ACTIVATION OF PLATELET ADENYLATE CYCLASE BY 2-AZIDOADENOSINE: A POTENTIAL PHOTOAFFINITY LABEL

By

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ABSTRACT

2-Azidoadenosine has previously been shown to be a potent activator of adenylate cyclase in human platelet particulate fractions. In the present study, the activation of adenylate cyclase was enhanced by: (a) reducing Mg^{2+} concentration in the assay mixture, (b) adding excess adenosine deaminase during the incubation, and (c) using rabbit platelets instead of human platelets. Under these optimal conditions a 200% increase in adenylate cyclase activity was achieved with 10 μM 2-azidoadenosine.

2-Azidoadenosine was stable in aqueous buffer systems in the absence of light. At pH 7.4, 2-azidoadenosine exists as two tautomers, the azide form and the tetrazole form which were shown to interconvert in a first order fashion with a half-life of 15.3 min at 22°C. These two tautomers appear to be equipotent in their activation of platelet particulate fraction adenylate cyclase. However, on irradiation at 313 nm only the azide form appears to be photolysed to a mixture of products, presumably via a nitrene intermediate.

Although the photolysis of 2-azidoadenosine in the presence of platelet particulate fraction produced activation of platelet adenylate cyclase, evidence was obtained that this was not the result of photoaffinity labeling. The individual effects of pre-exposure to 2-azidoadenosine and irradiation at 313 nm were additive.

Activation of adenylate cyclase by 2-azidoadenosine added to the enzyme assay was reduced if the particulate fraction had been exposed to UV radiation. The same reduction in activation by 2-azidoadenosine added to the assay was seen when the prior irradiation of the enzyme was carried out in the presence of 2-azidoadenosine.

The ^3H covalently bound to the platelet particulate fraction upon irradiation in the presence of 2-azido[^3H]adenosine was shown to be radiation dependent. However, pre-irradiation of 2-azido-[^3H]adenosine generated a product that covalently labeled platelet particulate fraction as effectively as irradiation of 2-azido[^3H]-adenosine in the presence of particulate fraction. This indicates that affinity labeling rather than photoaffinity labeling had occurred. Some inhibition of this labeling was produced by the addition of unlabeled 2-azidoadenosine, 2-chloroadenosine and theophylline. However, neither adenosine nor N^6-cyclohexyladenosine caused inhibition of labeling when present during photolysis, suggesting that adenosine receptors were ~~not~~ detected. Inhibition of labeling by dipyridamole and coformycin suggested reactions with adenosine transport proteins and adenosine deaminase, respectively. Further studies with various combinations of ligands are required to determine the nature of sites labeled by the photolysis products of 2-azido[^3H]-adenosine.

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AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
cyclic AMP	3',5'-adenosine monophosphate
cyclic GMP	3',5'-guanosine monophosphate
DMSO	dimethyl sufoxide
DTT	dithiothreitol
GTP	guanosine 5'-triphosphate
NaMgEGTA	sodium magnesium ethylene glycol-bis-(β-aminoethylether)NNN'N'-tetraacetate
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
POPOP	p-bis(2-(5-phenyloxazole))benzene
TXA_2	thromboxane A_2
SED	standard error of the difference
SEM	standard error of the mean
SDS	sodium dodecyl sulfate

Dedicated to my wife Patricia . . . .

CHAPTER 1

Introduction

INTRODUCTION

1.1 *The Blood Platelet*

Injury to a blood vessel wall causes changes in the structure of the endothelial surface which allow platelets to adhere to the injured site. Platelets adhere to subendothelial structures such as basement membranes, collagen fibres and microfibrils. These platelets release their granule contents and further platelets adhere to these activated platelets. Of the vessel wall constituents only collagen has been shown to cause release of platelet granule contents (Baumgartner et al., 1976). These three characteristic responses of platelets, i.e. adherence, aggregation and secretion of granule contents, the latter known as "release reaction", are essential for the formation of a hemostatic plug or the development of an arterial thrombus (Mustard et al., 1974).

The formation of prostaglandin endoperoxides and thromboxane A_2 and the release of granule contents, in particular, those of the amine storage granules, promote further aggregation and release. Contents of these amine storage granules include the potent aggregating agent ADP, ATP, vasoactive amines (serotonin) and divalent cations (Ca^{2+} in human platelets)(Feinstein, 1978). Activated platelets change shape from a disc-like structure to a more rounded form with pseudopodia. This changed shape allows for further platelet aggregation either to each other or, to other platelets already attached to the exposed collagen.

In addition to release of stored substances, collagen and thrombin-stimulated platelets rapidly synthesize compounds that produce further aggregation. Arachidonate acid is produced by phospholipase C and diacylglycerol lipase from phosphatidylinositol (Bell and Majerus, 1980). The arachidonate is converted enzymatically to the prostaglandin endoperoxides, PGG_2 and PGH_2 and thromboxane A_2. These short-lived compounds cause further aggregation and release (Hamberg et al., 1975; Malmsten et al., 1975).

With the formation of thrombin, fibrinogen is converted to fibrin. This fibrin stabilizes the platelet aggregates to form a hemostatic plug. Thrombin, besides converting fibrinogen to fibrin, causes further platelet aggregation by stimulating platelets to produce the prostaglandin derivatives,(PGG_2, PGH_2 and TXA_2), and the release of platelet ADP (Packham et al., 1977) or by a third mechanism involving mobilization of cellular Ca^{2+} stores (Feinstein, 1978) or possibly the formation of platelet aggregating factor (Chignard et al., 1979).

In addition to responding to the activating effects of agonists that cause aggregation and the release reaction, platelets can also respond via a negative pathway with reversal or inhibition of aggregation. These inhibitory agonists, in order of decreasing potency, are the inhibitory prostaglandins (PGI_2, PGD_2, PGE_1), adenosine and beta-adrenergic agonists (Haslam et al., 1979a). The mechanism of inhibition by these agonists is via an increase in platelet cyclic AMP concentration (Wolfe and Shulman, 1969; Mills

and Smith, 1971; Salzman, 1972; Haslam, 1973; McDonald and Stuart, 1973; Haslam, 1975; Haslam et al., 1978a; Haslam et al., 1978b).

1.1.1 *Platelet Cyclic AMP and Regulation of Platelet Function*

It is well established that platelet cyclic AMP is the second messenger in the inhibition of platelet aggregation by prostaglandins, however this relationship is much more qualitative than quantitative (Mills and Smith, 1971; Haslam, 1973; McDonald and Stuart, 1973; Haslam, 1975; Wang et al., 1978). Discrepancies arose when a given amount of inhibitory agonist inhibited by differing degrees the aggregation caused by various aggregating agents. One example is the effect of PGE_1 on ADP or vasopressin-induced aggregation. At the concentration producing the same extent of aggregation by both ADP and vasopressin, PGE_1 inhibits ADP-induced aggregation much less than vasopressin-induced aggregation (Haslam, 1975). The proposed reason for the discrepancy is the failure to take into account the effect of the aggregating agent on the increase in platelet cyclic AMP (Haslam, 1973; Haslam, 1975; Haslam et al., 1979a). ADP lowered the PGE_1-stimulated increase in cyclic AMP while vasopressin did not.

