solomons and Masson (1984b) used Waters HPLC equipment with the detector set at 254 nm. A C-18 Rad-Pak column was used with a radial

compression modual, and a flow rate of 4-8 ml/minute was achieved. Elution proceeded isocratically with 0.1 M KH_2PO_4.

For the application of this procedure to the current research, System B above was used with the same solvent system as Solomons and Masson.

3.3 RESULTS.

3.3.1 Evaluation of Pre-column Treatment.

To avoid contamination of the column used in this work for the separation of nucleotides from platelet extracts, it was necessary that the latter be first passed through a short pre-column (Sep-Pak) to remove tightly-bound contaminants.

The following procedure was employed to prepare, run, and regenerate the Sep-Pak;

-wash with 80% methanol
-wash with distilled, deionised water
-wash with mobile phase
-apply sample
-elute with mobile phase
-wash with distilled, deionised water (also reverse direction)
-wash with 80% methanol.

To ensure that nucleotides could be recovered from the Sep-Pak the absorbance spectrum of an aliquot of a mixture of nucleotide standards (in mobile phase) was determined (UNICAM SP 800 UV Spectrophotometer). The mixture was eluted from the Sep-Pak with further mobile phase and the absorbance spectrum of the eluate was analysed. The absorbance at 254nm was identical with that previously determined, which suggested that nucleotides could pass through the Sep-Pak with a near total recovery.

For platelet extracts, 100 µl of sample was applied to a Sep-Pak and eluate (mobile phase) was collected in fractions of approximately 100 µl each. The radioactivity of each fraction was determined and the elution profile is shown in Figure 3.1. Fractions 2 to 11 were pooled (containing 97% of the total radioactivity applied to the Sep-Pak) and a portion of this was analysed by paper chromatography. The distribution of radioactivity among the separated components was unchanged from that determined prior to passing the extract through the Sep-Pak.

3.3.2 Standards.

One per cent solutions of ATP, ADP, and AMP were prepared in mobile phase (since extraneous peaks were detected if they were prepared in distilled water) and a mixture of the adenine nucleotides was applied to the column. Figure 3.2 shows the separation of adenine nucleotides achieved.

FIGURE 3.1

Elution of a normal platelet extract from a Sep-Pak (short precolumn).

The extract was eluted with mobile phase in fractions of approximately 100 µl and the radioactivity of each fraction was determined.

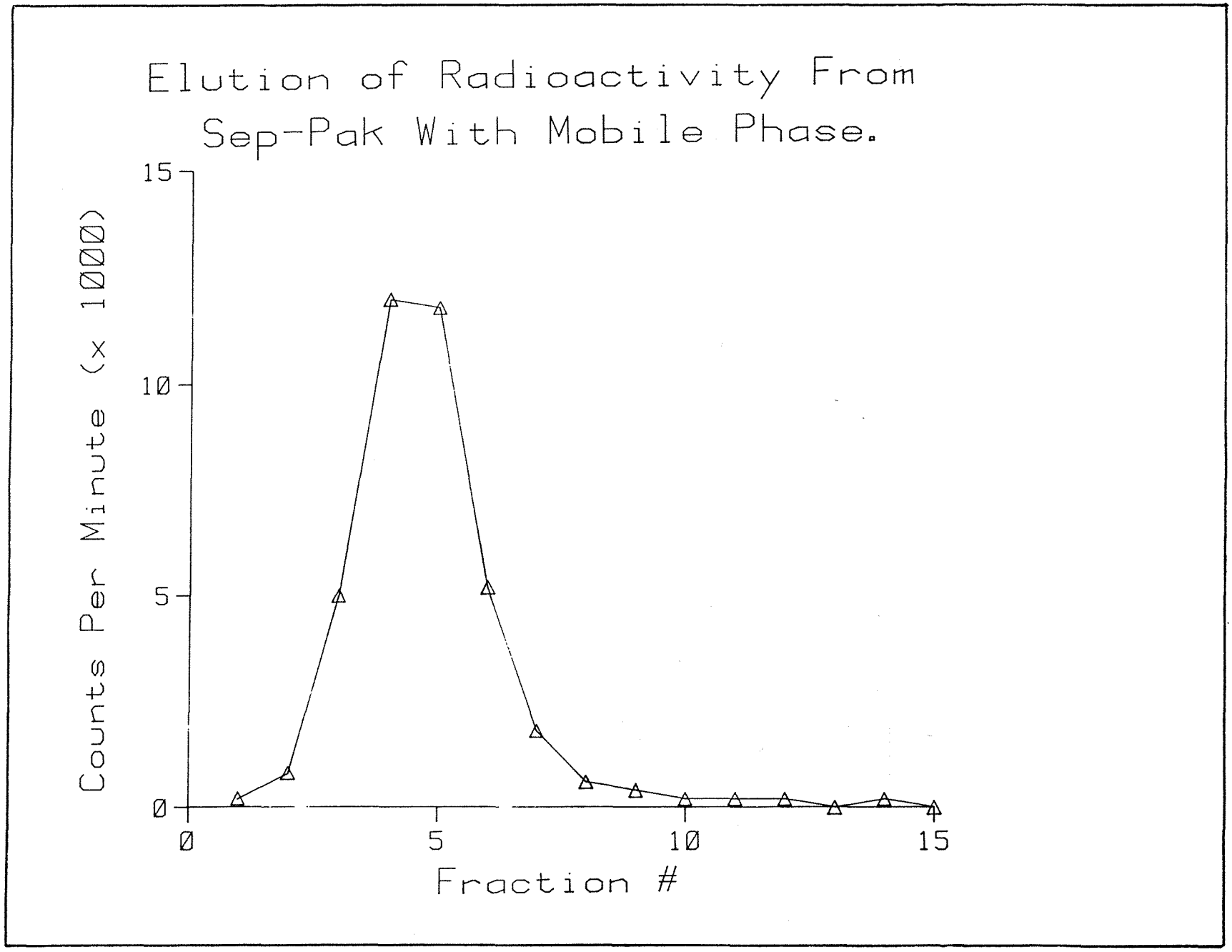
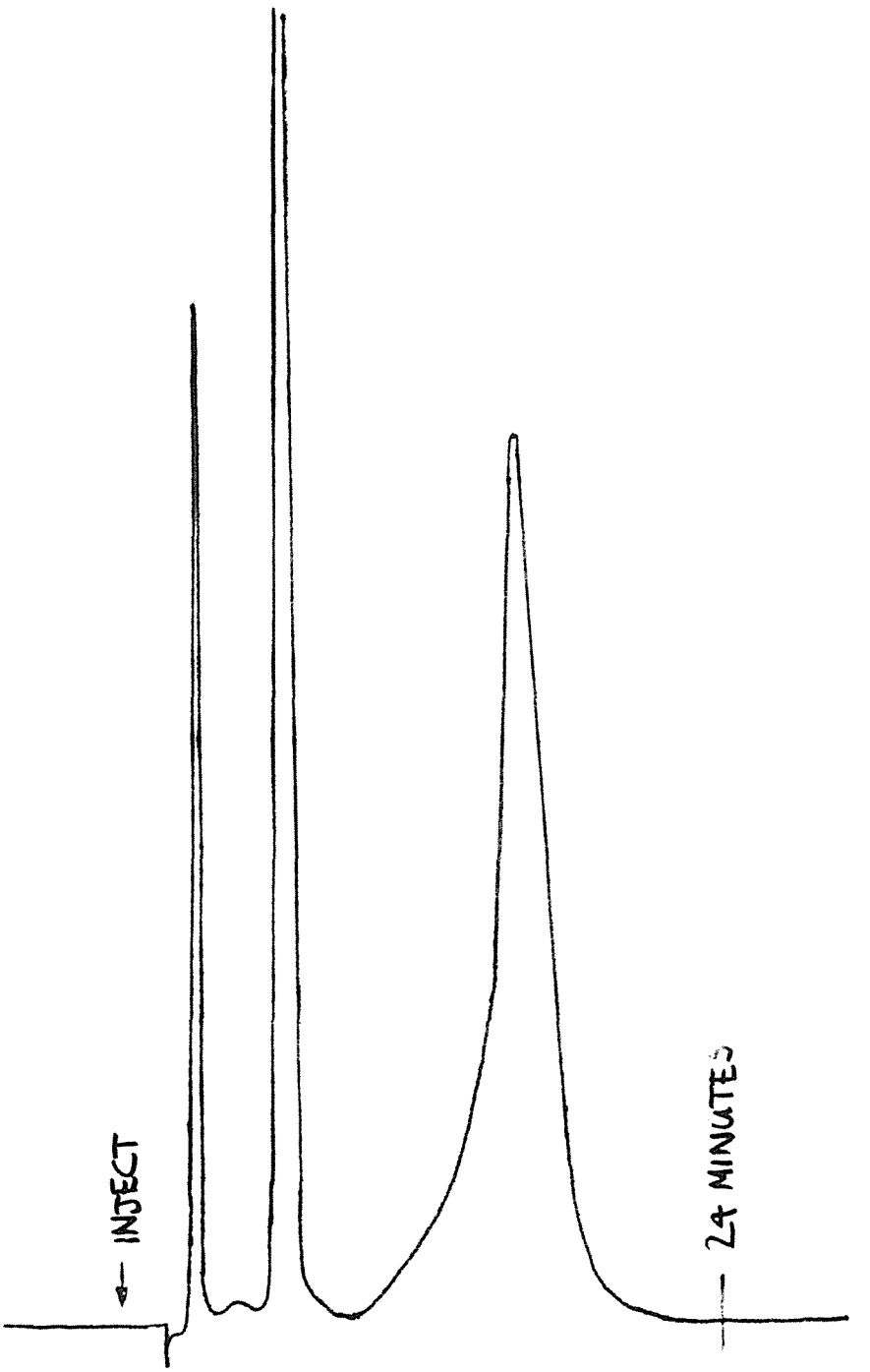

Elution of Radioactivity From
Sep-Pak With Mobile Phase.
Counts Per Minute (x 1000)
15
10
5
0
0
5
10
15
Fraction #

FIGURE 3.2 Separation of nucleotide standards using System A and the method of Rao *et al.* (1981).

Peaks in order of elution are;
(i) AMP
(ii) ADP
(iii) ATP

Chromatographic conditions;
Flow Rate - 2 ml/min
Meter Range - 0.2 AUFS
Chart Speed - 20 cm/hour
Injection Volume - 30 µl


INJECT
24 MINUTES

A range of standard solutions prepared in an identical manner was analysed using system B resulting in the separation presented in Figure 3.3. The separation of standards varied slightly between runs and should, therefore, be redetermined before each unknown sample is analysed.

3.3.3 HPLC of platelet extracts.

3.3.3.1 Rao *et al.* (1981).

An aliquot of platelet extract applied to the HPLC system did not produce peaks of sufficient magnitude to be integrated by the data processor. Therefore, aliquots of platelet extracts were added to equal volumes of mixtures of nucleotide standards (in mobile phase) before injection onto the Radial-Pak C18 column. Elution proceeded at the maximum flow rate possible without excessive back-pressure building up (up to 2 ml/minute).

Figure 3.4 shows that the first half of each elution profile (from two separate experiments) is very similar to that of the corresponding nucleotide mixture. A feature of the platelet extract traces is the non-appearance of peaks corresponding to ATP, and the appearance of two additional peaks in each profile; neither peak eluting with exactly the same R_f as ATP.

FIGURE 3.3

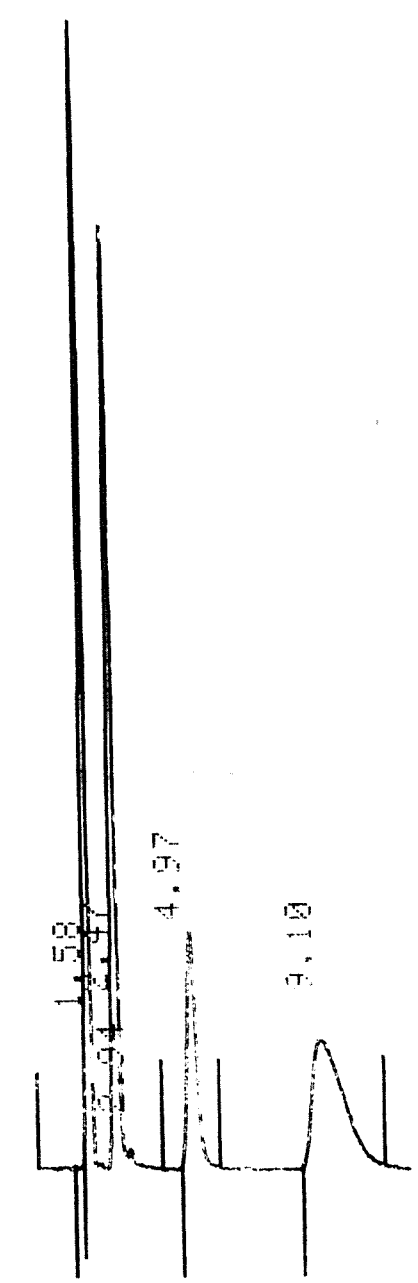
Separation of nucleotide standards using System B and the method of Rao *et al.* (1981).

Peaks in order of elution are;

(i) Adenine
(ii) AMP
(iii) ADP
(iv) ATP

Chromatographic Conditions;

Flow Rate - 3 ml/min
Meter Range - 0.20 AUFS
Chart Speed - 0.25 cm/hour
Injection Volume - 10 µl


INJECT
1.58
4.97
9.10

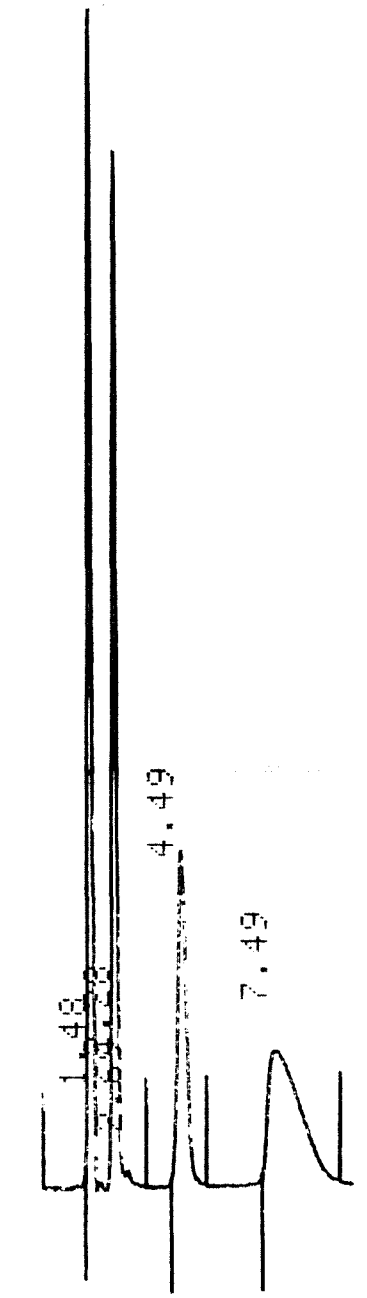

INJECT
1.48
4.49
7.49

FIGURE 3.4

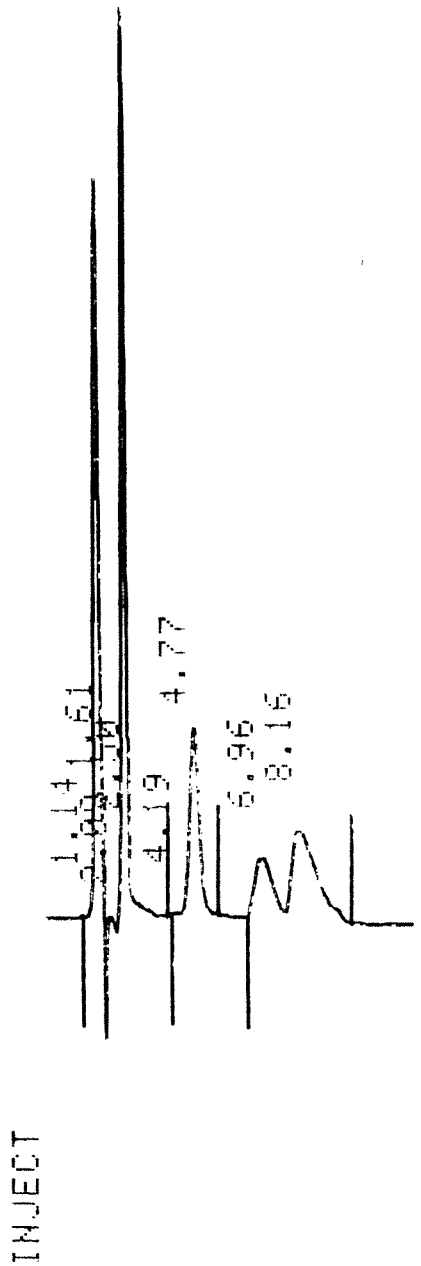
Elution profiles of normal platelet extracts (to which nucleotide standards had been added) using System B and the method of Rao *et al.* (1981).

Peaks in order of elution are;

(i) Adenine
(ii) AMP
(iii) ADP
(iv) ?
(v) ?

Chromatographic conditions;

Flow Rate - 3 ml/min
Meter Range - 0.20 AUFS
Chart Speed - 0.25 cm/hour
Injection Volume - 20 µl


INJECT
1.14
4.19
4.77
6.96
8.15

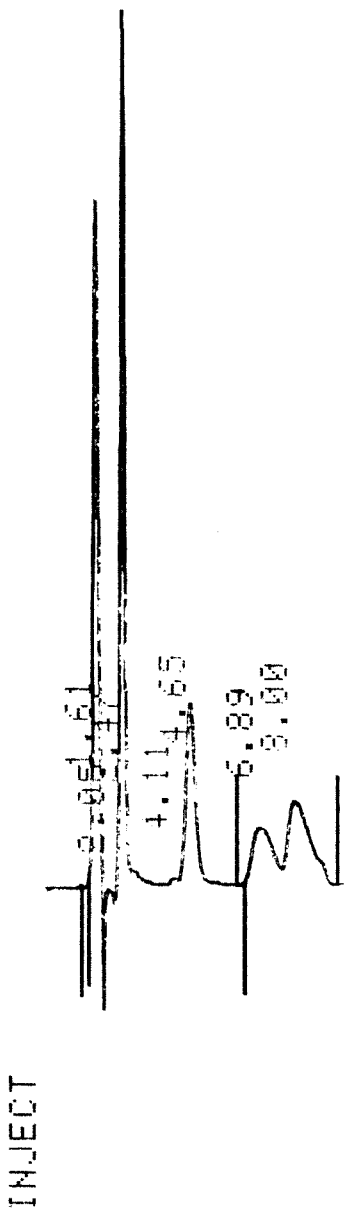

INJECT
4.11
4.65
6.89
8.00

FIGURE 3.4

Elution profiles of normal platelet extracts (to which nucleotide standards had been added) using System B and the method of Rao *et al.* (1981).

Peaks in order of elution are;

(i) Adenine
(ii) AMP
(iii) ADP
(iv) ?
(v) ?

Chromatographic conditions;

Flow Rate - 3 ml/min
Meter Range - 0.20 AUFS
Chart Speed - 0.25 cm/hour
Injection Volume - 20 µl

To determine the distribution of radioactivity among the separated components, two procedures were tried;

(i) Eluate corresponding to the duration of integration of each peak by the data processor was collected and radioactivity determined for each fraction. Only 26% of the radioactivity applied to the column was recovered in the peaks. Most of this was recovered from the AMP fraction (53%), while 38% was from adenine and 8.6% was from ADP. Other peaks contained negligible radioactivity.

(ii) An identical sample was injected onto the column, and consecutive fractions of approximateley 100 µl each were collected. Most of the radioactivity was in the AMP fraction (45%), while 31% was from adenine and 12% from ADP. This time, however, 7.4% was recovered from peak A and 4.2% from peak B. The total recovery was 34% and only background levels of radioactivity were detected between the integrated peaks.

The results presented above indicate that;

(i) platelet extracts could be passed through a pre-column (Sep-Pak) with almost total recovery of radioactivity and maintenance of the pattern of distribution of this radioactivity among adenine nucleotides. Nucleotide standards could also be passed through the pre-column with total recovery (determined by absorbance at 254nm)

(ii) nucleotide standards could be consistently and unequivocally separated on the column

(iii) the concentration of nucleotides in platelet extracts was not high enough to produce integratable peaks

(iv) the addition of nucleotide standards to platelet extracts resulted in the non-appearance of an identifiable ATP peak, and the appearance of two unidentified peaks

(v) less than half of the radioactivity applied to the column was recovered in the eluate and most of this was from the adenine and AMP fractions.

High levels of radioactive adenine and AMP may indicate that ATP and ADP are being dephosphorylated under the chromatographic conditions employed.

3.3.2 Method 2: Solomons and Masson (1984a).

Later in the current research, the HPLC method of Solomons and Masson (1982a) was briefly considered. Platelet extracts were passed through a pre-column as before, and aliquots were injected onto the main column in series with a guard column. Since the nucleotides in the platelet extract were not present at high enough concentrations to provide integratable peaks, the extract was mixed with nucleotide standards before application to the main column.