Since cyclic AMP was clearly involved in the inhibition of aggregation, investigators attempted to determine its role in aggregation. Salzman (1972) suggested that a decrease in cyclic AMP, caused by aggregating agents, might promote platelet aggregation. As epinephrine (Marquis et al., 1970) and ADP (Cole et al., 1971) inhibited the increase in platelet cyclic AMP caused by PGE_1, the concept of bidirectional regulation of platelet function by cyclic

AMP appeared feasible. The main piece of evidence against the type of control was the fact that aggregation could still occur in the presence of elevated cyclic AMP levels (Haslam, 1975). Subsequently another cyclic nucleotide was proposed as a mediator of platelet aggregation or the release reaction and this was cyclic GMP (Haslam, 1975; Goldberg et al., 1974). However, further work by Haslam et al. (1978c, 1979a) indicated that increases in cyclic GMP may be an effect rather than a cause of aggregation. More recent evidence has suggested that Ca^{2+} is the primary intracellular messenger for aggregation (for review see Feinstein, 1978). One action of cyclic AMP may be to decrease the concentration of Ca^{2+} in the platelet cytosol (Haslam et al., 1978a, 1978c) thus inhibiting aggregation.

Various lines of evidence have now reinforced the concept that cyclic AMP is a unidirectional regulator in platelet, being responsible for only inhibition of aggregation (Haslam et al., 1978a, 1978b, 1978c). Haslam et al. (1978b) provided evidence for this concept with the use of deoxyadenosine derivatives. Thus, the potent inhibitors of adenylate cyclase, 2',5'-dideoxyadenosine and 9-(tetrahydro-2-furyl) adenine (SQ22536) failed to potentiate aggregation induced by ADP, vasopressin, collagen, arachidonic acid or cause aggregation by themselves (Haslam et al., 1978b). Salzman et al. (1978) has also found, contrary to an earlier report by this group, that SQ22536 does not potentiate aggregation. Harris et al. (1979) has shown reversal of PGE_1 inhibition of ADP-induced aggregation by 9-substituted adenine derivatives.

1.2 *Adenylate Cyclase*

Hormone sensitive adenylate cyclase (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1), is a complex system of intrinsic membrane proteins that contains at least five distinct loci for the interaction of various regulatory ligands. In addition to the catalytic site there are sites for the actions of (a) guanine nucleotides (Rodbell et al., 1971; Lad et al., 1977), (b) divalent cations (Londos and Preston, 1977a), (c) hormones, and (d) adenosine which acts as 1) a local hormone and 2) an intracellular regulatory ligand (probably not the physiological ligand) (Fredholm, 1978; Arch and Newsholme, 1978; Fox and Kelly, 1978).

1.2.1 *Guanine Nucleotides and Adenylate Cyclase*

It is now clear that guanine nucleotides (GTP is probably the physiologically important one) (Johnson and Mukku, 1979) are essential for adenylate cyclase activation (Rodbell et al., 1971; Rodbell, 1975; Levitzki, 1977).

The guanine nucleotide site or sites (Lad et al., 1977) are distinct from the catalytic site. It has been suggested that both the catalytic unit and the receptor component each contain a guanine nucleotide site (Welton et al., 1977) and now Rodbell (1980) suggests the GTP-regulatory protein acts as a bridge between the receptor and the catalytic component.

Activation of adenylate cyclase by guanine nucleotides is dependent on the presence of the triphosphate. Hydrolysis of GTP at the nucleotide regulatory site results in deactivation of the enzyme

back to a resting state (Cassel and Selinger, 1976). Analogs of GTP that are resistant to hydrolysis at the terminal phosphate such as guanyl-5'-yl imidodiphosphate (Gpp(NH)p) induce a stable and highly active adenylate cyclase (Londos et al., 1974). This hydrolysis of GTP is highly specific with the GTPase activity being associated with the enzyme complex. GTPase activity increases in proportion to the GTP-enzyme activated complex concentration (Pfeuffer and Helmrich, 1975; Cassel and Selinger, 1976). Thus, the adenylate cyclase system oscillates between states of low and high activity depending on the rate of turnover of GTP at the GTPase site and the availability of GTP to the system. The GTPase system can be regarded playing an intergral part in the control of adenylate cyclase.

An actual evaluation of the physiological role of GTP has been reported (Johnson and Mukku, 1979), in which intracellular GTP levels were decreased. The use of inhibitors of IMP dehydrogenase reduced GTP concentrations by 80% and ATP by only 10 to 15% in intact, cultured NRK cells. When GTP levels were reduced, the ability of PGE_1 and isoproterenol to raise cyclic AMP levels was decreased by 50 to 70%, yet basal cyclic AMP levels were unaffected. It is possible that GTP activation of basal activity and hormonal stimulation are under different controls.

Guanine nucleotides not only activate adenylate cyclase in the presence of beta adrenergic agonists and stimulatory prostaglandins but is also essential for the membrane receptor action of adenosine. In the turkey erythrocyte, adenosine stimulates adenylate

cyclase in the presence of GTP (Sevilla et al., 1977). In the fat cell, Londos et al. (1978) have shown GTP-mediated adenosine inhibition of adenylate cyclase. In both these systems adenosine is postulated to exert its effects through a receptor.

1.2.2 *Divalent Cations and Adenylate Cyclase*

The activity of adenylate cyclase depends on the presence of Mg^{2+} or Mn^{2+}. Magnesium is the physiological divalent cation but cyclase activation by Mg^{2+} requires concentrations 50 to 100-fold greater than with Mn^{2+} (Londos and Preston, 1977a). The mechanism of cation regulation has been a point of controversy for the last several years. Sutherland and Rall (1962) first showed adenylate cyclase systems were activated by divalent cations at concentrations in excess of the metal-ATP substrate. They postulated two actions of magnesium from their data. Magnesium either, (a) acted upon a site independent of the catalytic site, or, (b) lowered the ratio of free ATP (ATP^{4-} or $HATP^{3-}$). Free ATP could have a higher affinity for the catalytic site although $MgATP^{2-}$ is the substrate; ATP^{4-} would be a competitive inhibitor. Thus, an increase in cation concentration would appear to activate adenylate cyclase by decreasing the concentration of ATP^{4-}.

Rodbell et al. (1971) and De Haen (1974) both indicated free ATP^{4-} was a competitive inhibitor. However, Drummond et al. (1971) and Garbers and Johnson (1975), who questioned Drummond's kinetic approach, all suggested free ATP^{4-} was not a potent inhibitor.

More recently (Londos and Preston, 1977a; Williams et al., 1978; Johnson et al., 1979), cation activation has been recognized to result from the interaction of the cation with its own regulatory site. Londos and Preston (1977a) also showed that guanine nucleotides lower the requirement for divalent cation, so that in the absence of GTP, half-maximal activation was obtained with 5 to 10 mM Mg^{2+} whereas the concentration of Mg^{2+} needed for half-maximal activation in the presence of GTP was about 1 mM.

Divalent cations can also inhibit hormone-stimulated adenylate cyclase. In the presence of increasing concentrations of Mn^{2+}, Londos and Preston (1977a) found inhibition of glucagon-stimulated liver adenylate cyclase. Similarly in the platelet, increasing Mg^{2+} concentration reduces the apparent stimulation produced by adenosine (Londos and Wolff, 1977). In the presence of other hormones, the concentration of Mg^{2+} required for maximum activation is reduced (Alvarez and Bruno, 1977).

Another effect of divalent cations concerns the binding of agonists to their receptors, e.g. β-adrenergic agonists to β-adrenergic receptors or prostaglandins to their receptors, in which divalent cations cause an increase in the amount of agonist bound (Williams et al., 1978). Magnesium ion was found to be the most potent ion out of those tested (Mg^{2+}, Mn^{2+} and Ca^{2+}). Antagonist binding was not enhanced by divalent cations, however, a partial shift in binding affinity was observed for partial agonists.

1.2.3 *Adenosine and Adenylate Cyclase*

Adenosine is gaining acceptance as a hormone-like regulator of adenylate cyclase (Daly, 1976; Arch and Newsholme, 1978; Fain and Malbon, 1979). In the three years encompassing 1976 to 1978, at least 617 articles were published in which adenosine was the main focus of the article (Fain and Malbon, 1979).

Adenosine affects adenylate cyclase activity in tissues such as brain (Sattin and Rall, 1970), platelets (Haslam and Lynham, 1972), mouse neuroblastoma (Blume and Foster, 1975), cultured human cells (glioma cell line) (Clark and Seney, 1976), ventricular myocardium (Huang and Drummond, 1976), bone cells (Peck et al., 1976), coronaries (Schrader et al., 1976), Leydig tumor cells and rat liver cells (Londos and Wolff, 1977), rabbit intestine (McKenzie et al., 1977), rat striatal homogenates (Prémont et al., 1977), turkey erythrocytes (Sevilla et al., 1977), adipocyte tissue (Trost and Stock, 1977) and thyroid tissue (Wolff et al., 1978).

The first demonstration of the activation of adenylate cyclase in a subcellular preparation (platelets) by a receptor-dependent mechanism was by Haslam and Lynham (1972). It was later shown (Haslam and Rosson, 1975) the stimulatory site was a membrane receptor but higher concentrations of adenosine inhibited adenylate cyclase activity. This inhibitory site was suggested to be intracellular.

Adenosine has been observed to inhibit adenylate cyclase activity in the fat cell, liver and thyroid; stimulation has been observed in the turkey erythrocyte and murine neuroblastoma, while

in the platelet, bone cell and cultured human cells, the effect is biphasic, that is adenosine both stimulates and inhibits adenylate cyclase activity with the net effect being concentration dependent.

The surface membrane receptor of adenosine accepts purine substituted analogs of adenosine as agonists (Londos and Wolff, 1977; Haslam et al., 1978b; Prémont et al., 1977) but is antagonized by methylxanthines (Haslam and Lynham, 1972; Blume and Foster, 1975; Clark and Seney, 1976; Londos et al., 1978; Tolkovsky and Levitzki, 1978). The response induced by the receptor, whether it be inhibition (Londos et al., 1978) or stimulation (Sevilla et al., 1977), requires GTP to mediate its action.

Londos and Wolff (1977) introduced the concept of an "R" site responsible for activation of adenylate cyclase by adenosine analogs containing an intact ribose residue. Antagonism by methylxanthines was required to be exhibited by this site. Recently (Londos et al., 1979; Haslam et al., 1979b; Fain and Malbon, 1979), the concept of activation by the "R" site has been markedly modified. The "R" site is now believed responsible for the inhibition or stimulation of adenylate cyclase, i.e. by analogy with adrenergic hormones, adenosine exerts both "alpha" and "beta" effects (van Calker et al., 1979).

Earlier it had been mentioned adenosine exerted an inhibitory effect upon a site which was intracellular. Londos and Wolff (1977) called this the "P" site since inhibition required an intact purine ring. It is distinguished from the "R" site because it is located

on the intracellular surface (Haslam and Rosson, 1975; Londos et al., 1979) of the plasma membrane, it always mediates inhibition of cyclase activity, requires an intact purine ring for activity and does not interact with methylxanthines (Fain et al., 1972; Cuatrecasas and Sahyoun, 1976; Trost and Stock, 1977; Londos and Wolff, 1977; Haslam et al., 1978b). The "P" site of adenylate cyclase is probably a functional part of all adenylate cyclases (Londos et al., 1979).

Adenosine activation of turkey erythrocyte adenylate cyclase is a typical "R" site effect. However, recently it has been suggested that the adenosine receptor is precoupled to the cyclase (Braun and Levitzki, 1979). Both adenosine and β-agonists activate the same adenylate cyclase yet the β-receptor, in contrast to the adenosine receptor, appears to act via a diffusion controlled process within the membrane (collision coupling of the receptor and adenylate cyclase) (Hanski et al., 1979). Both ligands also activate the adenylate cyclase through a common guanyl nucleotide regulatory site (Tolkovsky and Levitzki, 1978). Adenosine appears to activate only 80% of this pool of enzyme whereas the β-receptor can activate the entire pool.

With the use of a ribose-altered adenosine analog,(adenosine bisaldehyde), Braun and Levitzki (1979) suggested that a proportional decrease of the maximum specific activity, without a change in the rate constant of irreversable cyclase activation, followed a kinetic model which describes the adenosine receptor as being permanently attached to adenylate cyclase. This was further suggested by the observation that an increase in membrane fluidity has no effect

on the rate of adenylate cyclase activation by adenosine (in contrast with activation by β-adrenergic agonists and the collision coupling model (Hanski et al., 1979).

The apparent affinity labeling of the adenosine receptor by the bisaldehyde has several areas of inconsistency. First, adenosine activates this system and activation has only been shown to occur via "R"-type sites. The "P" site always mediates inhibition but Levitzki has never reported any inhibition by adenosine. Yet with the use of a ribose-altered adenosine analog, which would be expected to be more specific for a "P" (inhibitory) site, he claims to have labeled the "R" site. It is quite possible the decrease in activation by catecholamines, in the presence of adenosine could be the result of inhibition by adenosine similar to that observed when adenylate cyclase is activated by prostaglandin E_1 in the platelet system, where only inhibition by adenosine is observed (Haslam and Lynham, 1972). This issue could be settled by studies of the effects of adenosine analogs, specific for the "P" and "R" sites, on the binding properties of the dialdehyde analog.

Further adenosine receptor studies have been reported by Malbon et al. (1978) using fat cell membranes. The adenosine receptor in fat cells inhibits accumulation of cyclic AMP (Fain et al., 1972) but the structure-activity relationship suggests an "R" site mediation of the inhibition (Londos et al., 1979). Malbon et al. (1978) characterized [^{3}H] adenosine binding to fat cell membranes employing vacuum filtration technique. Binding could only be detected

in the presence of an adenosine deaminase inhibitor, deoxycoformycin. [^{3}H] Adenosine binding was rapid and reversible. Analysis of Scatchard plots revealed two populations of adenosine binding sites with differing affinities for adenosine. Both theophylline and 2',5'-dideoxyadenosine inhibitied [^{3}H] adenosine uptake. N^6-Phenylisopropyl-adenosine, a potent inhibitor of norepinephrine-stimulated lipolysis and cyclic AMP accumulation in intact cells failed to inhibit adenosine binding. This all suggested that adenosine binding to the "R" sites had not been detected.

Adenosine receptors linked to adenylate cyclase are closely related to one of the two types of purinergic receptor proposed by Burnstock (1972). The purinergic nerve hypothesis was put forward when evidence was presented that a purine nucleotide, probably ATP, was the principal transmitter released from the non-adrenergic, non- cholinergic nerves supplying the gastrointestinal tract (Burnstock, 1972). Recently Burnstock (1978) postulated two types of "purinoceptors", P1 and P2. P1 purinoceptors have agonist potencies of adenosine $\geq$ AMP $\geq$ ADP $\geq$ ATP, cause changes in cyclic AMP levels and are blocked by methylxanthines. Burnstock (1978) suggests P1 purinoceptors activate adenylate cyclase and may or may not involve the same adenylate cyclases as "R" type adenosine receptors (van Calker et al., 1979). Structure-activity relationships for P1 purinoceptors require an intact ribose moiety for agonist activity; substitution at the 2-position enhances activity (e.g. 2-chloro-adenosine) but deoxyadenosines are very potent antagonists. P2

purinoceptors have agonist potencies of ATP ≥ ADP ≥ AMP ≥ adenosine and are not involved with adenylate cyclase.