As with the first HPLC method tried, unidentified material absorbing at 254nm appeared in the region of the ATP peak. Insufficient time was available to determine the distribution and recovery of radioactivity.

Figures 3.5 and 3.6 show separations of standard nucleotides and standard nucleotide/platelet extract mixtures respectively.

3.4 STABILITY OF ADENINE NUCLEOTIDES DURING STORAGE.

It had been intended to study the effect of storage under various conditions on the stability of adenine nucleotides in platelet extracts using HPLC since only one brief report on the subject was found (Brown, 1971). However, given the variability of the above HPLC techniques when applied to platelet extracts, it was decided to use solutions of commercially prepared ATP for this experiment.

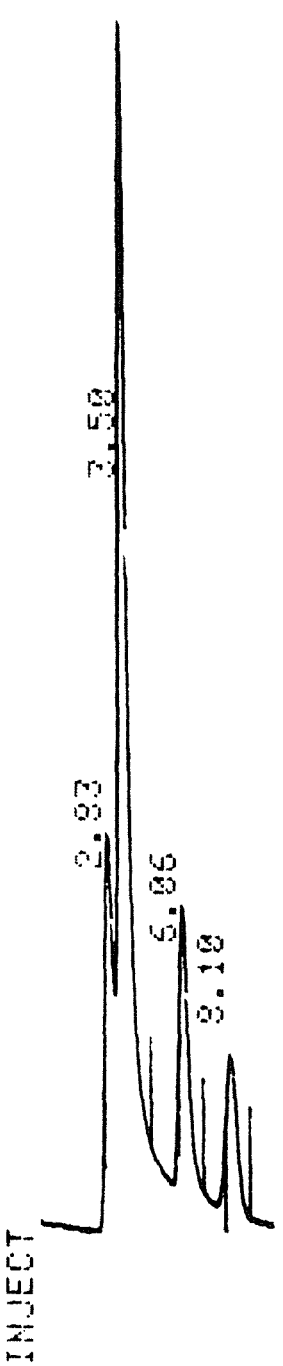
Fourteen solutions of ATP were prepared volumetrically in buffer (pH 4.3) and each solution was adjusted to a pH value between 1 an 13. Each solution was divided into two portions: one portion was stored at -20^{o}C for 40 days while the other was immediately analysed by HPLC. The response was determined again in 40 days using an identical method. Each response was calculated as a percentage of the initial response and this is shown in Figure 3.7. Maximum stability was obtained when ATP was stored at pH9-10.

FIGURE 3.5

Separation of nucleotide standards using System B and the method of Solomons and Masson (1984b).

Peaks in order of elution are;
(i) ATP
(ii) ADP
(iii) Hypoxanthine
(iv) AMP

Chromatographic conditions;
Flow Rate - 3 ml/min
Meter Range - 0.10 AUFS
Chart Speed - 0.25 cm/min
Injection Volume - 10 µl


INJECT
2.93
3.58
5.86
6.18

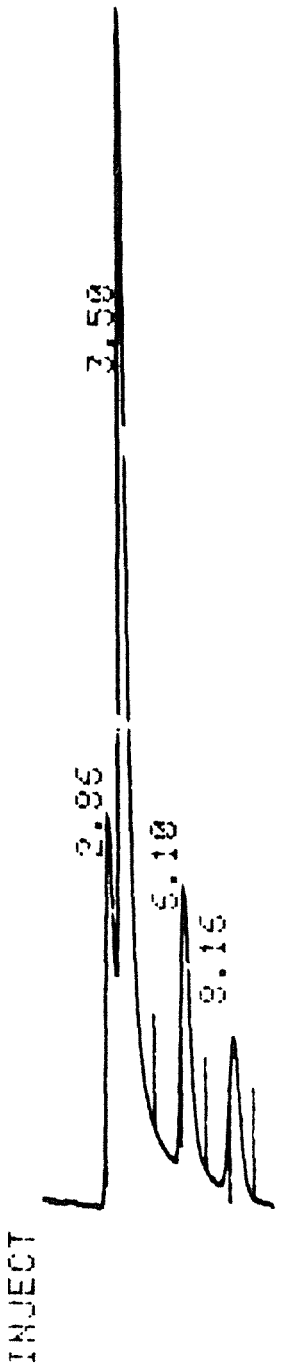

INJECT
2.95
3.58
6.18
6.15

FIGURE 3.6

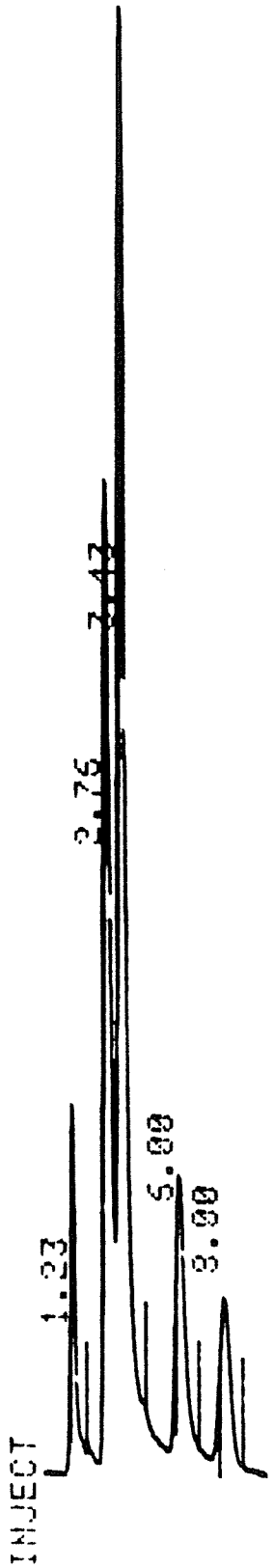
Elution profiles of normal platelet extracts (to which nucleotide standards had been added) using System B and the method of Solomons and Masson (1984b).

Peaks in order of elution are;

(i) ?
(ii) ATP
(iii) ADP
(iv) Hypoxanthine
(v) AMP

Chromatographic conditions;

Flow Rate - 3 ml/min
Meter Range - 0.20 AUFS
Chart Speed - 0.25 cm/hour
Injection Volume - 20 µl


INJECT
1.23
2.75
3.43
5.00
5.80

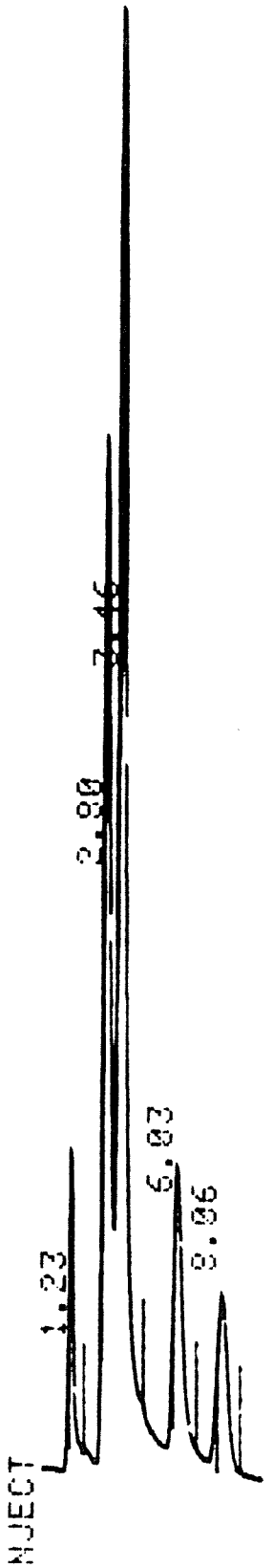
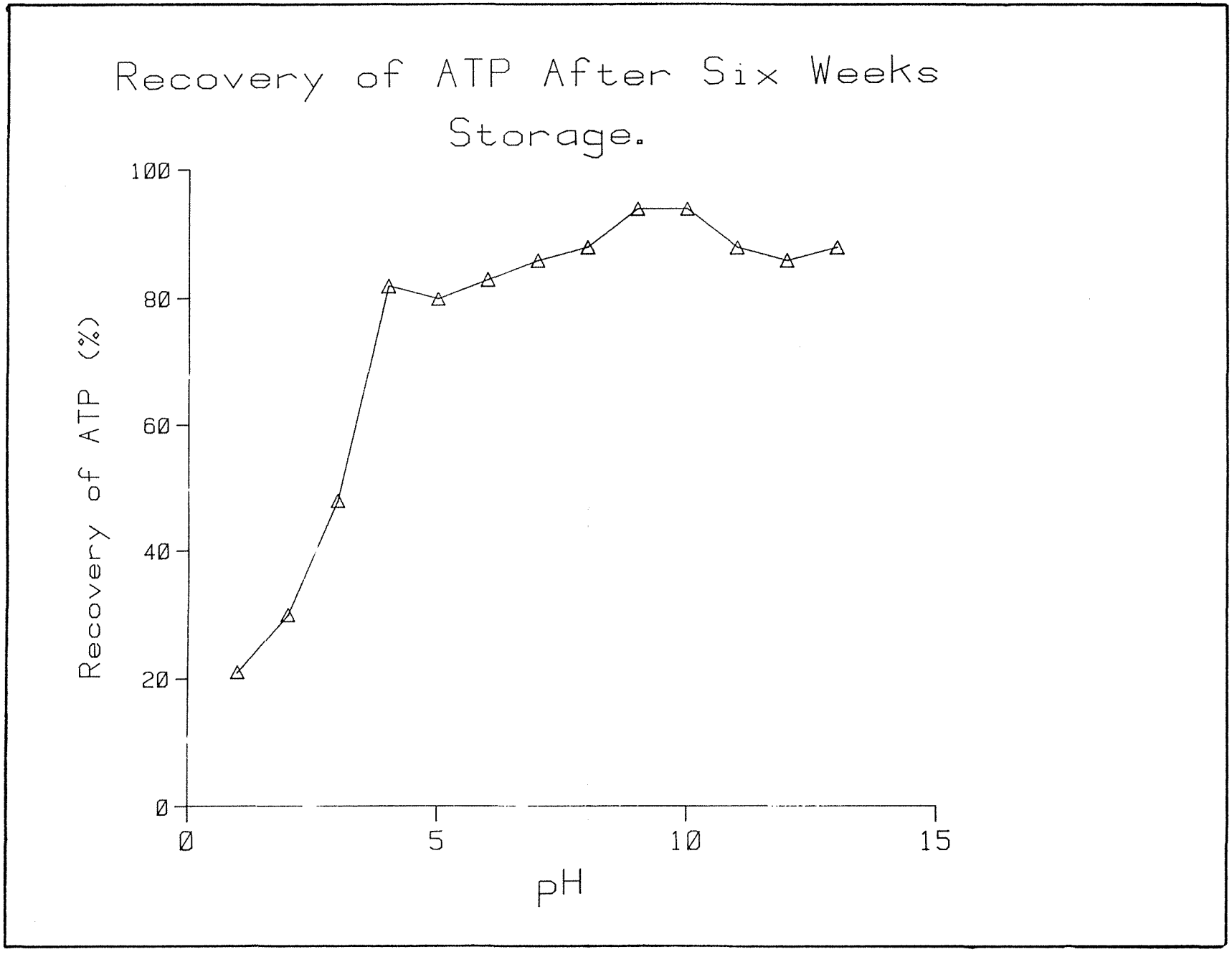

INJECT
1.23
2.80
3.46
5.07
5.86

FIGURE 3.7

Recovery of ATP stored at different pH values.

HPLC response (peak area) was determined for solutions of ATP that had been stored at -20°C for six weeks, and expressed as a percentage of the HPLC response prior to storage.


Recovery of ATP After Six Weeks Storage.
Recovery of ATP (%)
100
80
60
40
20
0
0
5
10
15
pH

CHAPTER 4: RESULTS.

4.1 ANALYSIS OF STOCK ADENINE.

The initial shipment of [8-^{14}C]adenine had been previously used in this department and was analysed for purity prior to use. A small amount of stock [8-^{14}C]adenine solution was spotted onto Whatman No. 1 chromatography paper, overlayed with 2 µl of a one per cent solution of adenine marker, and developed by descending chromatography. An autoradiogram (Osray M3 film, 3 weeks exposure) made of the area between the origin and the adenine marker on the chromatogram revealed a single spot corresponding to the adenine marker and no other spots were visible.

4.2 EVALUATION OF THE METHOD OF HOLMSEN AND ROZENBERG (1968a).

To assist in experimental design it was necessary to know the rate of formation of adenine nucleotides in the particular system being used. Holmsen and Rozenberg (1968a) reported that [8-^{14}C]adenine was taken up by platelets with a V_{max} of 100 picomoles/min/10^9 platelets and a K_m of 159 nM (compared with a V_{max} of 397 pmoles/min/10^9 platelets and a K_m of 453 nM for the uptake of adenosine). Mills (1973) reported that 85-95% of added radioactivity was incorporated into intracellular adenine nucleotides after 60 minutes, while Holmsen and Rozenberg (1968a) observed that this uptake was linear. The radioactivity remained in the platelets for 1-2 hours during storage at 37^{o}C with only slow loss at about 5%/hour after that (Mills, 1973).

However, for the purposes of this work, it was sufficient to determine the time at which most of the [^{14}C]adenine was incorporated into platelet adenine nucleotides. This was indicated by the presence of a minimum level of free radioactive adenine in the platelet extracts.

To obtain the desired information [8-^{14}C]adenine was added to a suspension of platelets samples of 200 µl each were taken from the incubating PRP as soon as possible after the addition of the radioactive material, and after 30, 60, and 90 minutes The platelets extracted with the method of Holmsen and Rozenberg (1968a) and an aliquot of each neutral extract was applied to Whatman no.1 chromatography paper which was developed by descending chromatography. The chromatogram was cut up into 1 cm squares so that each "sample-lane" was subdivided both laterally and longitudinally and the radioactivity of each square was determined.

Results indicated that spreading of the labelled compounds occurred since labelled components were detected across the entire lateral axis and not just in the sample lanes. This was possibly due to the volume of sample loaded, and care must be exercised to keep streaks as narrow as possible and to allow adequate separation of the applied samples.

The time-course of adenine incorporation was repeated twice (this time with adequate separation of sample lanes) and the results are presented in Table 4.1 and Figure 4.1.

	EXPERIMENT #1				EXPERIMENT #2			
TIME OF INCUBATION (MINUTES)	0	30	60	90	0	30	60	90
A T P	3.0	53	56	58	8.2	54	52	51
A D P	0.71	13	17	14	3.0	17	13	15
A M P	0.14	0.55	0.81	0.80	1.2	1.5	2.3	4.0
H X	1.7	12	21	20	1.3	19	22	22
INOSINE	0.51	0.82	0.43	--	2.2	3.3	5.2	2.2
ADENINE	94	20	1.3	4.4	82	1.8	2.4	6.8
A T P/A D P	4.2	4.1	3.3	4.1	2.7	3.2	3.3	3.4
ADENYLATE ENERGY CHARGE	0.86	0.89	0.87	0.89	0.88	0.86	0.84	0.86

TABLE 4.1

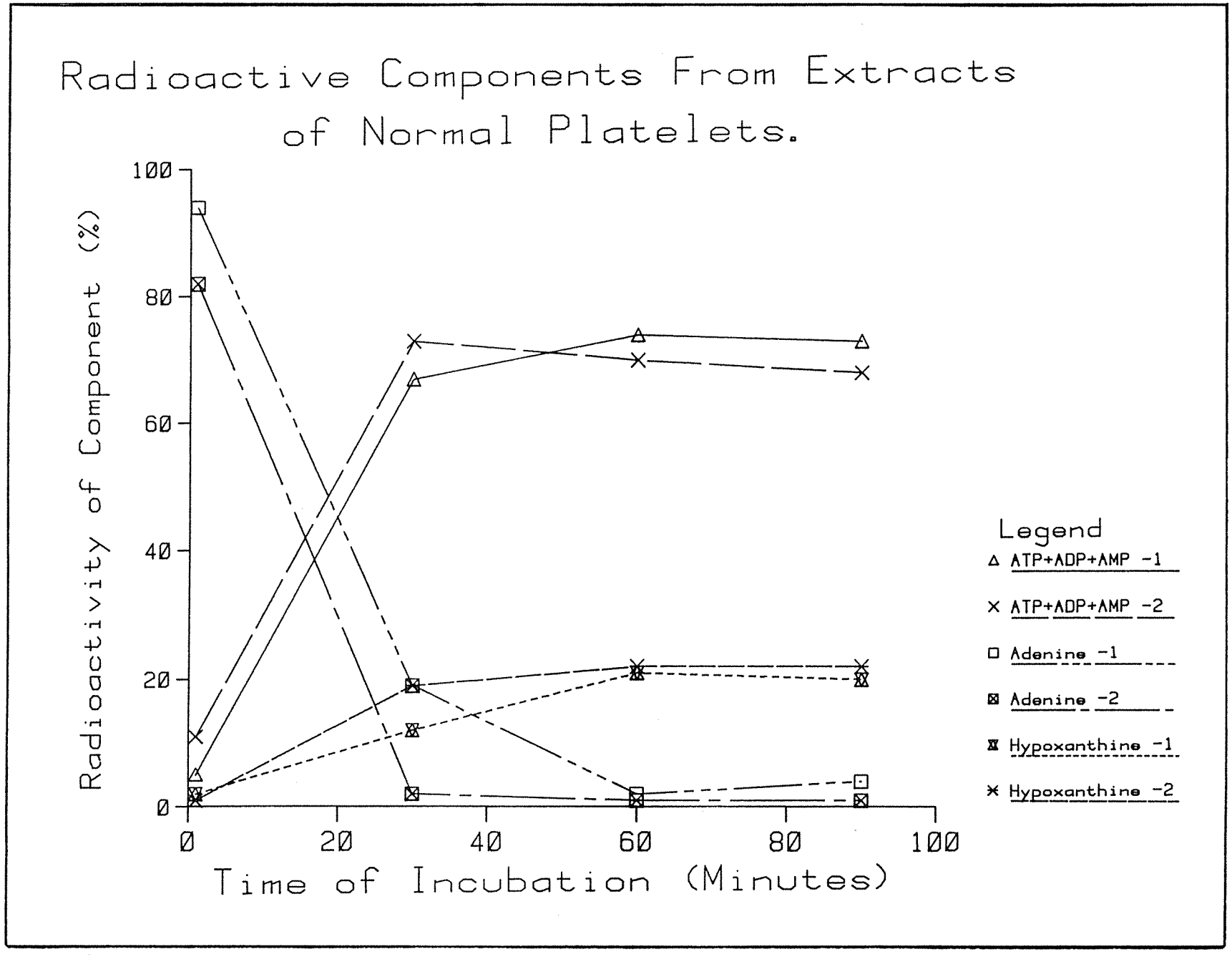
Radioactive components extracted from normal platelets incubated in the absence of halothane with the method of Holmsen and Rozenberg (1968a).

Numbers repesents radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

FIGURE 4.1

Radioactive components extracted from normal platelets incubated in the absence of halothane with the method of Holmsen and Rozenberg (1968a).

The vertical axis repesents radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract.


Radioactive Components From Extracts of Normal Platelets.
Radioactivity of Component (%)
100
80
60
40
20
0
0
20
40
60
80
100
Time of Incubation (Minutes)
Legend
ATP+ADP+AMP -1
ATP+ADP+AMP -2
Adenine -1
Adenine -2
Hypoxanthine -1
Hypoxanthine -2

The graph shows that radioactive free adenine was present at its highest level in platelets immediately after the addition of [8-^{14}C]adenine (0 minutes), after which the level decreased rapidly to a minimum between 30 and 60 minutes. Levels of radioactive ATP (the major labelled product) increased with equal rapidity to a maximum at 30 minutes and decreased only slightly after 60 and 90 minutes. Levels of radioactive ADP, AMP, HX, IMP, and inosine were also determined, but for the sake of clarity, they are not all shown separately in Figure 4.1.

While the disappearance of radioactive adenine occurred more rapidly in the second experiment approximately the same level of incorporation of label into adenine nucleotides as in the first experiment was reached. There appeared to be an inverse relationship between the levels of HX and free adenine which did not affect the level of total adenine nucleotides. In both experiments the level of radioactive HX approached 20% after 60 minutes. Other workers (e.g. Holmsen and Rozenberg, 1968a) have found that after incubation of platelets with [8-^{14}C]adenine for 60 minutes the radioactive HX comprises less than 1% of the radioactive components separated. Since adenine nucleotides are converted to HX via AMP (Holmsen and Day, 1971), the presence of abnormally high amounts of radioactive HX may indicate that catabolism of adenine nucleotides had occurred.

Normally, HX produced during catabolic processes is extruded into the extracellular medium (Akkerman et al., 1983). In the current procedure, however, there was no separation of platelets and plasma prior to platelet extraction. Therefore, the results represent

combined platelet and plasma radioactivity distribution profiles.

To investigate the possibility that apparent distribution of radioactivity among the separated components was dependent on the method of extraction employed, a second procedure was evaluated.

4.3 EVALUATION OF THE METHOD OF SOLOMONS et al. (1978).

Since the current research was inspired by the original work of Solomons et al. (1978), their extraction procedure was adopted. The procedure was a comparatively long process which required more manipulation of small amounts of sample than the previous method. After losing about 38 % of the added cpm in the $KClO_4$ precipitate nearly all of the remaining radioactivity was adsorbed onto charcoal. None was eluted by water and only 20 % by pyridine. About 40 % of the cpm were thus lost with the charcoal precipitate.

Table 4.2 shows the distribution of radioactivity among the separated components of extracts of duplicate 60 minute incubations of PRP. Columns 1 and 2 show the amounts of labelled nucleotides extracted from the platelet button (expressed as a percentage of total radioactivity extracted) while columns 3 and 4 apply to the supernatant plasma.

The apparent incorporation of [8-^{14}C]adenine into total adenine nucleotides was slightly higher in these experiments than with the extraction method of Holmsen and Rozenberg (1968a). This was partly due to decreased amounts of radioactive HX.

	PLATELET EXTRACT		SUPERNATANT PLASMA	
	EXPT #1	EXPT #2	EXPT #1	EXPT #2
ATP	32	45	16	30
ADP	39	24	17	12
AMP	5.4	13	10	8.1
HX	16	11	20	19
ADENINE	7.0	6.1	36	31
ATP/ADP	0.82	1.9	0.94	2.5
ADENYLATE ENERGY CHARGE	0.67	0.70	0.57	0.72

TABLE 4.2

Radioactive components extracted from normal platelets incubated in the absence of halothane with the method of Solomons et al. (1978).

Numbers repesents radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

The level of radioactive free adenine was higher after 60 minutes in these experiments than in the previous two. However, the results for the individual adenine nucleotides vary too widely for the method to be used with confidence as the basis for a test to distinguish abnormal values from normal ones.

Holmsen and Rozenberg (1968a) reported that after the separation of platelets from their incubation medium, there were no radioactive adenine nucleotides present. However, data presented in Table 4.2 indicate that 43-50% of labelled adenine in plasma was in adenine nucleotides.

The rate of transport of adenine into platelets is dependent on the condition of the membrane (Holmsen and Rozenberg, 1968a). The high levels of radioactive adenine in the extracellular medium in these experiments (31-36% of total radioactivity in the extracts) indicates that only two-thirds of the available adenine was taken up by platelets. These observations suggest that a degree of platelet membrane damage occurred either during the preparation of PRP or during incubation.

Both extraction methods thus far employed utilised PRP prepared by a procedure involving centrifugation of platelets at 1000 x g for short periods. Therefore, it was assumed that this was too harsh for the platelets and resulted in functional abnormalities and membrane damage. A survey of the literature revealed that an optimum force of 193.5 x g has been recommended for preparing PRP (Day, 1979).

However, as there are likely to be slight variations in functional integrity between PRP prepared by any method, a measure of this integrity would be useful in comparing results between experiments.

Many investigators have measured oxygen uptake by platelets.

4.4 ASSESSMENT OF PLATELET INTEGRITY.

A Clarke oxygen electrode was calibrated by placing 3 ml aerated water in a cell to indicate 100 % oxygen saturation, followed by a few mg of sodium dithionite to indicate 0 % oxygen saturation. The cell was then rinsed with distilled water and 3 ml PRP was added. A steady decrease in chart response was recorded, indicating uptake of oxygen by platelets. The cell was then rinsed with distilled water and the procedure repeated with a second 3 ml of PRP. The addition of various amounts of succinate had no effect on the rate of oxygen uptake observed.

However, since the PRP was not stirred, the observed uptake of oxygen may have been due to local effects at the membrane of the electrode and, therefore, not a true indication of the metabolic status of the entire 3 ml of PRP. When the stirrer was engaged the chart response levelled off indicating a cessation of oxygen uptake. A visual inspection of the platelets using phase-contrast microscopy revealed many fragmented and clumped platelets (probably due to the force of mechanical stirring).

To assess the requirement for stirring of the sample in the

electrode cell, a glucose/glucose oxidase system was utilised. When the stirrer was inoperative a constant rate of oxygen uptake was observed that was very similar to the rate for PRP, while engaging the stirrer caused a rapid increase in oxygen uptake in the system. This showed that stirring was necessary for substrate to be distributed throughout the system and, since this could not be performed without disrupting the platelets, the procedure could not be used as an indicator of platelet integrity.

Manometry in siliconised Warburg flasks may give the required evaluation (Estes et al., 1962) but this was not tried.

4.5 REANALYSIS OF STOCK ADENINE.

On most of the radiochromatogram scans, large peaks corresponding to the origin on the chromatograms were observed. This observation required that the composition of the stock adenine be reassessed. For this experiment 10 µl of stock [8-^{14}C]adenine was added to 1 ml plasma, and two 50 µl aliquots of this were immediately applied to separate strips of Whatman no.1 chromatography paper (along with non-radioactive markers) The chromatograms were then developed by descending chromatography and each strip was cut up, the radioactivity was counted, and the percentage of each separated component calculated.

Separated Component	Percentage (Average)
adenine	0.12
ATP	4.8
ADP	3.7
AMP	4.3
HX	14
origin	73

This showed that virtually none of the label was still in the form of free [8-^{14}C]adenine. Some had been converted to radioactive adenine nucleotides and radioactive HX, but most was in a form that did not move from the origin. It was assumed that bacterial contamination of the stock radioactive adenine occurred when the first experiments were performed, with subsequent metabolism of the base to HX and substantial incorporation into bacterial polynucleotide.

Since platelets cannot utilise ATP, ADP or AMP in the synthesis of adenine nucleotides (Holmsen and Day, 1971), HX must have been utilised for this purpose. This confirms the existence of a salvage pathway for HX in platelets (Rivard et al., 1974).

A fresh shipment of [8-^{14}C]adenine was checked for purity immediately upon arrival and found to contain the following percentages of labelled compounds when the above experiment was repeated:

Separated Component	Percentage (Average)
adenine	98
ATP	0.15
ADP	0.089
AMP	0.23
origin	1.1

A small amount of radioactivity did not move from the origin, but this has been observed by others (Akkerman et al., 1983).

Immediately prior to the receipt of a fresh shipment of stock [8-^{14}C]adenine an investigation into the use of HPLC in preference to the paper chromatographic method for nucleotide separation was begun (Chapter 3). To enable comparisons to be made between this study and those of Rao et al. (1981), on whose work the HPLC investigation was based, a similar method of platelet extraction must be used. Since only slight modifications of the method of Holmsen and Rozenberg (1968a) were necessary in the application of Rao's procedure, comparisons could also be made with the results obtained with the former method. This time, however, a more gentle method of PRP preparation was employed.

4.6 EVALUATION OF THE METHOD OF RAO et al. (1981).

For each experiment described in this section 20 ml blood was

drawn from separate donors and PRP produced from each sample using Method 2. Incubation was for 60 minutes after which the nucleotides were extracted and separated by paper chromatography. The relative amounts of each nucleotide are shown in Table 4.3.

(For the calculation of results, the amount of each component was initially expressed as a percentage of the total radioactivity recovered in the platelet extract. The level of free adenine was then subtracted and the amounts of ATP, ADP, AMP, and HX were expressed as percentages of the new total - the second figure appearing in the Table).

The level of radioactive ATP averaged 53% in the five experiments reported, while the level of radioactive ADP was 40% and radioactive AMP 5.5%. The level of radioactive HX was always less than 3% indicating that very low catabolism of adenine nucleotides occurred either during preparation and incubation of PRP or during the extraction of platelets. The level of free radioactive adenine in the extracts varied between 4.7 and 17%; with no apparent relationship between the level of adenine and any other nucleotide. However, the only time traces of IMP (a product of adenine nucleotide catabolism) were detected was on the two occassions that the level of radioactive adenine reached 17%.

The adenylate energy charge (AEC) was 0.74 ± 0.03, which is well below the value of 0.90 reported for PCA extracted platelets by Daniel et al. (1980), and the ATP/ADP ratio was 1.3 ± 0.02.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	EXPERIMENT #					MEAN
	1	2	3	4	5	
A T P	45	40	48	47	49	53 ±1.9
A D P	30	38	37	37	35	40 ±3.7
A M P	6.6	2.1	7.6	3.4	4.7	5.5 ±2.2
H X	1.1	1.6	0.69	2.2	2.9	2.0 ±0.87
ADENINE	17	17	4.7	11	5.5	11 ±5.3
A T P/A D P	1.5	1.1	1.3	1.3	1.4	1.32 ±0.13
ADENYLATE ENERGY CHARGE	0.74	0.74	0.72	0.75	0.75	0.74 ±0.011

TABLE 4.3

Radioactive components extracted from normal platelets incubated in the absence of halothane with the method of Rao et al. (1981).

Numbers repesent radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

The effect of the level of radioactive AMP on the AEC can be assessed by considering experiments 3 and 4. The levels of ATP and ADP were very similar in these experiments but a difference of 4.2% in the level of AMP caused a difference of 0.03 in the AEC. A difference in AEC of this magnitude may relect a large difference in the metabolic adenine nucleotide pools of platelets (Mills, 1973). A measure of platelet integrity (such as oxygen consumption) would be useful in determining whether such variability is to be expected between experiments or is the result of alterations in the energy status of platelets in suspension. Metabolic stress has been shown to lead to a small drop in AEC causing a decreased total concentration of adenine nucleotides (Ashby and Holmsen, 1983a and 1983b). In two of the experiments, the radioactive components of the supernatant plasma were separated and their proportions determined. Most of the label was free adenine (average 67%), while an average of 25% was incorporated into HX and very low levels of radioactive adenine nucleotides were detected (average 1.4%). Most of the remaining label was incorporated into inosine (average 4.1%).

Rao's method was further investigated with a view to using it in the remainder of this research.

4.7 EFFECT OF ADENINE.

In previous experiments, and most published work the specific activity and total activity of the added [8-^{14}C]adenine was poorly controlled; 10 µl [8-^{14}C]adenine of variable specific activity stock

being added to an unknown pool of cold adenine in the PRP.

It was, therefore, important to investigate the effects of varying the amount of radioactive adenine added to the PRP on the incorporation of label into platelet adenine nucleotides.

Separate 1 ml aliquots of PRP were incubated for 60 minutes with added radioactive adenine varying from 5 to 30 µl. The volumes were adjusted with 0.9% NaCl so that all the tubes were identical in this respect. The platelets were extracted (using Rao's procedure) and the radioactive components were separated by paper chromatography. Figure 4.2 and Table 4.4 show that there was very little effect on the relative amounts of nucleotides as the volume of [8-^{14}C]adenine added to PRP increased.

There was a roughly linear decrease in the level of radioactive ATP of 6% between five and 30 µl with a concomitant increase in the level of radioactive ADP and no significant change in other separated components. The AEC was unchanged as increasing amounts of radioactive adenine were added, indicating that the total adenylate pool was constant (Ashby and Holmsen, 1983a and 1983b).

The radioactive ATP contributed 58% of the radioactivity in separated components at 10 µl of radioactive adenine which further extends the range of normal values.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	VOLUME OF RADIOACTIVE ADENINE ADDED (MICROLITRES)			
	5	10	20	50
A T P	60	58	57	54
A D P	35	37	38	39
A M P	1.9	1.9	2.4	2.2
H X	0.97	2.8	1.3	1.2
ADENINE	1.1	0.32	0.84	2.7
A T P/A D P	1.7	1.6	1.5	1.4
ADENYLATE E N E R G Y C H A R G E	0.80	0.79	0.78	0.77

TABLE 4.4

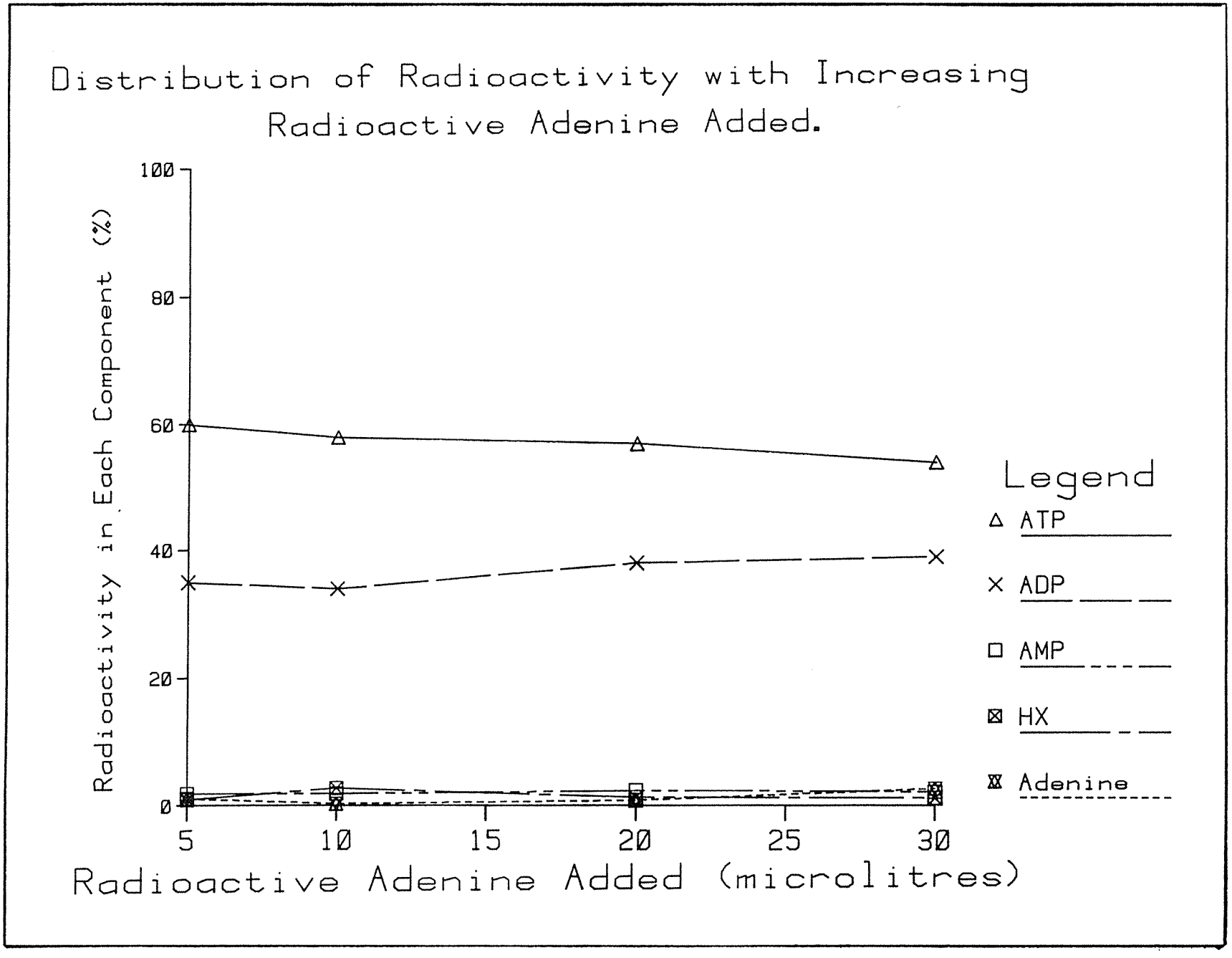
Radioactive components extracted from normal platelets incubated in the absence of halothane, but with increasing amounts of radioactive adenine, with the method of Rao *et al.* (1981).

Numbers repesent radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

FIGURE 4.2

Radioactive components extracted from normal platelets incubated in the absence of halothane, but with increasing amounts of radioactive adenine, with the method of Rao *et al.* (1981).

Numbers repesent radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.


Distribution of Radioactivity with Increasing Radioactive Adenine Added.
Radioactivity in Each Component (%)
100
80
60
40
20
0
5
10
15
20
25
30
Radioactive Adenine Added (microlitres)
Legend
ATP
ADP
AMP
HX
Adenine

Whether this range is a real indication of the variation in metabolic capacity between various PRP preparations or a reflection of slight differences in technical procedure remains to be determined. The tendency for later values to be higher than initial ones raises the possibility that increasing technical expertise in performing the experiments was a factor in the spread of results.

The addition of 10 µl of radioactive adenine was retained in future incubations both to conform with other workers and to conserve [8-^{14}C]adenine. This volume contributed enough radioactivity to provide an adequate amount in the final extract.

4.8 RECOVERY OF RADIOACTIVITY.

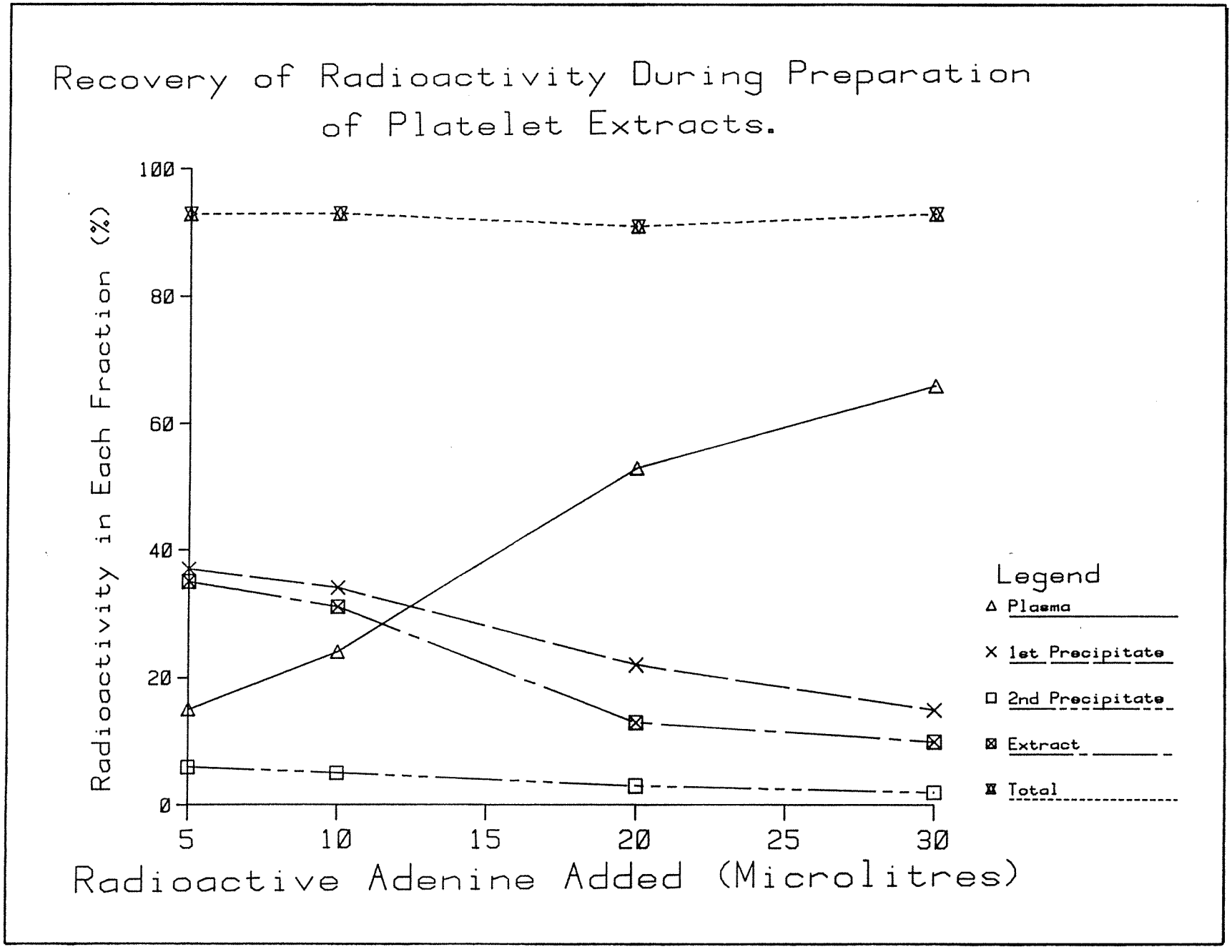
The [8-^{14}C]adenine dose-response experiment described above was also used to determine the recovery of radioactivity in platelet extracts. The total radioactivity in the extract and the supernatant plasma was calculated and the total cpm in the acid-insoluble material and in the $KClO_4$ precipitate were determined directly.

As shown in Figure 4.3, when the amount of [8-^{14}C]adenine added to the PRP was increased the amount of radioactivity found in the extract decreased, while the amount of radioactivity in the supernatant increased possibly reflecting the limited uptake of [8-^{14}C]adenine into platelets. Holmsen and Rozenberg (1968a) determined that the uptake of adenine by platelets is a linear and saturable process. The results of the above experiment are probably a reflection of this observation.