1.3 *Adenosine and Blood Platelets*

In their search for analogs of ADP that might inhibit platelet aggregration, Born and Cross (1963) found adenosine and AMP inhibited ADP-induced aggregation. Adenosine was ten times more effective than AMP. If the pre-incubation time with adenosine or AMP was increased before the addition of ADP, the inhibitory effect was greater. Born and Cross hypothesized that adenosine and AMP competed for a putative ADP receptor, though they did not adequately explain the enhancement of inhibition by pre-incubation. However, the effect of ADP in causing platelet aggregation, and the effect of adenosine and AMP in inhibiting this aggregation were shown to be highly specific. Adenosine was also found to inhibit aggregation induced by thrombin, serotonin, epinephrine and vasopressin suggesting competition for the ADP receptor was not a feasible theory (O'Brien, 1964; Haslam and Rosson, 1972). However, this only became clear when it emerged that primary aggregation was not dependent on released ADP (Mills et al., 1968; Haslam and Rosson, 1972).

Rozenberg and Holmsen (1968), in studying adenine nucleotide metabolism showed that after removal of adenosine by adenosine deaminase and subsequent addition of ADP, inhibition of aggregation still occured. Holmsen proposed that inhibition of aggregation was associated with the process of adenosine uptake by the platelet. Since adenosine is phosphorylated once it crosses the platelet membrane by

an adenosine kinase to AMP, the ATP consumed during transport and/or phosphorylation of adenosine would then not be available for platelet aggregation. Depletion of ATP by metabolic inhibitors also showed inhibition of aggregation suggesting that ATP is consumed during aggregation. However, partial reduction of ATP by the metabolic inhibitors produced complete inhibition of aggregation, although phosphorylation of adenosine still occured. Holmsen admitted that this might not be compatible with the 'energy competition theory' (Rozenberg and Holmsen, 1968).

1.3.1 *Effect of Adenosine and Analogs on Platelet Aggregation*

Investigation of adenosine-related compounds suggested that the 6-amino group of the adenosine moiety and the 9-β-D-ribofuranosyl residue were necessary for inhibition of platelet aggregation (Born and Cross, 1963; Born et al., 1965). These studies revealed that a substitution at the 2 position of adenosine did not always markedly reduce inhibitory activity; 2-chloroadenosine was found to be as potent as adenosine.

Kikugawa et al. (1972, 1973a, 1973b) synthesized a wider range of adenosine analogs. None of the 5'-substituted adenosines or the 8 substituted adenosines showed significant inhibitory activity but substitution at the 2 and/or 6 position gave compounds many of which had inhibitory activity similar to that seen with adenosine. For example, 2-aminoadenosine was as potent as adenosine in a study with rabbit platelets (Kikugawa et al., 1972)

though in human platelets 2-aminoadenosine had been reported to have little inhibitory activity (Born et al., 1965). It was suggested that this was due to species differences (Kikugawa et al., 1972). Monosubstitution at the N^6-position such as in N^6-cyclohexyl- or N^6-phenyladenosine, gave compounds that appeared to be equipotent with adenosine (Kikugawa et al., 1973a). 2-Thioadenosine derivatives such as 2-benzylthioadenosine were also found to have similar potency to adenosine with rabbit platelets.

Agarwal and Parks (1975) studied the effect of adenosine analogs on platelet nucleotide pools and aggregation. After incubation with 2-fluoroadenosine, they found significant increases of 2-fluoroadenine nucleotides in the platelet nucleotide pool; 2-fluoroadenosine also strongly inhibited ADP-induced aggregation. However, this inhibition was detectable within minutes of the analogs, well before significant quantities of analog nucleotides could be detected. N^6-Phenyladenosine, which is an inhibitor of adenosine kinase and is not metabolized to 5'-phosphates within the platelet, also inhibited ADP-induced aggregation.

Recently, Cusack and Born (1977) have studied a photolysable analog of adenosine, 2-azidoadenosine. This 2-substituted analog, like 2-chloroadenosine, inhibited aggregation by ADP as effectively as adenosine itself; moreover, 2-azido ADP was about five times more potent than ADP in causing aggregation. An earlier study (Cusack and Born, 1976) showed that 2-azidoadenosine irreversibly inhibited adenosine deaminase upon photolysis, showing that the analog can bind specifically to the active centre of this enzyme.

1.3.2 *Effects of Adenosine and Analogs on Platelet Cyclic AMP*

Following the observations that adenosine increased cyclic AMP levels in brain slices (Sattin and Rall, 1970) and that PGE_1, one of the most potent inhibitors of platelet aggregation, activated platelet adenylate cyclase (Wolfe and Shulman, 1969), studies were carried out on the effect of adenosine on platelet cyclic AMP (Mills and Smith, 1971) and adenylate cyclase (Haslam and Lynham, 1972). Mills and Smith (1971) found that in the presence of cyclic AMP phosphodiester inhibitors the effects of low concentrations of PGE_1, adenosine and 2-chloroadenosine on platelet aggregation and on platelet cyclic AMP were similar. They suggested that these nucleosides inhibit aggregation by the same mechanism as does PGE_1, namely an increase in platelet cyclic AMP.

Haslam and Lynham (1972), using the particulate fraction of lysed platelets, showed adenosine and 2-chloroadenosine had a biphasic effect on adenylate cyclase. At low concentrations of adenosine (1-40 μM), adenylate cyclase was stimulated while at high concentrations of adenosine ($>$ 100 μM) inhibition of adenylate cyclase occured. Haslam postulated that these findings represented the sum of two independent opposing actions with different concentration dependencies. The inhibitory action was postulated to be intracellular while the stimulatory action of adenosine was thought to be due to an extracellular receptor.

Evidence in support of the stimulatory site of adenosine being extracellular was obtained by the use of an adenosine transport

inhibitor, p-nitrobenzylthioguanosine (NBTGR) (Paterson and Oliver, 1971), that does not inhibit cyclic AMP phosphodiesterase. NBTGR was shown to inhibit adenosine incorporation into intact platelets. Only the stimulatory action of adenosine was seen as the inhibitory effects were blocked. It was concluded that incorporation of adenosine was not required for the increase in platelet cyclic AMP and that the site of action of adenosine must be on the external surface of the plasma membrane (Haslam and Rosson, 1975).

In the presence of papaverine, a potent inhibitor of both platelet cyclic AMP phosphodiesterase activity and of adenosine uptake by platelets, cyclic AMP accumulation was enhanced by adenosine. With the use of papaverine the rates of increase of platelet cyclic AMP were linear with time in the presence or absence of adenosine. Double reciprocal plots of the activation of adenylate cyclase in intact platelets by adenosine were linear, indicating that adenosine interacts with a single population of saturable sites. In the presence of papaverine, in intact platelets, increasing adenosine concentrations did not result in any inhibitory action on adenylate cyclase (Haslam and Rosson, 1975) yet, in broken platelets in the presence of papaverine, high concentrations of adenosine were inhibitory (Haslam and Lynham, 1972). This evidence suggested that the inhibitory site of adenosine was intracellular.

1.3.3 *Effects of Adenosine and Analogs on Platelet Adenylate Cyclase*

Haslam and Lynham (1972) were the first to show the effects of adenosine and 2-chloroadenosine on adenylate cyclase in the particulate fraction of lysed human platelets. The effect was difficult to observe due to its lability, simultaneous inhibition of adenylate cyclase by adenosine and the rapid metabolism of adenosine.

Adenosine appeared to have enhanced inhibitory activity when adenylate cyclase was activated by PGE_1, and further activation was apparently impossible. In the presence of PGE_1, 25 μM adenosine inhibited the control activity by about 30%. This suggested, assuming PGE_1 and adenosine acted upon the same adenylate cyclase (as was later proved by Haslam and Rosson (1975)), that the biphasic action of adenosine was the sum of two independent opposing processes.

2-Chloroadenosine was also used in the initial study by Haslam and Lynham (1972) and was found to be at least as potent an activator as adenosine but was a weaker inhibitor. This suggested a difference in the structural requirements for the two actions of adenosine. Using two concentrations of adenosine, one (5 μM) that produced activation and the other (400 μM) that caused at least 50% inhibition, Haslam et al. (1978b) compared a range of adenosine analogs. At 400 μM, analogs of adenosine substituted at the 2-position showed an absence or decrease in the inhibitory action observed with 400 μM adenosine, yet considerable stimulatory activity was present. Examples of these compounds are 2-hydrozinoadenosine, 2-chloroadenosine and 2-azidoadenosine. Activation by 2-azidoadenosine

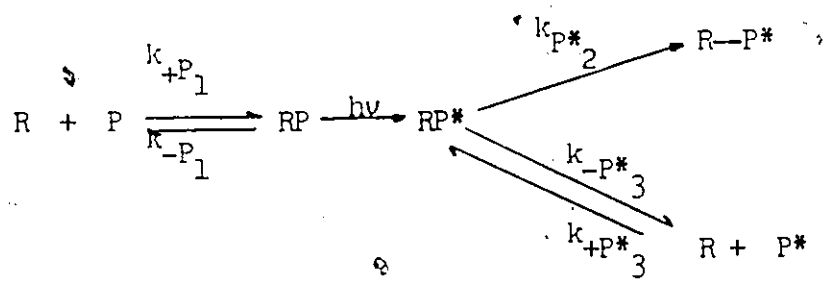
was equal to that observed with adenosine at 5 μM (30%) and at 400 μM, the azido compound still showed marked activation (40%). Analogs with a single substitution at the N^6-position were stimulatory at 400 μM also but showed less stimulatory activity than adenosine at 5 μM. Examples were N^6-phenyladenosine and N^6-benzyladenosine. The structure-activity relationships for activation by adenosine analogs of adenylate cyclase were similar to those reported for the effect of these compounds on cyclic AMP levels in slices of brain (Huang et al., 1972) and myocardium (Huang and Drummond, 1976) and other platelet studies (Londos and Wolff, 1977).

Analogs of adenosine that had one or more oxygen atoms removed from the ribose moiety were inhibitory to platelet adenylate cyclase (Harris et al., 1975; Londos and Wolff, 1977; Haslam et al., 1978b; Salzman et al., 1978). Examples include 9-(tetrahydro-2-furyl) adenine (SQ22536), 2',5'-dideoxyadenosine and 2'-deoxyadenosine-3'-monophosphate. Harris et al. (1975, 1979) showed that the increase in cyclic AMP caused by 0.5 μM PGE_1 could be blocked by SQ22536 and that this reversed the inhibition of ADP-induced aggregation by PGE_1. Haslam et al. (1978b) also showed 2',5'-dideoxyadenosine prevented the inhibition of aggregation of PGE_1 whereas 2'-deoxyadenosine-3'-monophosphate, a more potent inhibitor of particulate adenylate cyclase, did not. Since nucleoside phosphates would not be expected to penetrate the platelet plasma membrane, this evidence further supports the hypothesis that the inhibitory site is intracellular.

1.4 *Photoaffinity Labeling with Azidopurines*

Since the introduction of photolysable reagents to labeling studies by Westheimer and colleagues in 1962 (Singh et al., 1962), numerous investigations have used this approach (Westheimer, 1978). Aryl azides are common photolysable reagents because they are readily synthesized, chemically stable and have the ability to produce a highly reactive nitrene on photolysis. Nitrenes will form a covalent bond with any amino acid R-group by a variety of reactions such as nucleophilic addition, direct insertion and abstraction (normally a hydrogen from carbon) (Knowles, 1972).

In mechanistic terms photoaffinity labeling can be shown as this:



k_{+P_1}, k_{-P_1} = rate constants for label: receptor reversible complex formation and dissociation

k_{P*_2} = rate constant for formation of covalent label: receptor complex

k_{+P*_3}, k_{-P*_3} = rate constants for activated label: receptor reversible complex formation and dissociation

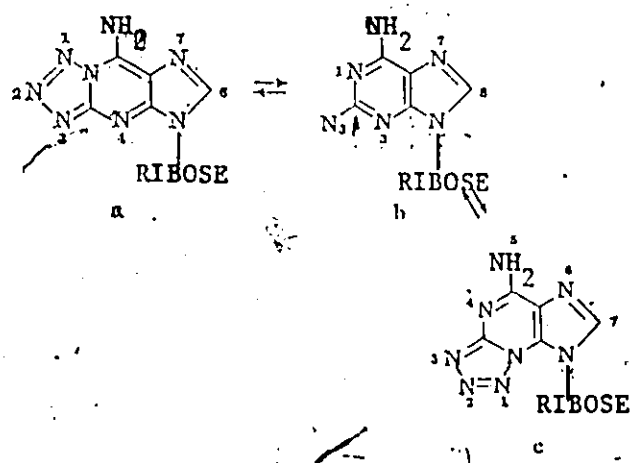
"R" is the receptor, "P" is the photoaffinity label, "P*" reactive species of the label, and "k" are the appropriate rate constants. "True" photoaffinity labeling occurs when rate constant $^{k}P^{*}_{2}$ is much greater than $^{k}{-P^{*}}_{3}$, so that the desired receptor is labeled. "Pseudo" photoaffinity labeling occurs when $^{k}{-P^{*}}_{3}$ is greater than $^{k}P^{*}_{2}$. P* will be produced by photolysis in free solution and may bind reversibly and dissociate from the receptor many times before it reacts to form a covalent bond. In principle, pseudo photoaffinity labeling is the same as ordinary affinity labeling except P* is produced in a photolytic reaction.

Azidopurine derivatives are useful photoaffinity labels and have been employed in studies with adenosine deaminase (Cusack and Born, 1976), adipocyte plasma membranes (Rosenblit and Levy, 1977) and cyclic AMP binding studies (Skare et al., 1977).

Rosenblit and Levy (1977) used 8-azido-[2-^{3}H]adenosine to attempt to identify adenosine binding components and their specific function in the fat cell. Photolysis produced several labeled protein components, however the specificity of the components labeled was not determined.

As already mentioned, Cusack and Born (1976) used 2-azido-adenosine to inhibit adenosine deaminase competitively in the absence of 365 mm light and noncompetitively after photolysis. Their later results (Cusack and Born, 1977) indicated that 2-azido-ADP and 2-azidoadenosine had high affinities for their respective receptors on the platelet membrane and might, therefore, be capable of binding irreversibly after photolysis.

The chemical structure of the azidopurine cannot be viewed as the usual azido resonance, due to the fact that at position 2 or 8 on the purine ring, the azido group is ortho to a nitrogen atom. As a result, a mixture of the azide structure and tetrazole tautomers will exist in solution. An azide at position 8 will have one tetrazole tautomer while, an azide at position 2 can potentially produce two tetrazole tautomers. The possible tautomers of 2-azidoadenosine are shown below:



The position of the tetrazolo-azido equilibrium is solvent and pH dependent. At low pH or in the presence of electron-withdrawing substituents, the azido tautomer is stabilized, and at high pH or with electron-donating groups, the tetrazolo form is stabilized (Temple et al., 1966). The tetrazolo tautomer is much less sensitive to photolysis than the azido derivatives (Bayley and Knowles, 1977). Knowledge of the equilibirum mixture is, therefore, important in attempts to use azidopurines as photoaffinity labels.