FIGURE 4.3

Recovery of radioactivity from normal platelets during extraction with the method of Rao *et al.* (1981).

Numbers represent the recovery of radioactivity in each fraction as a percentage of the total radioactivity added to the PRP.


Recovery of Radioactivity During Preparation of Platelet Extracts.
Radioactivity in Each Fraction (%)
100
80
60
40
20
0
5
10
15
20
25
30
Radioactive Adenine Added (Microlitres)
Legend
Plasma
1st Precipitate
2nd Precipitate
Extract
Total

The fraction of radioactivity in the first and second precipitates decreased with increasing [8-^{14}C]adenine.

The percentage of radioactivity in the extract and in the acid-insoluble material decreased at a similar rate. The cpm in the second precipitate also decreased roughly linearly but at an order of magnitude lower than the other fractions determined. The total recovery of cpm added to the 1 ml PRP was 89-96 %.

4.9 EFFECT OF HALOTHANE.

4.9.1 Dose-Response Experiments.

The effect of adding increasing volumes of halothane to 1 ml aliquots of PRP (volume of PRP adjusted with 0.9% NaCl) is shown in Table 4.5 and Figure 4.4. The incorporation of radioactive adenine into platelet ATP decreased rapidly up to 50 µl halothane with a slower subsequent decrease. The levels of radioactive ADP and AMP increased up to 50 µl halothane and then decreased. In contrast to the adenine dose-response experiment (see Figure 4.2), where the level of radioactive ADP displayed a dependence on the level of radioactive ATP, in the halothane dose-response experiment no such dependence was observed. Associated with the decrease in ATP levels was an increase in free radioactive adenine levels, with a concomitant increase in levels of radioactive HX.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	VOLUME OF HALOTHANE ADDED (MICROLITRES)					
	0	5	10	20	50	100
A T P	60	61	58	41	15	5.3
A D P	35	34	36	37	42	23
A M P	1.1	0.96	1.2	5.0	11	6.3
H X	1.5	2.9	2.8	7.9	9.3	6.1
ADENINE	1.9	3.0	2.9	4.4	20	48
A T P/A D P	1·71	1.8	1.6	1.1	0.36	0.23
ADENYLATE ENERGY CHARGE	0·80	0.81	0.81	0.72	0.53	0.48

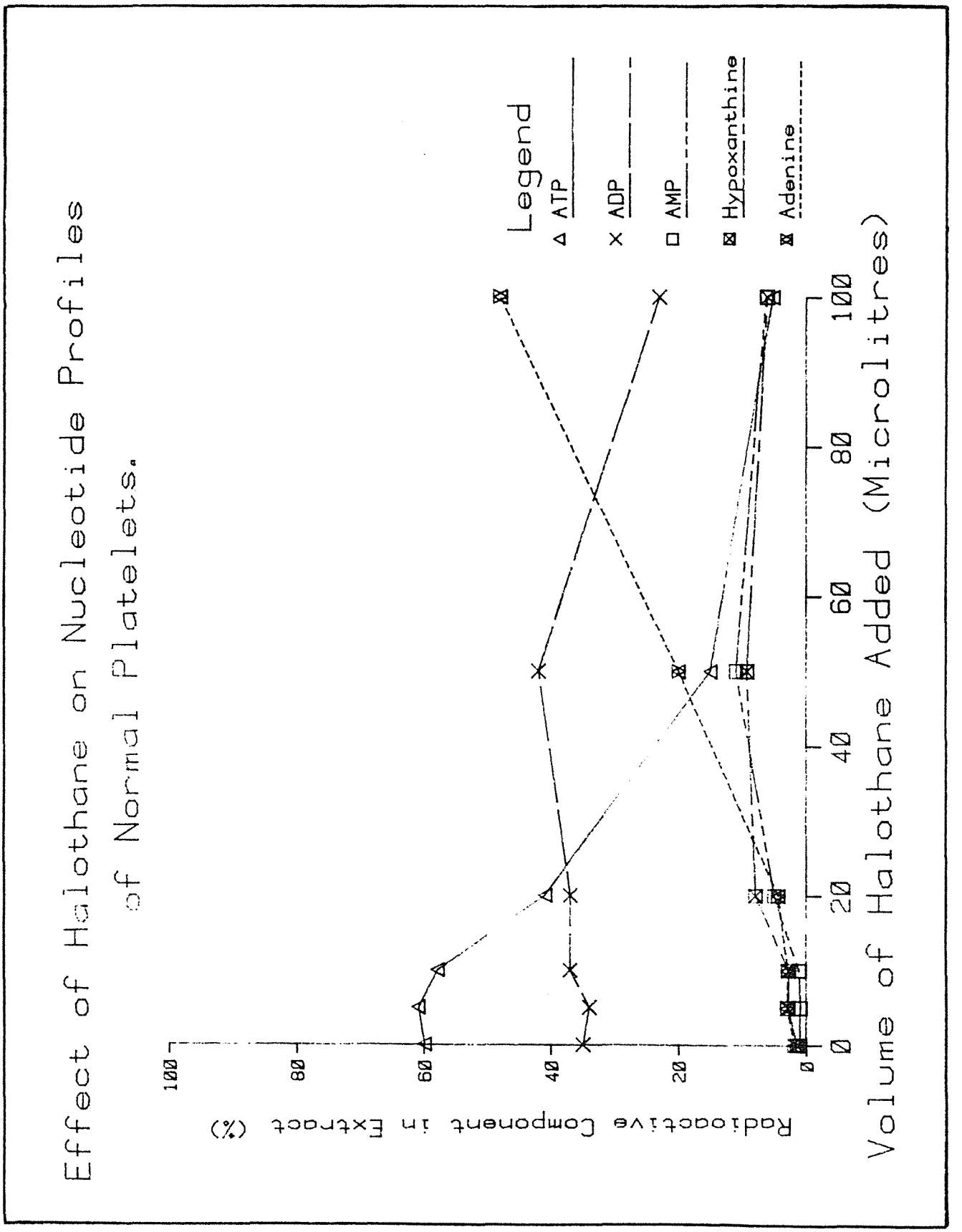
TABLE 4.5

Radioactive components extracted from normal platelets incubated in the presence of increasing amounts of halothane with the method of Rao et al. (1981).

Numbers represent radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

FIGURE 4.4

Radioactive components extracted from normal platelets incubated in the presence of increasing amounts of halothane, with the method of Rao *et al.* (1981).


Effect of Halothane on Nucleotide Profiles
of Normal Platelets.
Legend
ATP
ADP
AMP
Hypoxanthine
Adenine
Radioactive Component in Extract (%)
Volume of Halothane Added (Microlitres)
0
20
40
60
80
100

Metabolic stress results in a drop in the AEC which leads to a decrease in the total adenylate pool (Ashby and Holmsen, 1983a and 1983b). In the above series of experiments, increasing volumes of halothane caused the AEC to drop by more than two-thirds. This is consistent with the fact that the extract of platelets incubated in the presence of 100 µl halothane, contained less than one-third of the radioactivity that was present in the platelets incubated with five µl of halothane. This indicates that a sharp decrease in the total adenylate pool had occurred.

When platelets incubated in the presence of 100 µl of halothane were extracted, only three per cent of the radioactivity added to the PRP was recovered in the extract. Most of the radioactivity was present in the extracellular medium as free adenine. This indicates that transport of adenine into platelets was severely impaired. It has previously been reported that local and general anaesthetics, at high concentrations, irreversibly inhibit platelet function (Anderson et al., 1981; Dalsgaard-Nielsen and Gormsen, 1980).

The rapid fall in the level of radioactive ATP with small increases in the volume of halothane added, demonstrates the need for accuracy in the addition of halothane to PRP. All additions of halothane were made with a low volume Hamilton syringe and the sample was drawn well up the barrel of the syringe during transfer from container to the PRP to eliminate any chance of evaporation. Discrepant volumes of halothane may contribute to variability in experimental results.

4.9.2 Effect on Normal Platelets.

The effect of halothane on the incorporation of [8-^{14}C]adenine into platelet adenine nucleotides was assessed by adding 10 µl of liquid halothane to each 1 ml aliquot of PRP and then preparing a platelet extract using the extraction procedure of Rao et al. (1981). The radioactive components were separated using paper chromatography and the results are presented in Table 4.6. The level of radioactive ATP was higher and the level of ADP was lower than in extracts of platelets not incubated with halothane, while the levels of radioactive AMP and HX were about the same. Very little radioactive free adenine was present in the platelet extracts but small amounts of radioactive inosine were detected. Halothane, therefore, appeared to have a small stimulatory effect on the incorporation of adenine into ATP at the expense of ADP.

The AEC was higher and exhibited greater variability than in the absence of halothane, and the ATP/ADP ratio was slightly higher.

Therefore, the paper chromatographic method for separation of nucleotides from platelet extracts prepared by the procedure of Rao et al (1979) had been investigated and modified to the extent that technical inconsistencies would not contribute significantly to any variability observed between normal and MHS platelet nucleotide profiles.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	EXPERIMENT #					MEAN
	1	2	3	4	5	
A T P	52	59	61	70	72	66 ±7.9
A D P	37	27	30	19	21	28 ±6.5
A M P	6.2	6.6	2.1	2.1	1.6	3.9 ±2.3
H X	0.65	1.2	0.95	2.0	0.70	1.2 ±0.52
INOSINE	0.71	1.7	1.6	1.6	1.4	1.5 ±0.39
ADENINE	1.7	1.4	1.2	1.2	1.6	1.4 ±0.20
A T P/A D P	1.4	3.4	2.0	3.7	3.	2.77 ±0.90
ADENYLATE ENERGY CHARGE	0.72	0.81	0.82	0.87	0.87	0.82 ±0.055

TABLE 4.6

Radioactive components extracted from normal platelets incubated in the presence of halothane with the method of Rao et al. (1981).

Numbers repesent radioactivity of each component as a percentage of the total radioactivity recovered in the platelet extract, unless indicated.

This confidence was founded on the following facts;

(i) relative independence of incorporation of radioactive adenine into platelet adenine nucleotides on the amount of radioactive adenine added to the PRP was demonstrated

(ii) dependence of incorporation of radioactive adenine into platelet adenine nucleotides on the amount of halothane added to the PRP was demonstrated

(iii) titration of perchlorate extracts by 1 microlitre aliquots of KOH to ensure that a pH as close as possible to the end-point for optimum nucleotide recovery was introduced

(iv) storage of nucleotides (if absolutely necessary) was at the optimum pH

(v) loading of chromatographic standards was optimised to afford discrete visual (UV) separation of nucleotides

(vi) separation of lanes by an appropriate distance eliminated crossover of radioactivity.

4.10 TIME-COURSE EXPERIMENTS.

4.10.1 Normal Platelets.

Earlier (Figure 4.1), it was shown that the total level of radioactive adenine nucleotides had decreased only slightly after 90 minutes incubation at 37°C, after reaching a maximum at 30 minutes. This feature of consistency of incorporation of label after 30 minutes was used as an indication that an experiment was valid. Experiments which showed that incorporation of label varied markedly between 30 and 90 minutes were excluded on the grounds of loss of platelet integrity (for want of a better measure of integrity). Since each platelet extract in an experiment was from a separate aliquot of PRP from the same donor, each experiment incorporated its own controls.

Three normal donors each contributed 40 ml blood from which PRP was prepared and divided into aliquots of 1 ml each (2 for each time-point). Each aliquot received 10 µl of radioactive adenine and one aliquot from each pair received 10 µl of liquid halothane. All pairs of aliquots were incubated at 37°C in a shaking water-bath for 0, 5, 10, 15, 30, 60, or 120 minutes, after which platelet extracts were prepared. The results are presented in Tables 4.7 and 4.8 as well as graphically in Figures 4.5-4.9. The level of HX never rose above 3% which is consistent with earlier results and indicates that catabolism of adenine nucleotides was not occurring to a great extent.

For most components of normal platelets the level of incorporation

of label was relatively constant over the entire two hours of incubation. The exception was adenine which was present at higher levels at 0 minutes than at all subsequent determinations. This indicates that radioactive adenine entering the platelets requires at least five minutes to be converted to other compounds. Theoretically, there should be no radioactivity in platelets at 0 minutes and the detection of labelled nucleotides at this time represents the delay between adding adenine to the PRP and preparing the extract. Up to one minute usually elapsed from the addition of radioactive adenine to isolation of platelets by centrifugation, with a further 3 minutes before addition of PCA to disrupt the platelets. This delay was identical for each time sample but resulted in labelled nucleotides being detected at "0 minutes".

Nucleotide profiles from individual donors sometimes displayed considerable fluctuation up to 30 minutes but at times after this the levels of each nucleotide were reasonably constant. Fluctuations would probably still be observed at longer time intervals if more frequent samples were considered. Averaging the results smoothed out the curves somewhat but several rather large standard deviations remain a legacy of these fluctuations. Within the limits of these standard deviations there was very little difference between extracts of platelets prepared in the absence or presence of halothane for any adenine nucleotide, HX, or adenine.

Both the ATP/ADP ratio (Table 4.9 and Figure 4.10) and the AEC (Table 4.10 and Figure 4.11) remained relatively constant throughout the incubation period and were unaffected by the addition of halothane.

4.10.2 Malignant Hyperthermia-Susceptible Platelets.

Three donors who had previously been determined MHS on the basis of a muscle contracture test, each contributed 40ml blood from which PRP was prepared and divided into 1 ml aliquots. Exactly the same procedure was performed on these samples as for the normal samples. The results for each experiment are presented in Tables 4.11 and 4.12 and graphically in Figures 4.5-4.9. The fluctuations observed in the early time samples of normal platelet extracts were also evident in extracts of MHS platelets. However, when the results were averaged, relatively smooth curves again resulted.

In unstimulated MHS platelets, the amount of radioactive ATP was higher than in normal platelets, and the amount of ADP was lower. There was a significant lag phase for the incorporation of adenine into ATP which corresponded to a lag phase of similar duration for the utilisation of adenine that had been transported into the platelets. ADP formation showed a lag phase of smaller magnitude. Normal platelets do not display as large a lag phase and presumably assimilate adenine into other products almost as rapidly as it enters the platelets.

When the amounts of adenine nucleotides were determined relative to a total radioactivity that excluded free adenine, Figures 4.12-4.15 resulted.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
A T P	51 ±9.7	55 ±8.7	54 ±9.5	56 ±6.1	57 ±7.3	59 ±1.9	55 ±4.5
A D P	31 ±1.9	36 ±5.6	38 ±6.7	35 ±5.0	36 ±3.9	34 ±2.5	36 ±2.9
A M P	3.1 ±2.3	2.6 ±1.9	3.8 ±2.4	4.5 ±2.8	4.3 ±2.5	3.8 ±0.83	6.3 ±1.4
H X	2.8 ±0.95	1.4 ±0.40	1.4 ±0.43	1.2 ±0.52	0.94 ±1.1	0.77 ±0.30	0.92 ±0.25
ADENINE	10 ±5.2	4.4 ±1.5	3.0 ±0.61	3.4 ±1.5	2.1 ±1.5	2.0 ±0.92	1.8 ±0.45

TABLE 4.7

Radioactive components separated from normal platelets incubated in the absence of halothane and extracted with the method of Rao *et al.* (1981).

Numbers represent the radioactivity of each component as a percentage of the total radioactivity recovered from the platelets, unless indicated.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
A T P	50 ±8.0	56 ±6.7	55 ±11	61 ±5.2	54 ±4.0	59 ±2.2	58 ±5.0
A D P	35 ±4.0	32 ±5.1	36 ±12	31 ±3.8	34 ±3 .4	32 ±1.7	34 ±4.0
A M P	2.4 ±1.9	2.6 ±0.99	3.5 ±1.1	4.1 ±2.6	3.9 ±1.0	4.5 ±1.8	4.8 ±1.0
H X	2.0 ±1.2	1.5 ±0.38	1.8 ±1.2	0.57 ±0.30	2.2 ±1.3	1.5 ±1.4	1.2 ±0.81
ADENINE	11 ±5.1	7.6 ±1.7	3.3 ±1.3	3.3 ±1.3	4.2 ±3.7	2.8 ±2.0	1.6 ±0.80

TABLE 4.8

Radioactive components separated from normal platelets incubated in the presence of halothane and extracted with the method of Rao et al. (1981).

Numbers represent the radioactivity of each component as a percentage of the total radioactivity recovered from the platelets, unless indicated.

FIGURE 4.5

Radioactive ATP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao et al. (1981).

Vertical axis repesents radioactivity of ATP as a percentage of the total radioactivity recovered in the platelet extract.

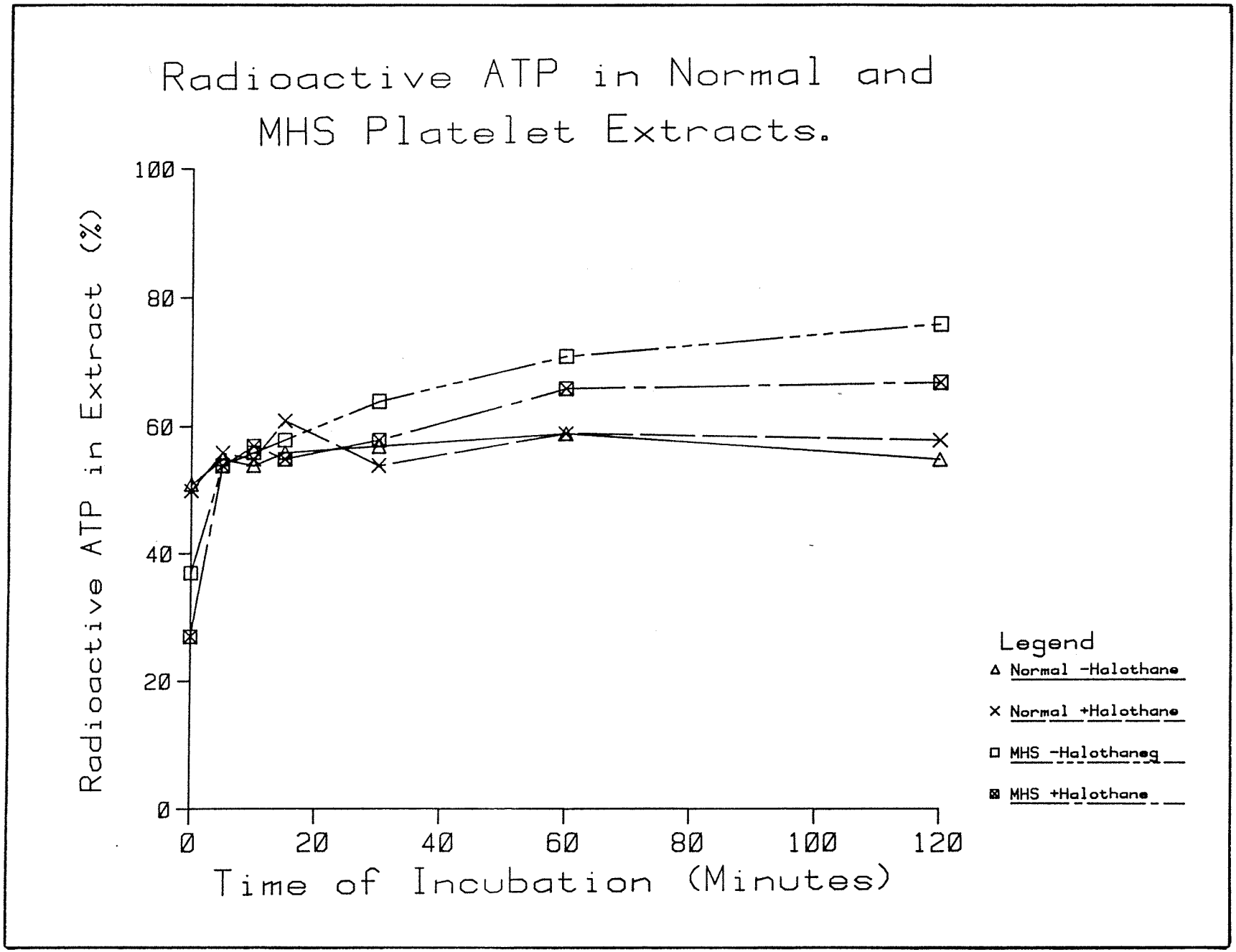

Radioactive ATP in Normal and MHS Platelet Extracts.
Radioactive ATP in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothaneg
MHS +Halothane

FIGURE 4.6

Radioactive ADP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of ADP as a percentage of the total radioactivity recovered in the platelet extract.