Photolysis of these azido compounds occurs most readily at wavelengths below 300 nm. Cusack and Born (1977) irradiated 2-azidoadenosine at 254 nm for 5 min or at 365 nm for 60 min; Skare et al. (1977) used 253.4 nm light for 8-azidoadenosine-3',5'-monophosphate. The effects of radiation of low wavelengths on these systems was not considered. However, platelets have been shown to aggregate in response to ultraviolet light below 300 nm (Dickson et al., 1971; Doery et al., 1973). Dickson et al. (1971) irradiated whole human platelets at various wavelengths from 313-220 nm; aggregation occurred from 302 nm to 225 nm with a maximum at 248 nm in the presence of fibrinogen. For this reason it is desirable to use wavelengths above 302 nm in attempts to carry out photoaffinity labeling of platelet receptors.

1.5 *Objectives of the Project*

1.5.1 *General Objectives*

The objectives of this thesis/study were, firstly, to investigate the activation of platelet adenylate cyclase by the adenosine receptor (R-site) using a potent adenosine analog, 2-azidoadenosine; secondly, using 2-azidoadenosine, a potential photoaffinity label, to attempt to identify the platelet adenosine receptor.

1.5.2 *Specific Objectives*

(1) To optimize the activation of platelet particulate fraction adenylate cyclase by 2-azidoadenosine by modifying the enzyme preparation and assay conditions.

(2) To define experimental conditions under which photoaffinity labeling of the adenosine receptor might be carried out and detected.

(3) To determine the effect of photolysis of unlabeled 2-azidoadenosine on the interaction of the adenosine receptor with platelet particulate fraction adenylate cyclase.

(4) To demonstrate photoaffinity labeling of the adenosine receptor with 2-azido[^{3}H]adenosine.

CHAPTER 2

Materials and Methods

MATERIALS AND METHODS

2.1 *Reagents*

Acrylamide, ammonium persulphate, ATP, adenosine, adenosine deaminase (Type 1), bovine serum albumin (crystallized and lyophylized), bromophenol blue, cyclic AMP, Coomasie Brilliant Blue R, creatine phosphate, creatine phosphokinase, dithiothreitol, ethyleneglycol-bis-(β-aminoethyl ether) NN,N',N'tetraacetic acid, glycerol, mercapto-ethanol, NNN'N-tetramethylethylenediamine. NN'-methylene bisacrylamide, papaverine, protein standard, theophylline, 2-chloroadenosine and TRIS base were all purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-25 (fine) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and sodium lauryl sulphate and sodium salicylate were obtained from BDH Chemicals Ltd., Poole, England, and 2-aminoadenosine and 2-azidoadenosine were gifts from B.E. McCarry, Chemistry Dept., McMaster University. Dipyridamole ("Persantin") was purchased from C.H. Boehringer Sohn, Ingelheim am Rhein. The following were also generous gifts, N^6-cyclohexyladenosine, Dr. K. Kikugawa, Kokjin Co. Ltd., Japan; coformycin, Dr. H. Umezawa, National Institute of Health, Kamiohsaki, Tokyo, Japan; 2',5'-dideoxyadenosine, Dr. H.B. Wood, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD, U.S.A.

2.2 *Radioactive Compounds*

[2-^{3}H] ATP (27 Ci/mmol) and cyclic [adenine-U-^{14}C] AMP (287 mCi/mmol) and liquid scintillation cocktail (ACS) were obtained from Amersham Co., Oakville, Ontario.

2-Azido-[8-^{3}H]adenosine was prepared via the hydrazine derivative from 2-chloro-[8-^{3}H]adenosine (9 Ci/mmol) purchased from Moravek Biochemicals, City of Industry, California, U.S.A. (Schaeffer and Thomas, 1958). 2-Azido[^{3}H]adenosine was separated and purified by high pressure liquid chromatography.

2.3 *[^{3}H] ATP Purification*

[^{3}H] ATP was purified by using a column prepared as for the isolation of cyclic [^{3}H] AMP isolation (1.5 ml packed vol of Bio-Rad resin AG50W-X8, 200-400 mesh). [^{3}H]ATP (1 mCi/ml, ethanol: water) from Amersham was dried by a stream of air and redissolved in 0.5 ml H_2O. The aqueous [^{3}H]ATP was applied to the column and eluted with 1 ml of distilled water. This 1 ml fraction was collected and then diluted (1:500) to a concentration of 100 μCi/ml.

2.4 *Preparation of $Ba(OH)_2$ Solution*

$Ba(OH)_2$ was dissolved to give a concentration slightly greater than 0.25 M $Ba(OH)_2$. This solution was titrated against a standard volume of 0.25 M $ZnSO_4$ and the volume of $Ba(OH)_2$ added to the $ZnSO_4$ was adjusted to give a final pH of 7.4 (in absence of 1 mM KH_2PO_4). Care was taken to prevent $BaCO_3$ from forming in any $Ba(OH)_2$ solutions.

2.5 *Platelet Preparations*

Human blood was collected by venipuncture from healthy volunteers who had taken no drugs containing aspirin for at least 1 week and rabbit blood was obtained by a carotid artery cannulation of animals anaesthetized with pentobarbital (64 mg/kg). The blood was collected in a vessel containing ACD anticoagulant (85 mM sodium citrate; 71.4 mm citric acid; 2% glucose), in a ratio of 6 parts blood to 1 part ACD.

Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 280 x g for 15 min at 22°C. The PRP was removed into siliconized conical tubes on ice and these tubes were then centrifuged at 1000 x g (5°C, 15 min). The plasma was removed and the platelet pellet resuspended in ACD washing fluid (13 mM sodium citrate; 5 mM glucose; 135 mM sodium chloride) leaving red and white cells behind. When all the red cells and white cells had been removed the washed platelets were finally resuspended in 150 mM TRIS-HCl, pH 7.4, at a concentration of 25 mg wet wt/ml.

This platelet suspension was rapidly frozen in a solid CO_2/acetone bath and remained emersed in the bath for at least one hour or was placed at -60°C overnight. The platelets were thawed at 37°C until all ice had just disappeared and centrifuged at 48,000 x g for 20 min at 0°C. The supernatant was discarded and the pellet was prepared for either an adenylate cyclase assay or a photoaffinity labeling experiment.

2.6 *Adenylate Cyclase Assay*

The 48,000 x g pellet was resuspended in a solution that contained 125 mM TRIS-HCl, pH 7.4; $MgCl_2$ (concentration dependent upon experiment); 1.66 mM cyclic AMP; 0.166% (w/v) bovine serum albumin; 6.66 mM creatine phosphate; 1.66 mM papaverine; 0.67 mM ATP; 0.67 mM dithiothreitol; 13.3 units/ml of creatine phosphokinase and 0.67 mM Mg ethylene glycol-bis-(β-aminoethylether)NNN'N'-tetra-acetate (Mg EGTA). Compounds to be tested were made up to a volume of 80 μl, 150 μl of the above enzyme suspension was added and 20 μl of [^{3}H]ATP (2 μCi/sample) was added 1 min afterwards. The final assay mixture (250 μl) contained 75 mM TRIS-HCl, pH 7.4; $MgCl_2$ (final concentrations of 0.8, 2.0 or 5.0 mM); 1 mM cyclic AMP; 1 mM papaverine; 0.4 mM ATP, 4 mM creatine phosphate; 8 units/ml of creatine phosphokinase; 1 mg/ml (0.1%) bovine serum albumin; 0.4 mM dithiothreitol and 0.4 mM Mg EGTA.

Incubations were usually for 10 min at 30°C at which time the assay was terminated by the addition of 100 μl of solution containing 1 μmol of unlabeled ATP and 0.2 μmol cyclic [^{14}C] AMP (1000 dpm/0.1 ml) added as a recovery marker. These samples were then placed in a boiling water bath for 3 min. Assays were carried out in triplicate.