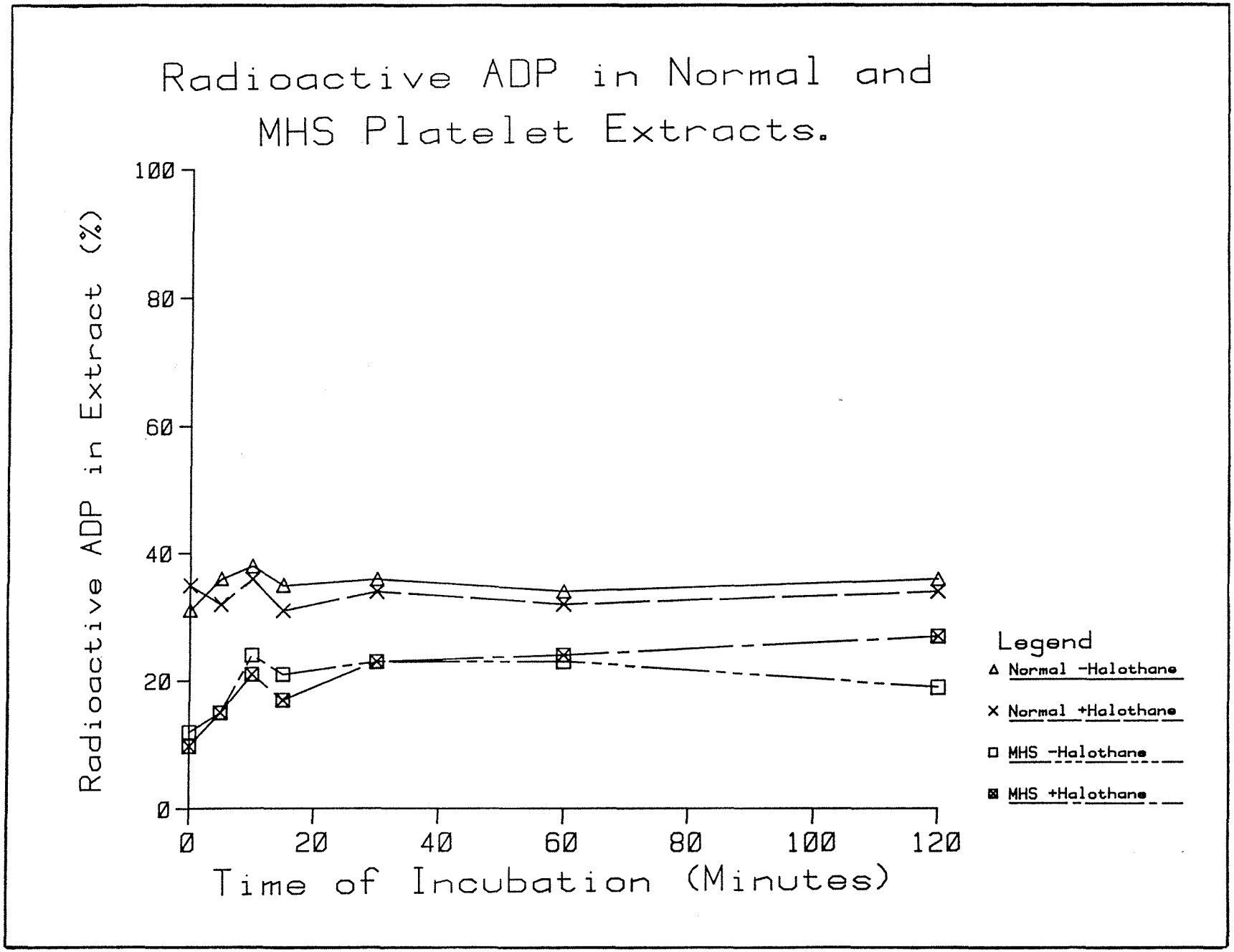

Radioactive ADP in Normal and MHS Platelet Extracts.
Radioactive ADP in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

FIGURE 4.7

Radioactive AMP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of AMP as a percentage of the total radioactivity recovered in the platelet extract.

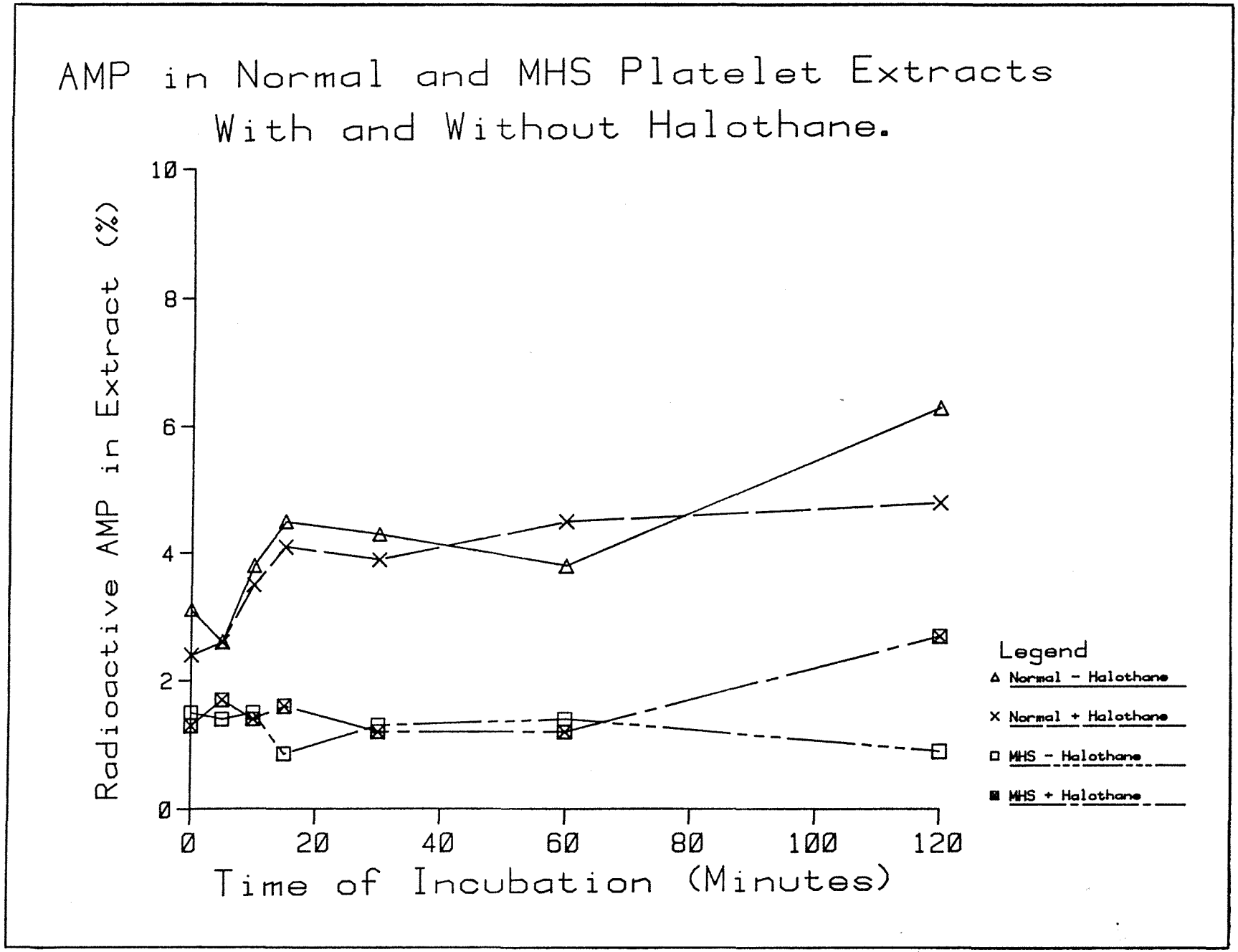

AMP in Normal and MHS Platelet Extracts
With and Without Halothane.
Radioactive AMP in Extract (%)
10
8
6
4
2
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal - Halothane
Normal + Halothane
MHS - Halothane
MHS + Halothane

FIGURE 4.8

Radioactive HX extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of HX as a percentage of the

Radioactive Hypoxanthine in Normal and MHS Platelet Extracts.

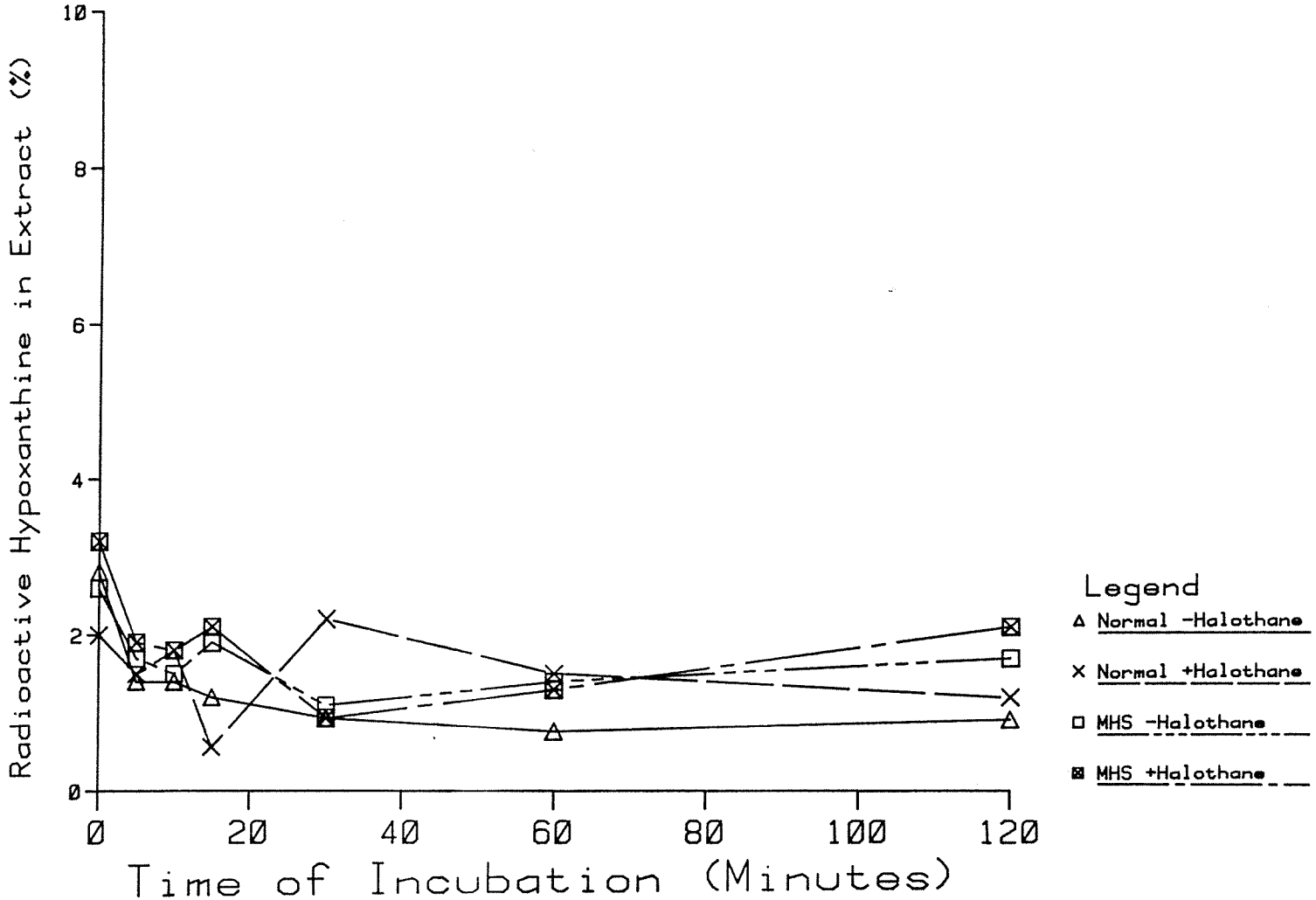
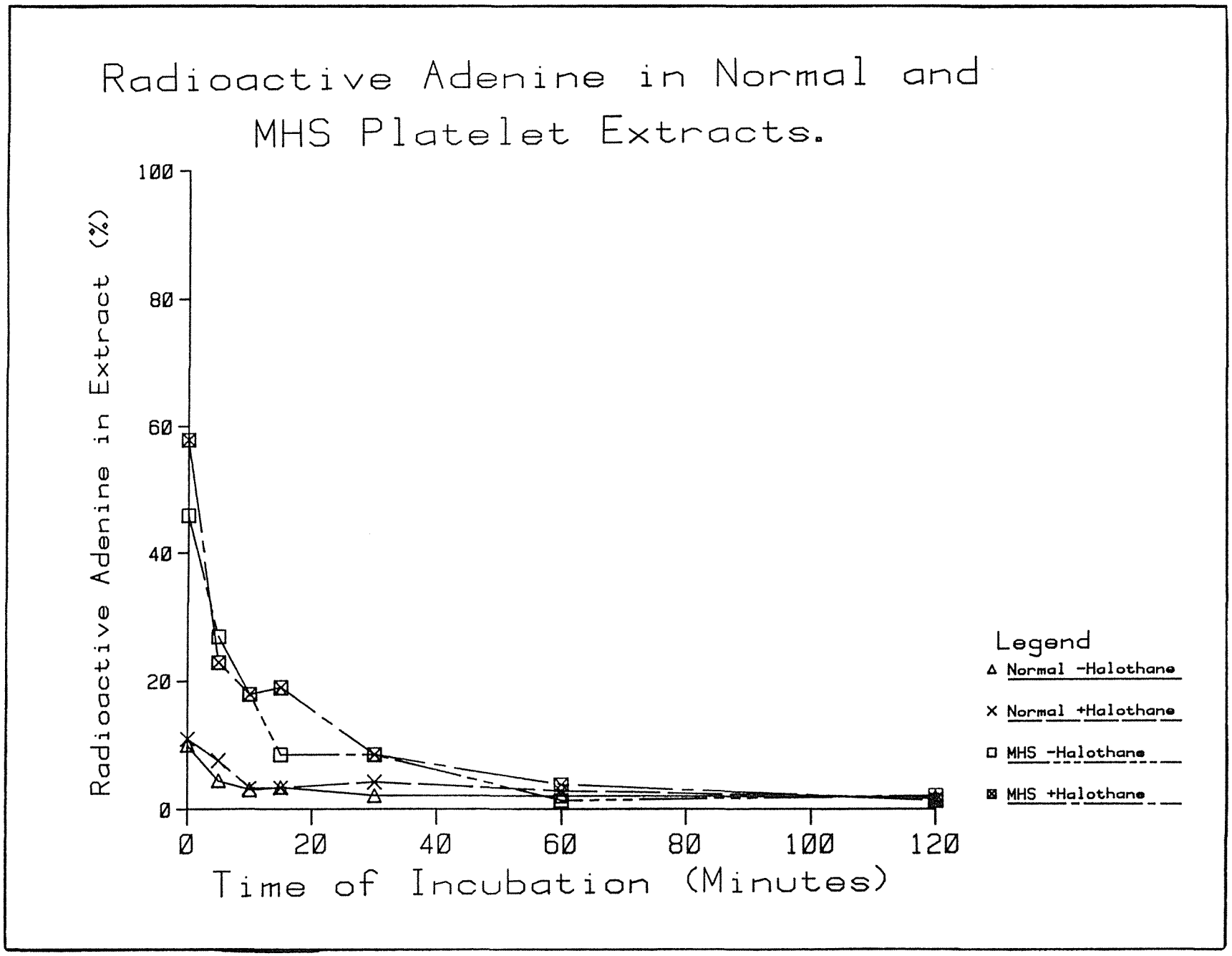


FIGURE 4.9

Radioactive adenine extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of adenine as a percentage of the total radioactivity recovered in the platelet extract.


Radioactive Adenine in Normal and MHS Platelet Extracts.
Radioactive Adenine in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

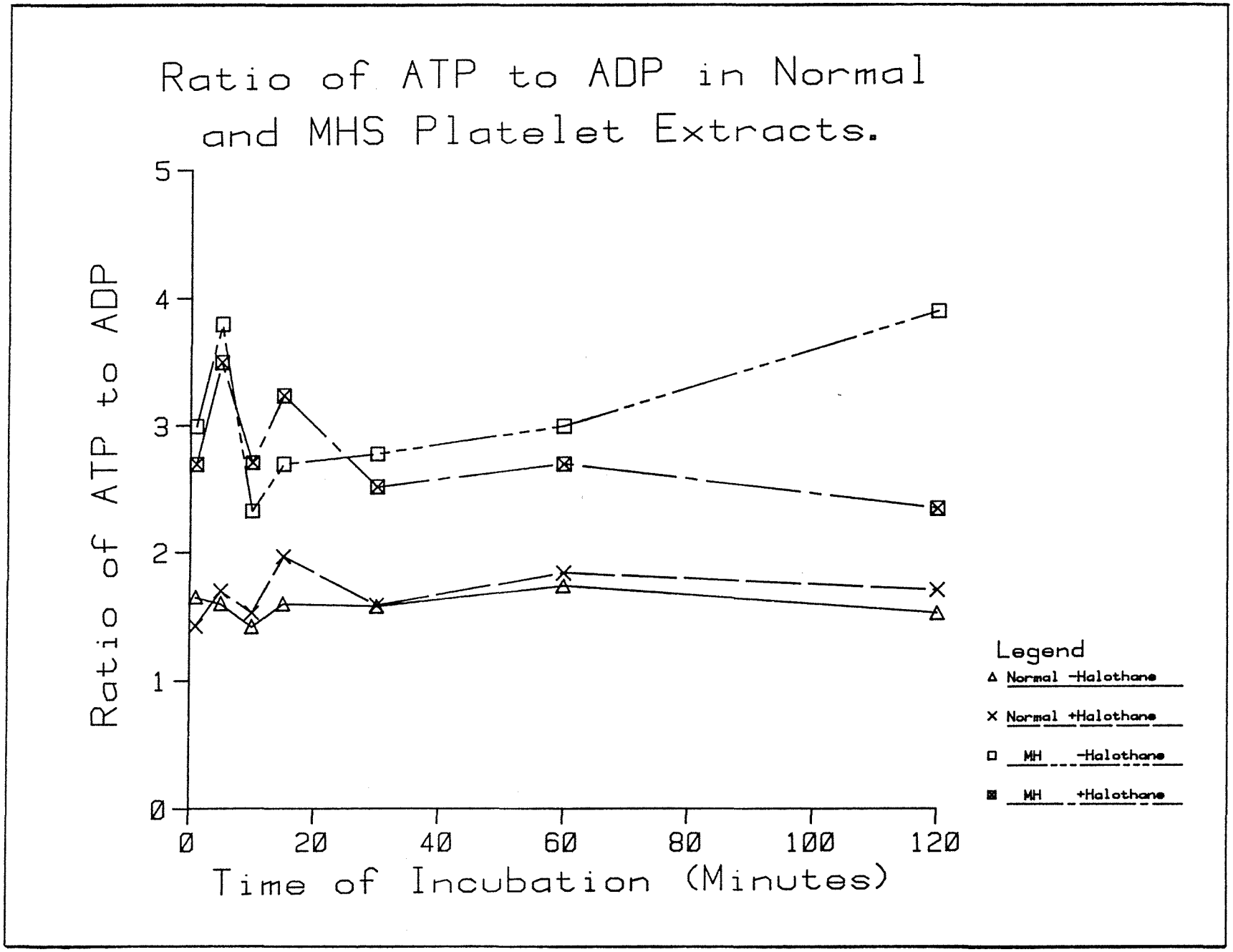
	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
N O R M A L - HALOTHANE	1.65 ±0.37	1.59 ±0.45	1.52 ±0.47	1.63 ±0.39	1.63 ±0.36	1.76 ±0.18	1.55 ±0.24
N O R M A L + HALOTHANE	1.47 ±0.32	1.84 ±0.46	1.76 ±0.73	1.99 ±0.44	1.39 ±0.44	1.83 ±0.11	1.73 ±0.32
M H S - HALOTHANE	3.04 ±0.50	3.79 ±0.73	2.48 ±0.54	2.85 ±0.38	2.83 ±0.13	3.09 ±0.44	3.99 ±0.25
M H S + HALOTHANE	2.63 ±0.73	5.46 ,±4.4	2.79 ±0.44	3.14 ±0.52	2.67 ±0.63	3.42 ±1.9	2.52 ±0.87

TABLE 4.9

The ratio of radioactive ATP/ADP extracted from normal and MHS platelets with the method of Rao *et al.* (1981).

FIGURE 4.10

The ratio of radioactive ATP/ADP extracted from normal and MHS platelets with the method of Rao et al. (1981).


Ratio of ATP to ADP in Normal and MHS Platelet Extracts.
Ratio of ATP to ADP
0
1
2
3
4
5
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MH -Halothane
MH +Halothane

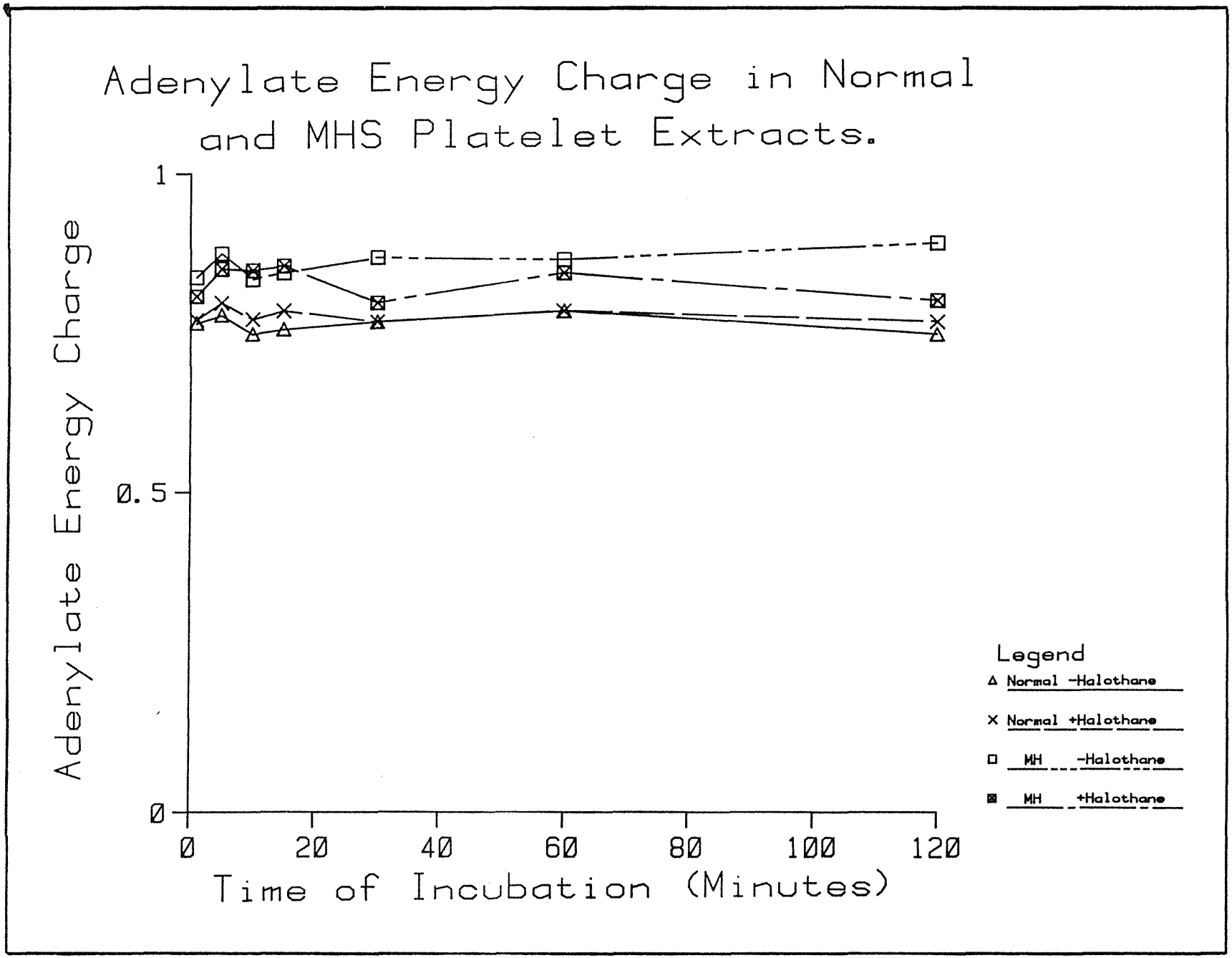
	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
N O R M A L - HALOTHANE	0.767 ±0.053	0.780 ±0.054	0.750 ±0.043	0.758 ±0.047	0.770 ±0.046	0.787 ±0.017	0.750 ±0.029
N O R M A L + HALOTHANE	0.773 ±0.045	0.800 ±0.033	0.773 ±0.067	0.787 ±0.033	0.770 ±0.022	0.787 ±0.019	0.770 ±0.028
M H S - HALOTHANE	0.840 ±0.036	0.877 ±0.017	0.837 ±0.021	0.847 ±0.017	0.870 ±0.029	0.867 ±0.012	0.893 ±0.005
M H S + HALOTHANE	0.810 ±0.079	0.853 ±0.092	0.850 ±0.016	0.857 ±0.026	0.800 ±0.080	0.847 ±0.045	0.803 ±0.058

TABLE 4.10

Adenylate Energy Charge determined from nucleotides extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

FIGURE 4.11

Adenylate Energy Charge determined from nucleotides extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).


Adenylate Energy Charge in Normal and MHS Platelet Extracts.
Adenylate Energy Charge
1
0.5
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MH -Halothane
MH +Halothane

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
A T P	37 ±13	54 ±5.8	56 ±2.5	58 ±2.1	64 ±2.6	71 ±2.8	76 ±1.7
A D P	12 ±2.6	15 ±2.6	24 ±5.3	21 ±3.1	23 ±1.3	23 ±2.6	19 ±0.82
A M P	1.5 ±0.40	1.4 ±0.89	1.5 ±0.53	0.86 ±0.58	1.3 ±0.70	1.4 ±0.66	0.90 ±0.055
H X	2.6 ±1.3	1.7 ±1.5	1.5 ±0.87	1.9 ±1.5	1.1 ±0.88	1.4 ±0.76	1.7 ±0.55
ADENINE	46 ±14	27 ±7.1	18 ±7.6	15 ±8.6	8.5 ±3.5	1.3 ±0.87	2.1 ±0.55

TABLE 4.11

Radioactive components separated from MHS platelets incubated in the absence of halothane and extracted with the method of Rao *et al.* (1981).

Numbers represent the radioactivity of each component as a percentage of the total radioactivity recovered from the platelets, unless indicated.

RADIOACTIVE COMPONENTS IN PLATELET EXTRACT	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
A T P	27 ±13	54 ±20	57 ±5.0	55 ±13	58 ±6.7	66 ±10	67 ±5.3
A D P	9.8 ±3.9	15 ±6.7	21 ±4.2	17 ±2.6	23 ±4.6	24 ±7.9	27 ±6.6
A M P	1.3 ±0.77	1.7 ±0.87	1.4 ±0.33	1.6 ±0.56	1.2 ±0.41	1.2 ±0.40	2.7 ±1.7
H X	3.2 ±3.2	1.9 ±1.5	1.8 ±1.6	2.1 ±1.9	0.94 ±0.48	1.3 ±0.76	2.1 ±1.1
ADENINE	58 ±12	23 ±12	18 ±6.4	19 ±13	8.5 ±5.3	3.8 ±1.4	1.3 ±1.3

TABLE 4.12

Radioactive components separated from MHS platelets incubated in the presence of halothane and extracted with the method of Rao et al. (1981).

Numbers represent the radioactivity of each component as a percentage of the total radioactivity recovered from the platelets, unless indicated.

FIGURE 4.12

Radioactive ATP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao et al. (1981).

Vertical axis repesents radioactivity of ATP as a percentage of the total radioactivity recovered in the platelet extract minus the amount of radioactive adenine present.

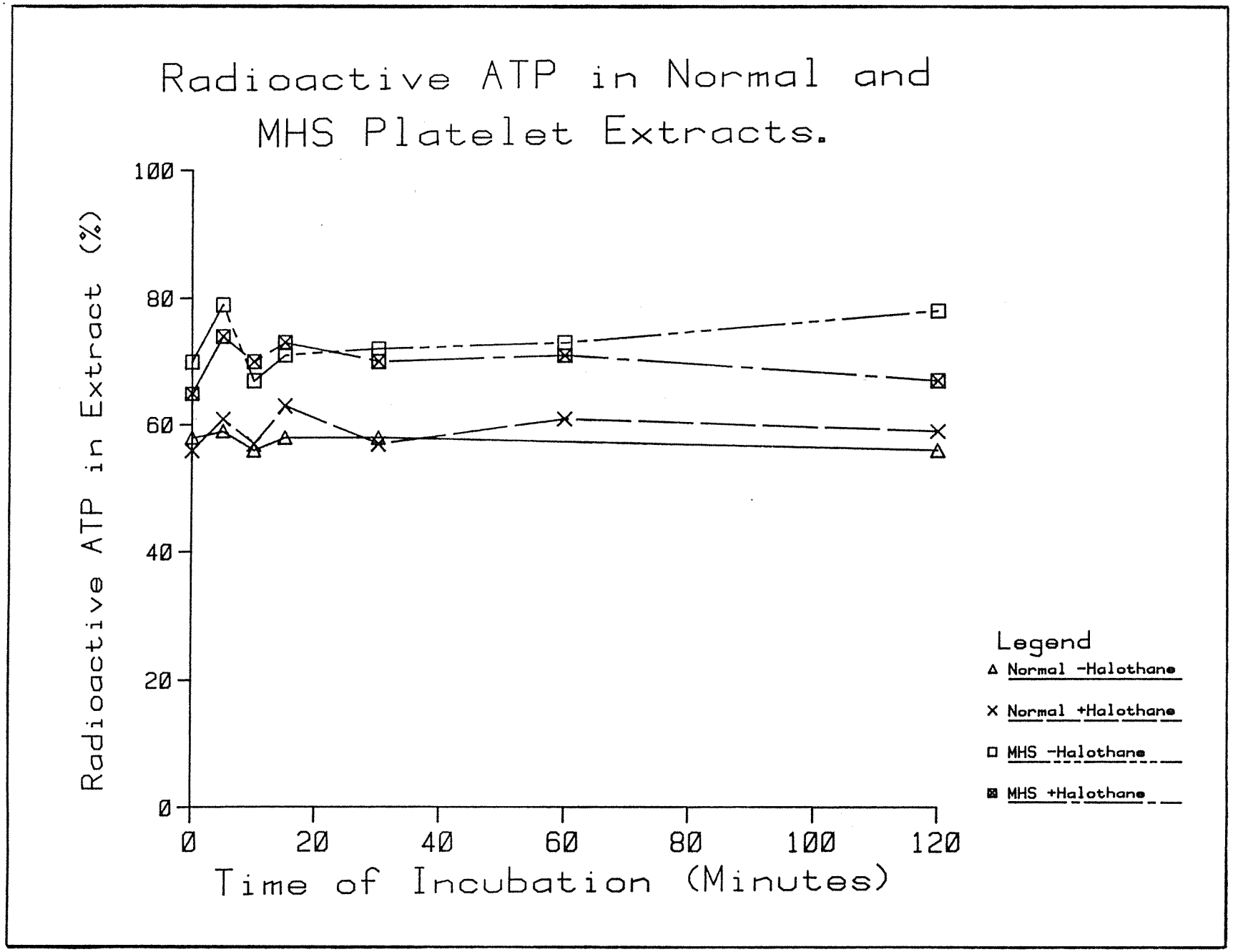

Radioactive ATP in Normal and MHS Platelet Extracts.
Radioactive ATP in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

FIGURE 4.13

Radioactive ADP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of ADP as a percentage of the total radioactivity recovered in the platelet extract minus the amount of radioactive adenine present.

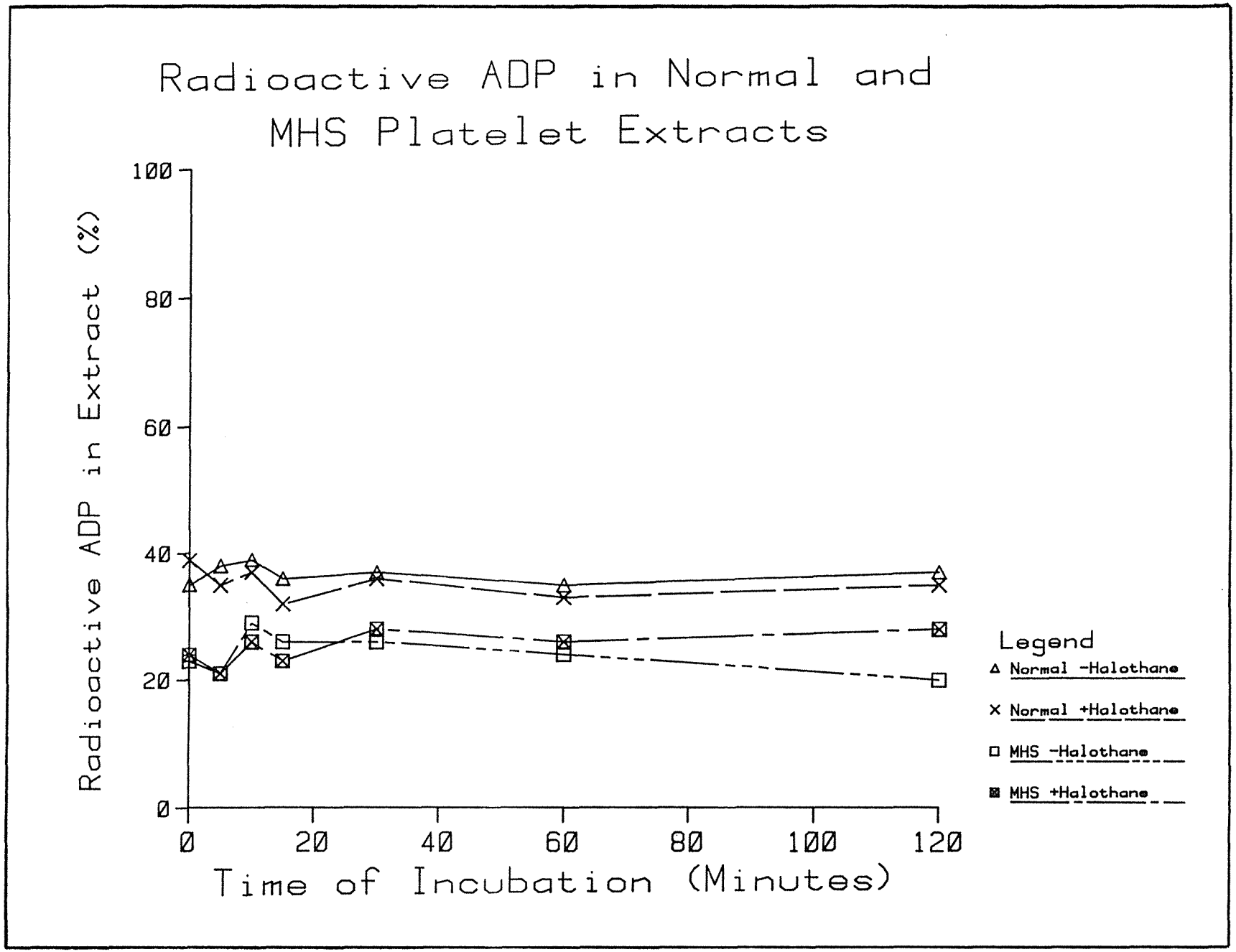

Radioactive ADP in Normal and MHS Platelet Extracts
Radioactive ADP in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

FIGURE 4.14

Radioactive AMP extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of AMP as a percentage of the total radioactivity recovered in the platelet extract minus the amount of radioactive adenine present.

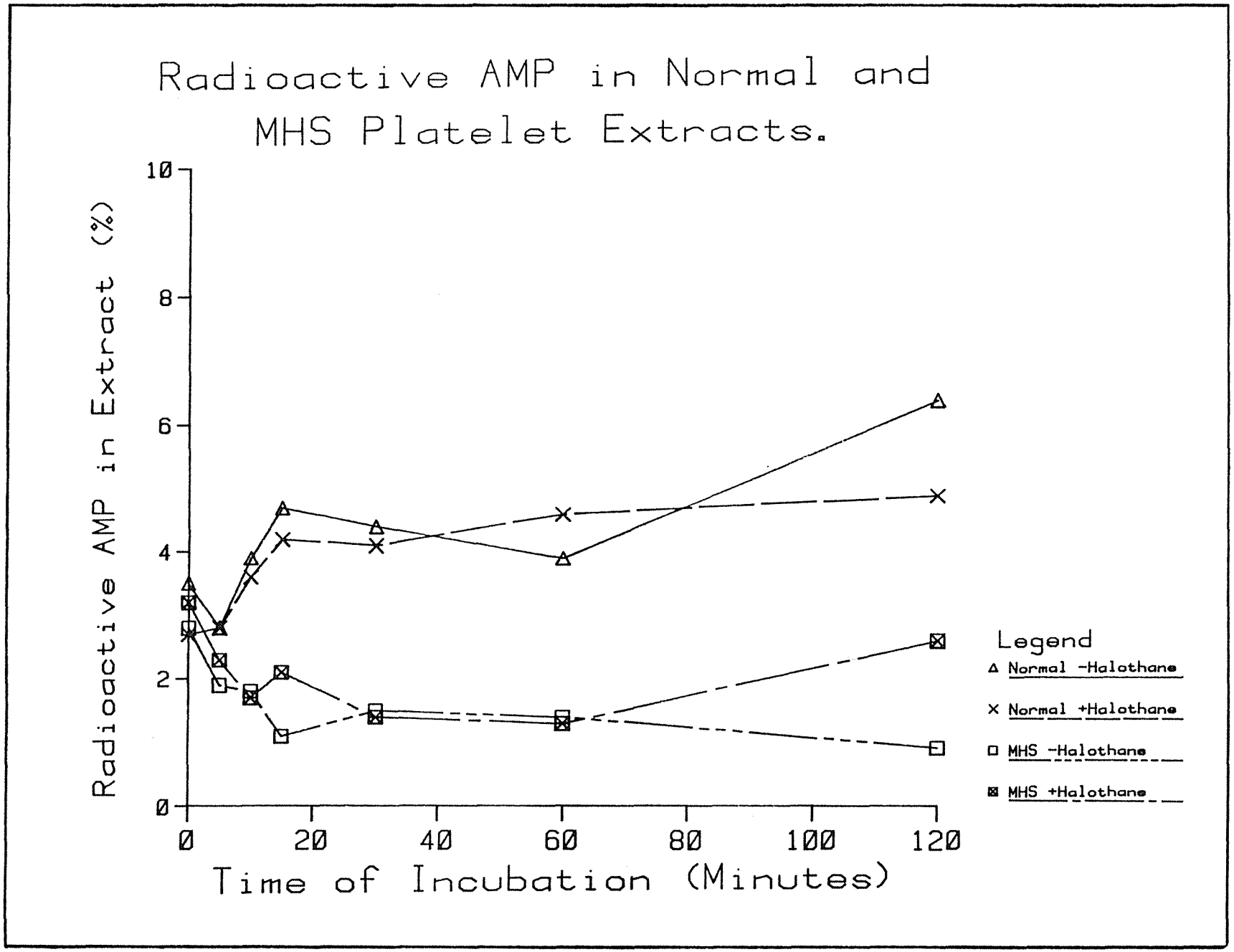
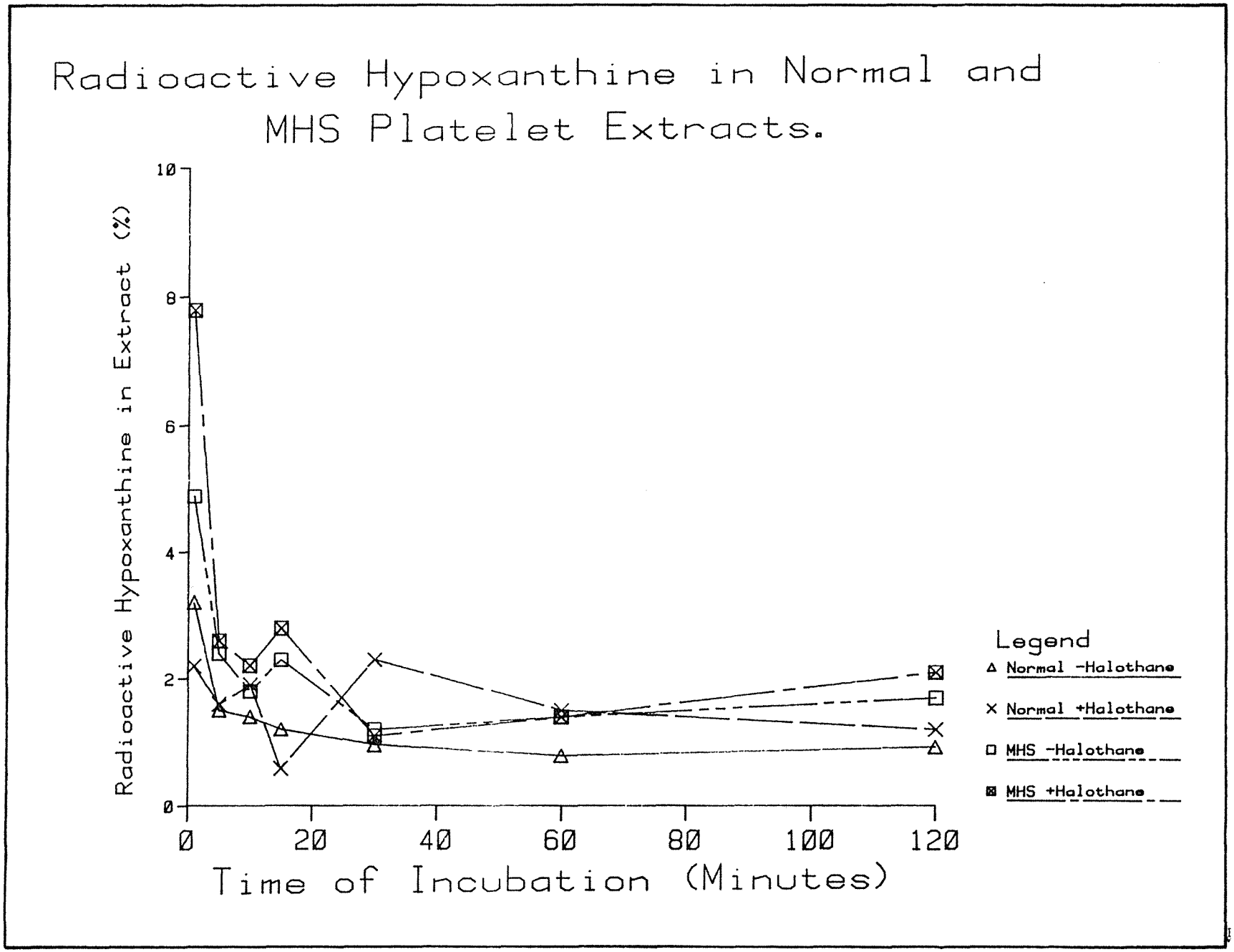

Radioactive AMP in Normal and MHS Platelet Extracts.
Radioactive AMP in Extract (%)
10
8
6
4
2
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

FIGURE 4.15

Radioactive HX extracted from normal and MHS platelets incubated in the absence and presence of halothane and extracted with the method of Rao *et al.* (1981).