The effects of the length of incubation time and the amount of platelet protein (rabbit) on basal and 2-azidoadenosine-stimulated adenylate cyclase activities at 30°C was determined in the presence of 0.8 mM and 5.0 mM $MgCl_2$. Figure 1 and 2 show that for 5, 10 and

20 min incubations and a protein range of 0.05 to 0.6 mg platelet protein/sample, basal and 2-azidoadenosine-stimulated adenylate cyclase activities approached linearity at both Mg^{2+} concentrations. The greatest departure from linearity with respect to protein was seen in the 20 min incubation with >0.4 mg platelet protein/sample. All assays were linear with respect to time up to at least 20 min.

2.7 *Photolysis Experiments*

The 48,000 x g pellet was resuspended in a medium containing 75 mM TRIS, pH 7.4, 0.8 mM $MgCl_2$; 0.4 mM DTT; 0.4 mM NaMg EGTA and 0.1% (w/v) bovine serum albumin with or without the appropriate amount of 2-azidoadenosine. A 1 or 2 ml volume was placed into a quartz cuvette and was stirred for 5 min prior to irradiation. The atmosphere in the cuvette was flushed with O_2-free N_2 during this time and throughout the photolysis experiment. The platelet protein value throughout these experiments was 0.76-1.5 mg platelet protein/ml.

The photolysis apparatus consisted of a Bausch and Lomb monochrometer using a super-pressure 200 watt mercury arc lamp. The collimated beam (1.6 cm^2) entered into a plastic vessel containing ice-water through a quartz window. The reaction cuvette was placed behind the quartz window. Photolysis began with the removal of a blind allowing light to strike the cuvette. A non-irradiated stirred control cuvette was also present in the photolysis apparatus but was shielded from light. Following photolysis, the control and irradiated platelet membrane suspensions were placed in centrifuge

Figure 1

Linearity of Adenylate Cyclase Assay with respect to Protein and Time at 5.0 mM $MgCl_2$

Values are means ± SEM from three determination on the same enzyme preparation (the SEM values are all within the symbols).

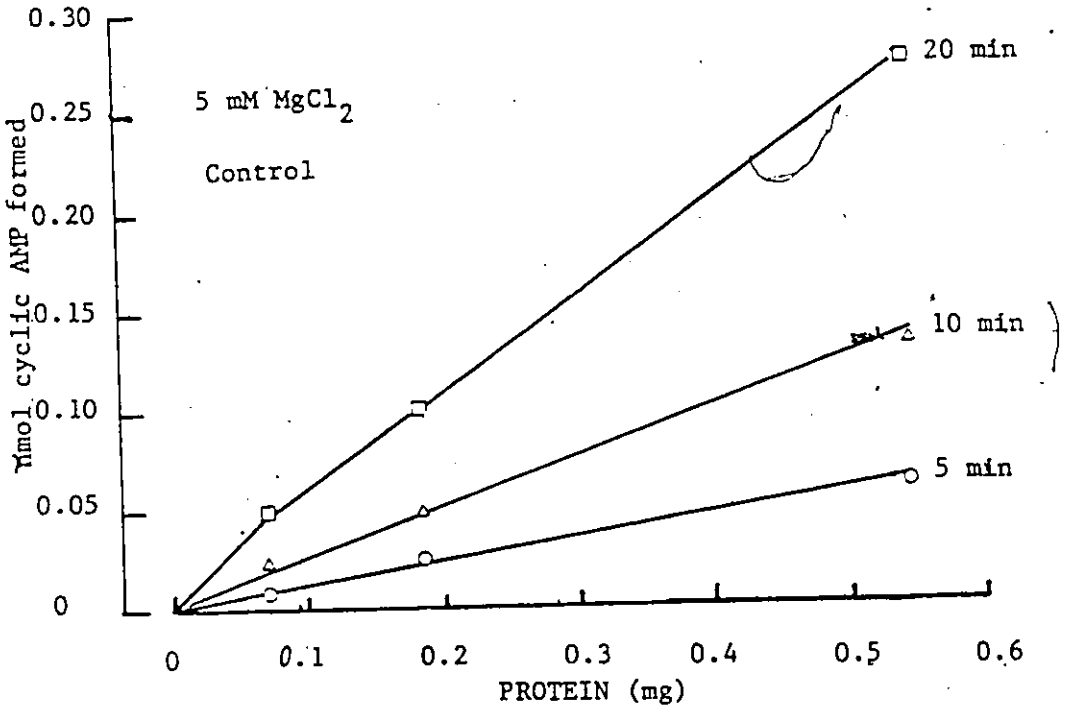
○,● ; nmol of cyclic AMP formed during a 5 min incubation

△,▲ ; nmol of cyclic AMP formed during a 10 min incubation

□,■ ; nmol of cyclic AMP formed during a 20 min incubation

(filled symbols indicate the presence of 10 μM 2-azidoadenosine)

Figure 1


0.30
0.25
0.20
0.15
0.10
0.05
0
nmol cyclic AMP formed
5 mM MgCl2
Control
20 min
10 min
5 min
0
0.1
0.2
0.3
0.4
0.5
0.6
PROTEIN (mg)

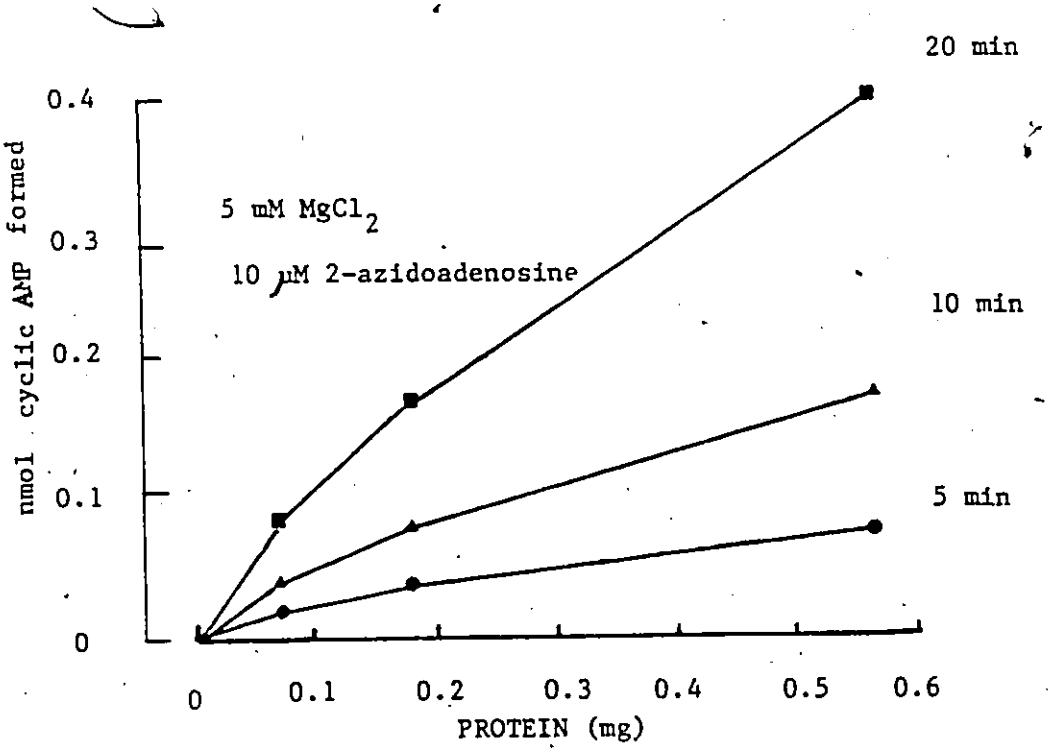

0.4
0.3
0.2
0.1
0
nmol cyclic AMP formed
5 mM MgCl2
10 μM 2-azidoadenosine
20 min
10 min
5 min
0
0.1
0.2
0.3
0.4
0.5
0.6
PROTEIN (mg)

Figure 2

Linearity of Adenylate Cyclase Assay with respect to Protein and Time at 0.8 mM $MgCl_2$

Values are means ± SEM from three determination on the same enzyme preparation (the SEM values are all within the symbols).