Vertical axis repesents radioactivity of HX as a percentage of the total radioactivity recovered in the platelet extract minus the amount of radioactive adenine present.


Radioactive Hypoxanthine in Normal and MHS Platelet Extracts.
Radioactive Hypoxanthine in Extract (%)
10
8
6
4
2
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

For ATP and ADP, the amount of each nucleotide was constant over the two hour incubation period. This showed that adenine nucleotides in the metabolic pool were in rapid equilibrium in both normal and MHS platelets under unstressed conditions. The level of ATP at equilibrium in MHS platelets is higher than in normal platelets, while the levels of ADP and AMP were lower than normal at equilibrium.

When MHS platelets were incubated in the presence of halothane, the level of ATP decreased at longer time intervals with a corresponding increase in the level of ADP. The level of labelled HX was higher in unstressed MHS platelets than in normal platelets immediately after the addition of radioactive adenine. The level of HX rapidly decreased in 5-10 minutes and remained constant thereafter.

The level of HX in platelets reflects the rate of catabolism of adenine nucleotides (Solomons et al., 1978) and the HX produced is exported from the platelet into the extracellular medium (Akkerman et al., 1983). If the rate of turnover of adenine nucleotides is accelerated even in unstimulated MHS platelets, then increased HX production would occur. The presence of higher amounts of HX at the shortest time sample may be a result of this increased metabolism; the rate of formation of HX initially being greater than the rate of its removal from the platelet.

In MHS platelets, the ATP/ADP ratio displyed marked fluctuations at short time intervals. These fluctuations would probably also be observed at longer time intervals if more frequent samples were considered. Halothane had the effect of significantly increasing the

ratio at longer time intervals.

In the absence of halothane, the AEC was constant but higher than that of normal platelets (Figure 4.11). Halothane decreased the AEC after 30 minutes which remained significantly lower than that observed in unstressed MHS platelets.

4.10.3 Recovery of Radioactivity.

More radioactive adenine was extracted from the platelets of MHS subjects than normals in the early stages of incubation. The observed difference could be due to;

(i) more rapid uptake of adenine by MHS platelets, or

(ii) decreased incorporation of adenine by MHS platelets into other products.

In order to distinguish between these possibilities, the total recovery of added radioactivity found in the acid soluble extract was measured in each case (Table 4.13 and Figure 4.16). The recovery was much less in the MHS platelets, suggesting that less [8-^{14}C]adenine was taken up by these cells. Consequently, the higher amounts of radioactive adenine found in the early stages of incubation must be due to a decreased conversion of [8-^{14}C]adenine to other products.

	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
N O R M A L - HALOTHANE	5.1 ±1.1	19 ±2.5	15 ±5.2	15 ±3.7	19 ±1.5	34 ±1.2	89 ±4.5
N O R M A L + HALOTHANE	6.4 ±0.98	9.8 ±4.4	10 ±4.7	18 ±7.1	25 ±8.2	30 ±8.2	69 ±12
M H S - HALOTHANE	4.2 ±2.7	6.3 ±1.6	9.2 ±2.7	12 ±4.3	15 ±3.1	23 ±6.6	33 ±12
M H S + HALOTHANE	4.8 ±3.0	6.3 ±1.7	7.6 ±1.3	8.1 ±0.82	10 ±1.7	14 ±4.1	29 ±14

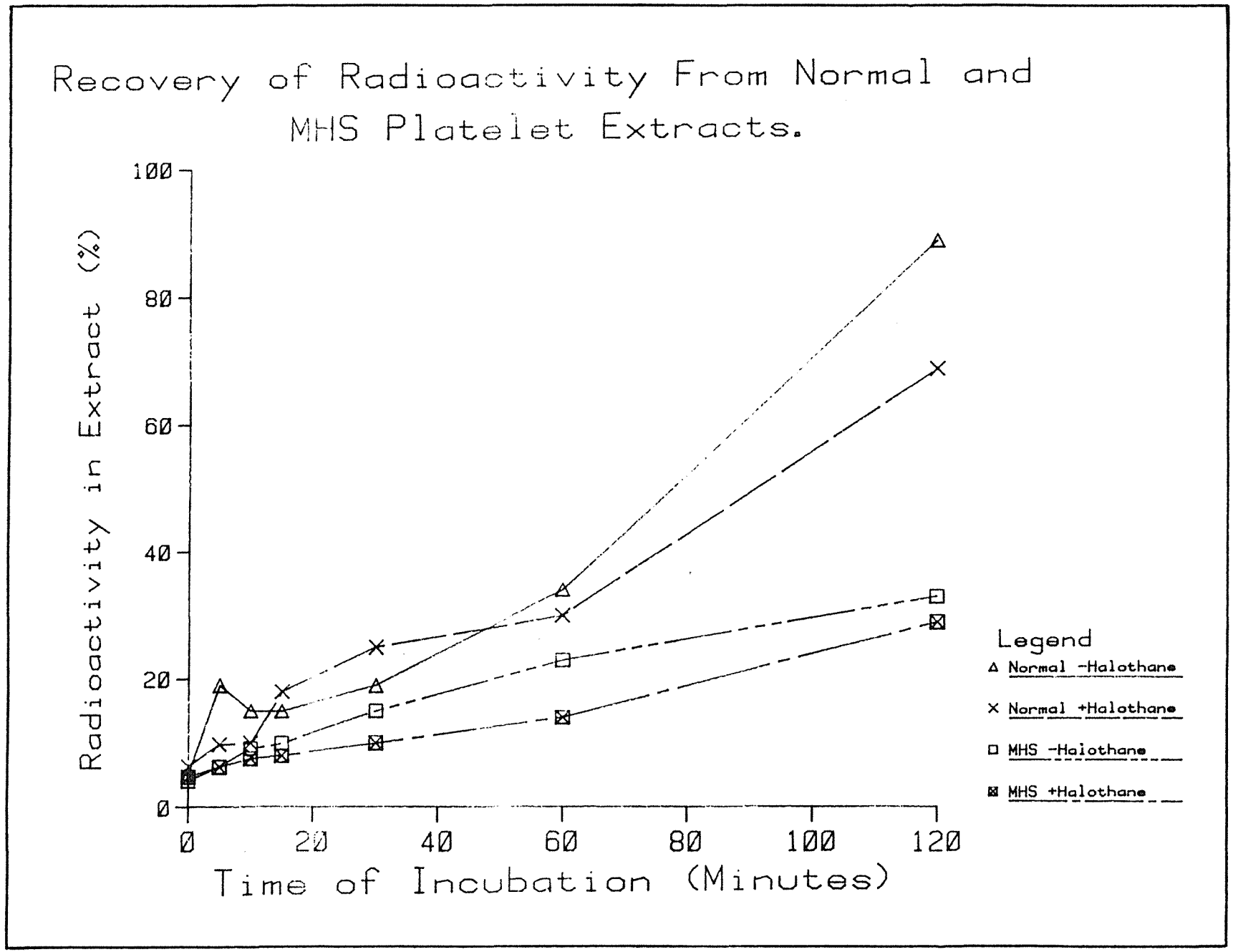
TABLE 4.13

Recovery of radioactivity from acid-soluble extracts of normal platelets prepared with the method of Rao *et al.* (1981).

Numbers represent the recovery of radioactivity as a percentage of the total radioactivity added to the PRP.

FIGURE 4.16

Recovery of radioactivity from acid-soluble extracts of normal platelets prepared with the method of Rao *et al.* (1981).


Recovery of Radioactivity From Normal and MHS Platelet Extracts.
Radioactivity in Extract (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

4.10.4 Acid-Insoluble Material.

Although the above data was obtained from acid extracts of platelets, some of the [^{14}C]adenine radioactivity was incorporated into acid-insoluble components (presumably polynucleotide and/or protein-bound material). Incorporation into this fraction in the incubation described above is shown in Table 4.14 and Figure 4.17. The incorporation proceeded approximateley seven times faster in normal subjects than MHS subjects, and halothane had little effect on either.

4.10.5 Supernatant Plasma.

The distribution of radioactivity among the separated components of the supernatant plasma was determined in several experiments. While the pattern varied between experiments, examples of such experiments are shown in Figure 4.18 for a normal individual, and Figure 4.19 for a MHS individual.

Inosine was detected at much higher levels than normal in MHS supernatant fractions. The amount of inosine mirrored that of HX. Very low levels of adenine nucleotides were detected in both normal and MHS supernatant fractions.

	TIME OF INCUBATION (MINUTES)						
	0	5	10	15	30	60	120
N O R M A L - HALOTHANE	688 ±302	910 ±196	1127 ±509	1380 ±681	3359 ±79	3907 ±1288	6415 ±2630
N O R M A L + HALOTHANE	224 ±81	574 ±313	743 ±434	1540 ±564	3907 ±1288	2974 ±887	7537 ±2460
M H S - HALOTHANE	67 ±16	103 ±21	180 ±31	259 ±61	256 ±39	548 ±78	947 ±158
M H S + HALOTHANE	63 ±11	112 ± 8.6	162 ±48	175 ±39	255 ±70	319 ±157	638 ±234

TABLE 4.14

Radioactivity recovered per milligram of acid-insoluble precipitate remaining after normal platelets were extracted with the method of Rao *et al.* (1981).

Numbers represent the recovery of radioactivity as a percentage of the total radioactivity added to the PRP.

FIGURE 4.17

Radioactivity recovered per milligram of acid-insoluble precipitate remaining after normal platelets were extracted with the method of Rao et al. (1981).

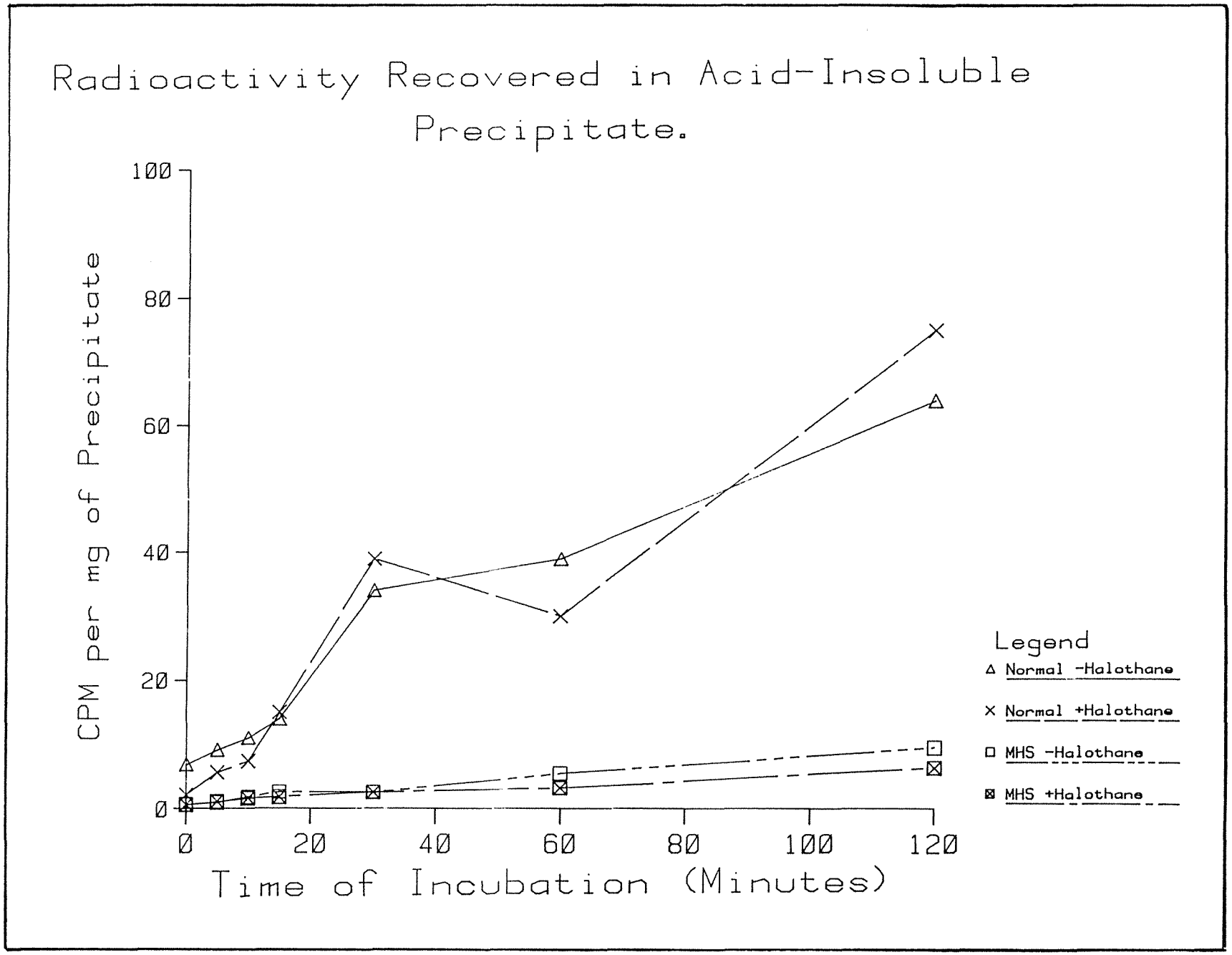

Radioactivity Recovered in Acid-Insoluble Precipitate.
CPM per mg of Precipitate
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Normal -Halothane
Normal +Halothane
MHS -Halothane
MHS +Halothane

FIGURE 4.18

Distribution of radioactivity among components of supernatant plasma from normal platelet suspensions.

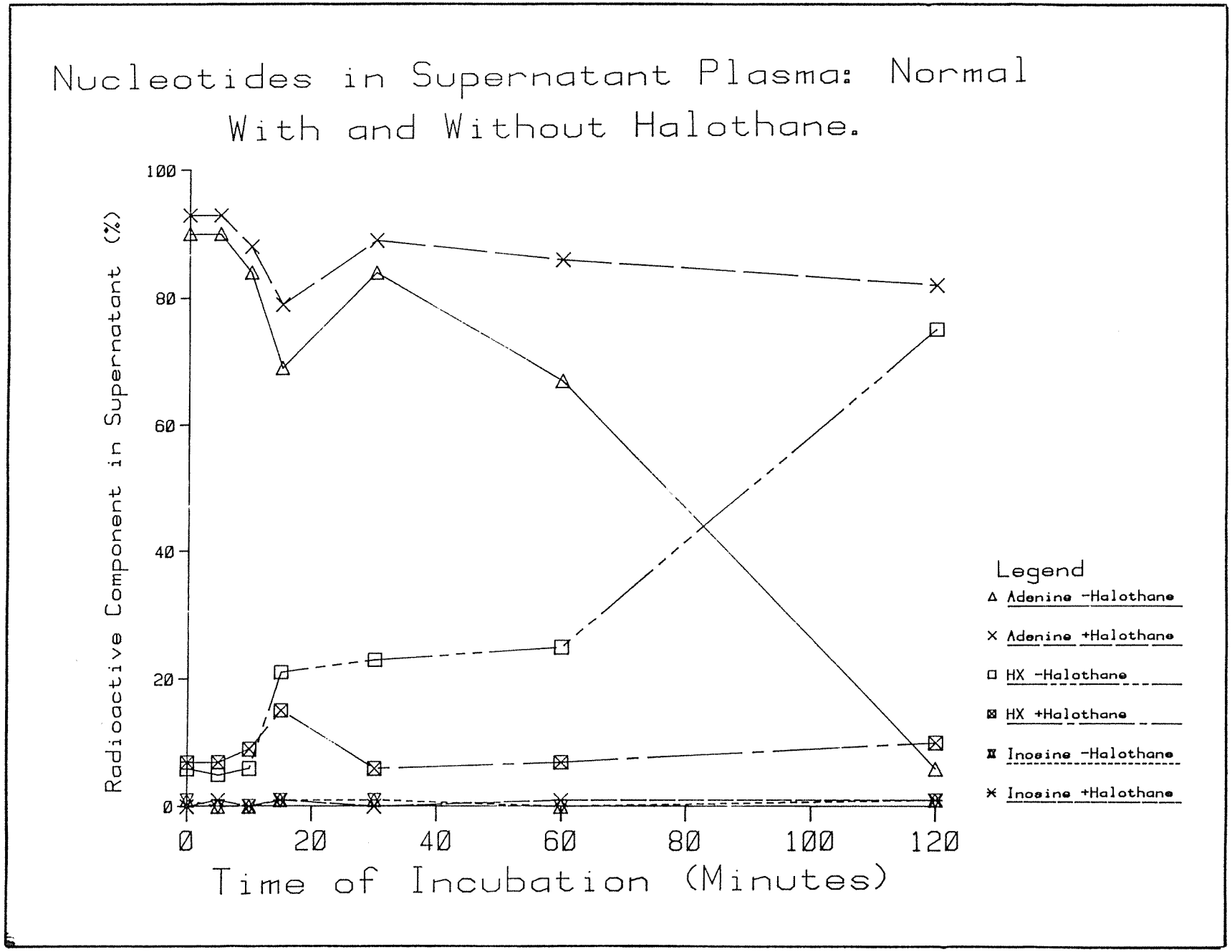
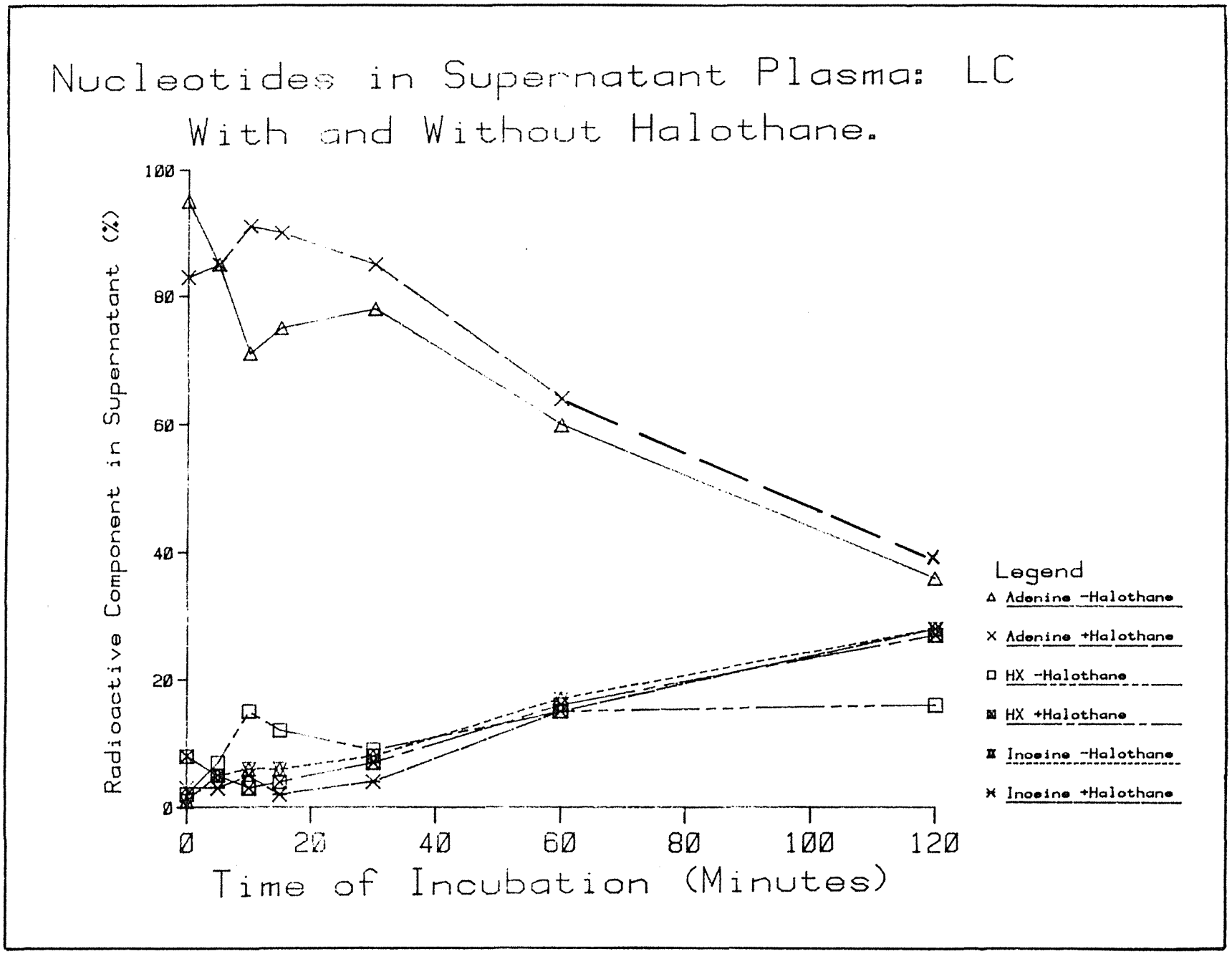

Nucleotides in Supernatant Plasma: Normal
With and Without Halothane.
Radioactive Component in Supernatant (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Adenine -Halothane
Adenine +Halothane
HX -Halothane
HX +Halothane
Inosine -Halothane
Inosine +Halothane

FIGURE 4.19

Distribution of radioactivity among components of supernatant plasma from MHS platelet suspensions.


Nucleotides in Supernatant Plasma: LC
With and Without Halothane.
Radioactive Component in Supernatant (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time of Incubation (Minutes)
Legend
Adenine -Halothane
Adenine +Halothane
HX -Halothane
HX +Halothane
Inosine -Halothane
Inosine +Halothane

CHAPTER 5: DISCUSSION

CURRENT DIAGNOSTIC TESTS FOR MALIGNANT HYPERTHERMIA.

Malignant Hyperthermia (MH) is an inherited muscle abnormality characterised by reactions to several kinds of drugs normally used during anaesthesia, and to extreme physical and emotional stresses. Such drugs and stresses may trigger an enormous acceleration of catabolic processes of muscle, along with hyperthermia and other serious complications (Britt, 1979a). At present there are three ways in which MH-susceptibility is diagnosed;

(i) Diagnosis following a severe MH reaction.

This is a rather unfortunate method of diagnosis for the patient but is an indication that his or her relatives should submit themselves for clinical testing by one of the following methods to determine the presence or absence of the Malignant Hyperthermia-susceptibility trait.

(ii) Blood serum test.

Measurement of Creatine Phosphokinase (CPK) has been used extensively for more than a decade in the investigation of MHS families. However, elevations in resting levels of this enzyme are only observed in 40-70% of persons susceptible to MH (Britt, 1979a) and, therefore, has limited diagnostic value. The probability that a person possesses the MH trait is increased if a skeletal muscle abnormality is present along with a high serum CPK level.

(iii) Skeletal muscle biopsy.

To date, a skeletal muscle biopsy provides the most specific test for MH-susceptibility, although some laboratories would dispute this. Muscle sections are subjected to a "contracture test" using caffeine or halothane or both as the triggering agent, or the "ATP-depletion test". Although the muscle biopsy test provides an accurate diagnosis of MHS, such surgical procedures are time-consuming and expensive, as well as inconvenient to the patient, and as such are unsuitable for large scale testing. Therefore, a simpler, less invasive test to detect susceptibility to MH is highly desirable.

Blood platelets have been described as "floating muscle cells" (Britt, 1979a), as they contain a calcium-activated contractile mechanism, a calcium-storing and releasing system, and an active ATP-generating system (Solomons et al., 1978). It is thus logical to assume that the same processes occur in platelets during MH-induced accelerated metabolism. Indeed, Solomons and co-workers proposed that [^{14}C]adenine incorporation into platelet nucleotides could form the basis of a Malignant Hyperthermia-susceptibility test, but did not proceed with this work beyond a preliminary report (1978). Solomons et al. (1980) went on to develop a "platelet-halothane bioassay", which they used to show a highly significant decrease in ATP in halothane-treated platelets from MH-susceptible individuals compared with non-susceptible controls. This finding appears to be analogous to the ATP-depletion test in muscle biopsy specimens. It was reported that no false negatives were observed and 100% concordance with muscle

biopsy findings was obtained. Using HPLC, the platelet nucleotide profile could be obtained within 45 minutes after venipuncture.

Although Giger and Kaplan (1983) failed to reproduce these results, further investigation of the method is warranted. The present research was an attempt to develop a platelet-based test for MH-susceptibility, and was started prior to the methodological controversy now in progress between Giger and Kaplan (1984) and Solomons and Masson (1984a).

NUCLEOTIDE TURNOVER IN MALIGNANT HYPERTHERMIA.

In the early stages of a MH reaction ATP turnover is markedly increased: elevated myoplasmic calcium activating both myosin ATPase and ATP-production by the phosphorylase $\longrightarrow$ glycogenolysis $\longrightarrow$ glycolysis and oxidative phosphorylation pathways.

As ADP and AMP are in equilibrium via the adenylate kinase reaction, and AMP is rapidly deaminated by adenylate deaminase in platelets, it is reasonable to suppose that the total adenine nucleotide pool has a shorter half-life in a MH reaction. If exogenous adenine is supplied it might be expected to be incorporated into adenine nucleotides more rapidly in the accelerated metabolic state of MH.

ADENINE UPTAKE BY BLOOD PLATELETS.

Adenine is not metabolised in plasma or in the extracellular phase

of platelet suspensions. Instead, adenine is taken up by blood platelets at a constant rate which depends on the number of platelets in suspension (Holmsen and Rozenberg, 1968a). Adenine uptake can, therefore, be measured as the rate of disappearance of total extracellular radioactivity during incubation with [^{14}C]adenine.

The uptake of adenine can be considered a unidirectional process as it is immediateley converted to adenine nucleotides upon entering the platelet. The high affinity of platelets for adenine (k_m = 159nM) is probably related to the important function of adenine nucleotides in platelet processes (platelets contain more nucleotides than any other cell), and to the lack of de novo synthesis of nucleotides in platelets.

Both the k_m and the V_{max} for adenine transport into platelets are influenced by the condition of the platelet membrane.

CHOICE OF STRESS-INDUCING AGENT.

Considerable debate surrounds the diagnosis of Malignant Hyperthermia-susceptibility by muscle contracture testing. A study of 122 MHS-patients distinguished three diagnostic contracture phenotypes (Nelson et al., 1982). The phenotype that was unequivocal for MH-susceptibility had an abnormal contracture response to halothane alone, to caffeine alone, and to caffeine plus halothane. Therefore, for the purposes of this research, it was considered that an investigation of the effects of halothane on platelet nucleotide turnover would distinguish between MHS platelets and normal ones, if

such a distinction exists.

The possibility also remains that even unstressed platelets from MHS individuals have an abnormal basal nucleotide turnover.

Three major assumptions, therefore, formed the basis of a working hypothesis for the current research;

(i) the muscle defect in MHS subjects is also reflected in the platelet contractile system,

(ii) under resting and/or halothane-stressed conditions the nucleotide turnover in MHS-platelets is significantly abnormal

(iii) the turnover abnormality is reflected in differences in adenine incorporation to platelet nucleotides via the salvage pathway.

ADENINE NUCLEOTIDE TURNOVER IN PLATELETS.

In human platelets, phosphorylation of free purine bases by specific enzymes requiring phosphoribosyl pyrophosphate as the ribose-phosphate donor, constitutes the major portion of the salvage pathway for nucleotide synthesis. Phosphorylation of purine nucleotides occurs to a small extent, and the combination of the two processes accounts for the entire production of platelet nucleotides.

In platelets, ADP is degraded by adenylate kinase to AMP from which ATP can be regenerated. AMP is also further degraded to HX which diffuses out of the platelet (Akkerman et al., 1983). Increases in the rate of ATP regeneration and the rate of appearance of HX in the extracellular phase of the platelet suspension should therefore be observed in accelerated platelet metabolism when more ADP is produced.

Preliminary work in this department on the direct chemical measurement of ATP and ADP using the firefly luciferase assay was shelved in favour of the assessment of ATP turnover by incorporation of radioactive isotopes into adenine nucleotides.

Although ^{32}P-orthophosphate incorporation into ATP and ADP would more accurately reflect increased cycling between the two nucleotides, indirect determination of ATP turnover in platelets by measuring the incorporation of [8-^{14}C]adenine was decided upon for the current research because of its more convenient half-life.

The selection of isotope was also influenced by the report of Solomons and Masson (1982a) that the amount of HX in platelet acid-soluble extracts was increased after platelets were stressed with halothane. Incorporation of [8-^{14}C]adenine allowed the measurement of five components of the extracts simultaneously: ATP, ADP, AMP, HX, and adenine. Therefore, the entire ATP-HX conversion process could be monitored.

Quantitation of the adenine nucleotide turnover rate would require measurement of the specific activity of ATP, ADP, and AMP. However, if

cycling between ATP, ADP, and AMP is rapid enough in the metabolic pool of nucleotides, the relative radioactivity of incorporated adenine should reflect the relative specific activities and yield the same information without introducing direct measurement of the amounts of nucleotides. Moreover, measurements of total nucleotides in platelet extracts, such as those of Solomons and Masson (1982a and 1984b) are inflated by the inclusion of granular nucleotides, particularly ADP, which are outside the metabolic pools of interest in this work.

However, when the granular pool is excluded, evidence that the specific activity of ATP, ADP (metabolic and protein-bound), AMP, HX, and inosine are equal in platelet extracts provided justification for equating concentration with per cent radioactivity of a separated component from a platelet extract (Daniel et al., 1980).

CONDITIONS OF PLATELET INCUBATION AND EXTRACTION.

The platelet extraction method of Holmsen and Rozenberg (1968a) had previously been investigated in this department but it had not been used to a great extent. Early in the current research, radioactive adenine transported into platelets was incorporated into adenine nucleotides after 30 minutes incubation of PRP at 37^{o}C, but the relatively high level of labelled HX indicated that the platelets were breaking down their ATP. This was a reproducible phenomenon. However, other investigators found that labelled HX constituted less than one per cent of the total radioactive components of platelets.

ATP levels were similar to normal values obtained later in this

research using the method of Rao et al. (1981), but ADP levels were low which contributed to a high ATP/ADP ratio and AEC. Apparently, the adenylate kinase reaction maintained ATP levels at the expense of ADP, with AMP being broken down to HX. The AEC was reasonably constant over 90 minutes despite this evidence of increased nucleotide turnover.

Using this platelet extraction method the total adenylate pool was approximately 70% of the total radioactivity extracted compared with more than 90% determined later for normal platelets. This supports the findings of Akkerman et al. (1983) that the AEC is maintained at the expense of the total adenylate pool.

Since this research was inspired by a publication by Solomons et al. (1978) the platelet extraction method used by this group was tried. In the two experiments performed, there were high levels of radioactive HX in the platelet extracts. Levels of other labelled components varied considerably, and about 50% of the radioactivity detected in the supernatant plasma was adenine nucleotides. However, Holmsen and Day (1971) reported that when platelets were separated from their suspension medium after incubation with radioactive adenine, there were no radioactive adenine nucleotides in the supernatant plasma. Therefore, the nucleotides must have come from the platelets, which may indicate membrane damage. The rate of transport of adenine into platelets is dependent on the condition of the membrane (Holmsen and Rozenberg, 1968a).

The level of free adenine in the supernatant plasma after 60 minutes incubation (about 33% of the total recovered radioactivity) was

higher than that observed in later experiments for normal platelets. This may also be a result of membrane damage. However, relatively low levels of intracellular free adenine in both experiments indicate that most of the adenine taken up by platelets was utilised.

For both the platelet extraction method of Holmsen and Rozenberg (1968a) and that of Solomons et al. (1978), PRP was prepared by a method that was probably too harsh for platelets. A subsequent investigation of methods for preparation of PRP revealed that an average force of 193.6 x g for 10 minutes was sufficient to give a satisfactory harvest of platelets (Day, 1975).

This stresses the need for the method of PRP preparation employed to be suitable for the particular investigation being performed. The various isolation procedures have different effects on platelet functionality, morphology, and metabolism (Lages et al., 1974).

PLATELET INTEGRITY.

A measure of the functional integrity of platelets in PRP would be a valuable aid in the assessment of the suitability of each preparation for radioisotope incorporation studies and in the comparison of results between such experiments. Oxygen uptake is a function of platelets that has been measured by several groups. While a careful study of this process using a Clarke Electrode did not give the required evaluations, manometry in siliconised Warburg flasks may be worthy of further investigation.

PLATELET EXTRACTION.

Most investigators use HPLC or enzymatic methods to determine the concentration of adenine nucleotides in platelet extracts. Of those who have studied radioisotope incorporation into platelet nucleotides none have presented data in a form that allowed direct comparison with the results of this research. Since the method of PRP preparation varies widely between investigators, each laboratory needs to establish a range of normal values for a particular experimental technique. A similar situation is therefore created to that existing among the laboratories routinely performing muscle contracture tests for Malignant Hyperthermia-susceptibility diagnosis.

Incorporation of [8-^{14}C]adenine into nucleotides of normal platelets was remarkably consistent between donors. In the earliest experiments using Rao's procedure, some platelet suspensions appeared to utilise adenine less efficiently than others but this was not observed in normal platelets in any future experiments. However,the ATP/ADP ratios and AEC were similar to those in later experiments.

Incubation of PRP in the presence of a MH-triggering agent, halothane, appeared to stimulate ATP production at the expense of ADP since the ATP/ADP ratio and AEC were increased. Similar amounts of AMP and HX were present compared with platelets incubated in the absence of halothane. Halothane introduced more variation between individuals than that produced by unstimulated platelets.

If ATP was turning over more rapidly in the presence of halothane,

with preservation of the total adenylate pool, then incorporation of [8-^{14}C]adenine into ATP would be expected to increase.

COMPARISON OF NORMAL AND MHS PLATELETS.

In this series, platelets from three normal subjects and from three volunteers known to exhibit positive responses to halothane in the muscle contracture test (performed by Dr. Anderson) were compared. PRP was incubated with [8-^{14}C]adenine both in the absence and presence of halothane and an acid-soluble extract of the platelets was prepared. Separate aliquots were incubated for approximateley 90 seconds (i.e. platelets were extracted as soon as possible after the addition of adenine) and at 5, 10, 15, 30, 60 and 120 minutes after this.

Platelets were extracted by the method of Rao et al. (1981), with the addition of three minutes sonication of the platelet extract after manual redispersal of the platelet button in PCA. This was an effort to free some of the radioactivity associated with the acid-insoluble precipitate which, in some cases, was equivalent to the amount of radioactivity in the extract itself.

Figures 4.5 to 4.9 summarise the time-course of incorporation of added [8-^{14}C] adenine into platelet acid-soluble ATP, ADP, AMP, HX and adenine. In each case the vertical axis represents the percentage of the total counts recovered in the acid-extract of the platelet sample. The observations on this limited number of subjects suggest that;

(a) More labelled adenine is extractable from the platelets of MHS

subjects than normals in the early stages of incubation. This was in spite of the fact that MHS platelets took up less adenine than normal platelets, and must therefore reflect a deranged conversion of adenine to other products in the early stages of the incubation.

(b) More radioactivity appears as ATP in MHS platelets at all incubation times.

(c) Less radioactivity appears as ADP in MHS platelets at all incubation times.

(d) Less radioactivity appears as AMP in MHS platelets after five minutes

(e) More radioactivity appears as HX in MHS platelets up to five minutes; thereafter, the levels are similar to those of normal platelets.

(f) The ATP/ADP ratio was higher for MHS platelets at all times

(g) The AEC was higher for MHS platelets at all times

(h) The presence of halothane had no apparent effect on the distribution of radioactivity in normal platelets. However, in MHS platelets, halothane caused a decrease in ATP levels at longer incubation times with a concommitant increase in ADP. Accordingly, an increase in the ATP/ADP ratio was observed at longer times. AMP and HX levels were essentially unaffected by halothane.

Although the above data was obtained from acid extracts of platelets, some of the [8-^{14}C] adenine radioactivity was incorporated into acid-insoluble components (presumably polynucleotide and/or protein-bound material). The incorporation proceeded approximateley 7 times faster in normal subjects and again halothane had little effect on either. Again, this reflects a decreased ability of MHS platelets to incorporate adenine into other products - a finding contrary to the initial hypothesis.

CHANGES IN THE ADENYLATE ENERGY CHARGE.

The AEC, which is increasingly being considered a better indication of a cells energy status than absolute ATP levels or ATP turnover (Akkerman et al., 1983), decreased after 30 minutes and after two hours had decreased by about 10%.

Chapman and Atkinson (1973) suggested a role for AMP deaminase in buffering against large fluctuations in AEC. If this enzyme were to become active when the AEC falls then a decrease in AMP buffers or protects the AEC value by increasing the mole fractions of ATP and ADP. Since this occurs at the expense of the total adenylate pool, it must be a short term phenomenon as the adenylate pool size is limited. Although Atkinson and Chapman used rat hepatocytes in their experiments, Holmsen and Robkin (1977) showed that human platelet metabolic ATP levels can decrease by up to 67% of the total metabolic ATP pool size provided the AEC does not drop by more than five per cent. A greater decrease in AEC results in inhibition of some platelet functions. Later, Daniel et al. (1980) confirmed that subjecting

platelets to metabolic stress led to a small drop in the AEC, which in turn led to a decrease in the total concentration of adenine nucleotides.

More recently, Giger and Kaplan (1982) followed the incorporation of radioactive adenine into normal and MHS human platelet nucleotides over 30 minutes in the presence of halothane. They found that ATP concentrations decreased by 43% in both normal and MHS platelets without a decrease in AEC (Calculations based on data provided in their publication). On the basis of evidence from the current research, in which the AEC drops quite suddenly after 30 minutes, Giger and Kaplan should have extended the time of incubation of their platelets.

CONCLUSION.

The present research indicates that the total adenylate pool is being depleted in MHS platelets for several reasons;

(i) AEC decreases after 30 minutes

(ii) AMP decreases after 5 minutes (indicating increased deamination of this nucleotide).

(iii) HX and Inosine appear in the extracellular medium at higher levels at longer incubation times.

Although HX was present in the extracellular medium of normal platelets, nearly all of the adenine supplied had disappeared (Figure

4.16). Therefore, a small actual pool of HX would register as a large pool when radioactive percentages are considered.

While no data are supplied by Solomons and Masson (1984b), visual inspection of their HPLC traces indicates that MHS platelets have higher resting levels of ATP than normal platelets. The decrease in the ATP pool is greater in MHS platelets incubated with halothane than in normal platelets treated in the same manner.

From the limited number of individuals screened in the current research, results suggest that MHS platelets have a higher basal ATP level than normal. When challenged with halothane the AEC decreased, causing an increased turnover rate which inturn led to a decrease in the ATP pool due to increased deamination of AMP. The appearance of more HX and inosine than normal in the extra-platelet medium is consistent with the above sequence of events.

FUTURE RESEARCH.

Many more individuals need to be screened before the efficiency of the assay described above, in distinguishing between normal and MHS platelets, can be assessed. Nevertheless, on the basis of the limited number of samples considered, the assay is worthy of further research and development.

The nature of the acid-insoluble material remains to be determined; radioactive nucleotides in this fraction may or may not be in the same proportions as those in the acid-soluble fraction.

The significantly decreased ability of MHS platelets to take up adenine, and the slower rate at which it occurs, indicates that differences in the platelet membrane between these groups may be found.

Methodological developments should include assessments of the effects of shaking and temperature on the incorporation of radioactive adenine into platelet nucleotides, and of varying the amounts of perchloric acid and time of sonication in the preparation of platelet extracts. A non-metabolisable internal standard should be employed to determine the efficiency of platelet extraction. this step, along with the determination of platelet oxygen consumption as a measure of their functional integrity, would identify platelet suspensions which are likely to deviate markedly from ranges of values established for normal and MHS platelets.

The assay could more closely approximate physiological conditions if halothane was delivered to the PRP from an anaesthetic machine. Preliminary investigations revealed that normal platelets prepared in this way have slightly different nucleotide profiles from those to which liquid halothane was added. Subjecting whole blood samples to halothane prior to the preparation of PRP may also provide valuable information.

Quantitation of the amount of halothane in the PRP sample may be of value. Preliminary investigations revealed that this can be achieved with success using extraction of PRP with heptane and determination of halothane spectrophotometrically, or using extraction of PRP with chloroform using a carbon tetrachloride internal standard

and determination of halothane using gas-liquid chromatography.

Carrying out radioactive adenine incorporation studies will be of most value if performed in parallel with muscle biopsy contracture tests. The predictive ability of the two tests could then be directly compared. Since the primary defect in MH is probably a generalised one involving the membranes of many tissues, an investigation of the uptake of radioactive adenine, and its subsequent metabolism in muscle biopsy specimens in the absence and presence of triggering agents may produce results similar to those for blood platelets.

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