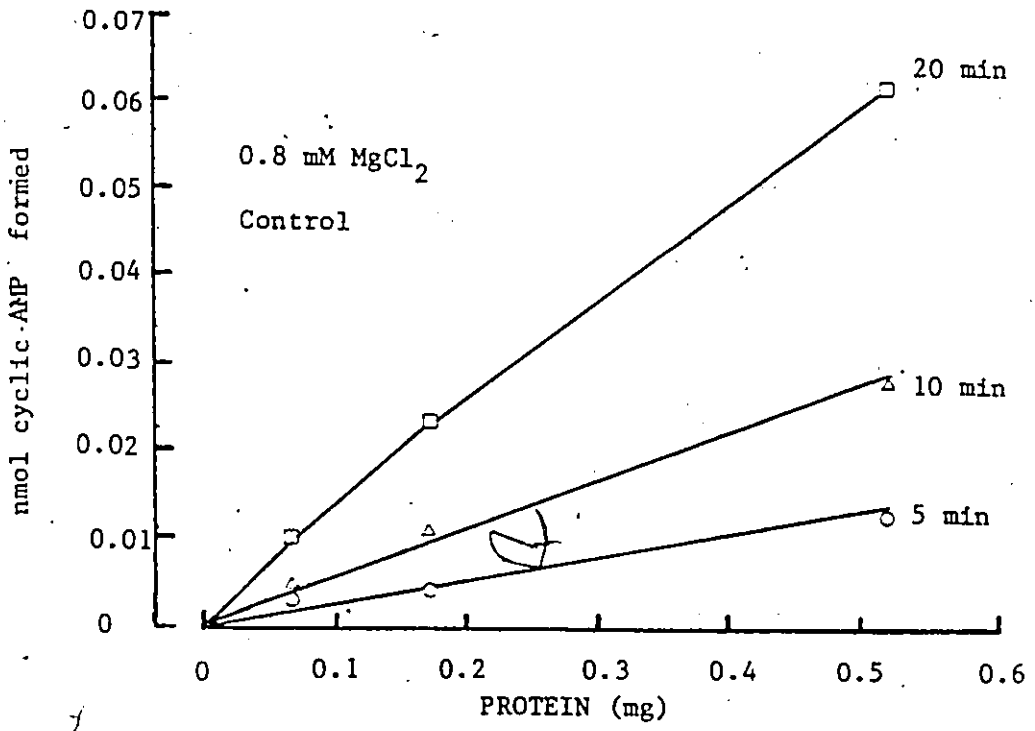
○,● ; nmol of cyclic AMP formed during a 5 min incubation

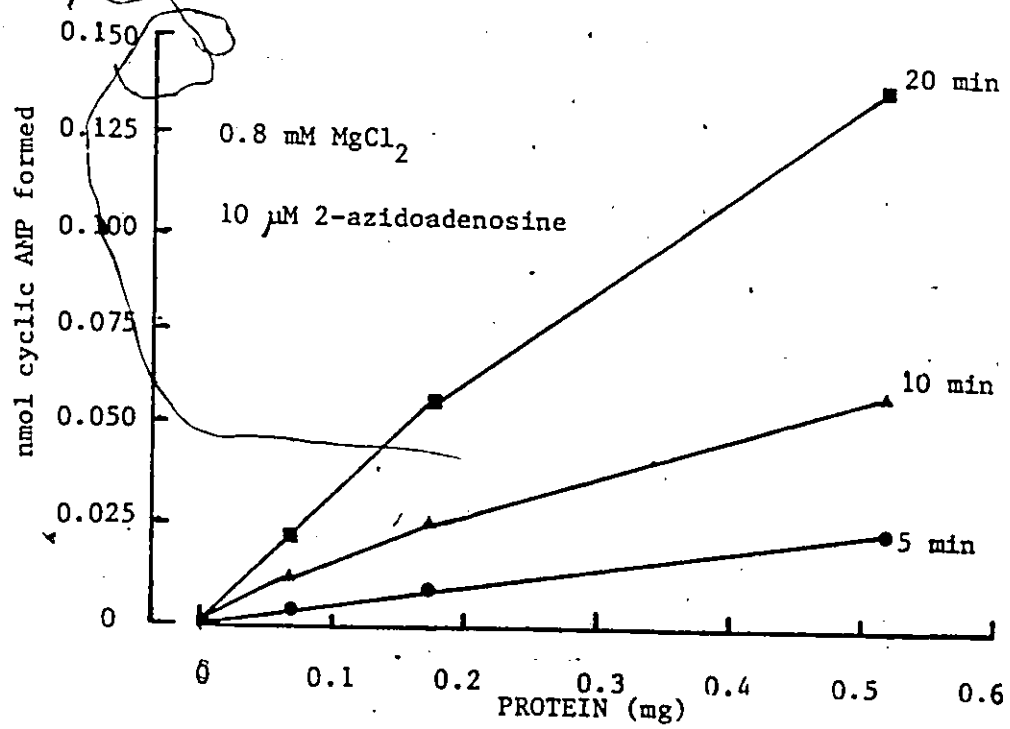
△,▲ ; nmol of cyclic AMP formed during a 10 min incubation

□,■ ; nmol of cyclic AMP formed during a 20 min incubation

(filled symbols indicate the presence of 10 μM 2-azidoadenosine)

Figure 2


0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
nmol cyclic AMP formed
0.8 mM MgCl2
Control
20 min
10 min
5 min
0
0.1
0.2
0.3
0.4
0.5
0.6
PROTEIN (mg)


0.150
0.125
0.100
0.075
0.050
0.025
0
nmol cyclic AMP formed
0.8 mM MgCl2
10 μM 2-azidoadenosine
20 min
10 min
5 min
0
0.1
0.2
0.3
0.4
0.5
0.6
PROTEIN (mg)

tubes, 5 ml of ice-cold basic medium without 2-azidoadenosine was added, and then each tube was centrifuged (48,000 x g, 20 min, $0^{o}C$). The pellet was resuspended again in 6 ml ice cold basic medium and the centrifugation repeated. This pellet was then used for adenylate cyclase assays as described previously.

During experiments using 2-azido-[8-^{3}H]adenosine, a modification of the above method was used. After photolysis the platelet membranes were diluted with 5 ml of ice cold buffer (75 mM TRIS-HCl, pH 7.4 containing 0.4 mM NaMg EGTA) and then centrifuged once at 48,000 x g for 20 min at $0^{o}C$. The supernatant was discarded and the pellet resuspended in 1 ml of the same buffer. The platelet membrane suspension, now with almost all bovine serum albumin removed, was placed into a 1.5 ml Eppendorf tube and acid-precipitated with 0.2 ml of 60% trichloroacetic acid. After standing on ice overnight the samples were centrifuged for 15 sec at 12,000 x g (Eppendorf centrifuge). The supernatant was discarded and the pellet washed with 10% trichloroacetic acid and centrifuged on the Eppendorf centrifuge as before. The samples were dissolved in 100-200 µl of 3% SDS + 0.6 M TRIS-HCl, pH 7.4. These samples were heated at $80^{o}C$ for 3 min. A sample was counted for ^{3}H and protein was determined by Folin-Lowry (see 2.12.1).

If a sample was to be used for electrophoresis the acid precipitated pellet was dissolved in electrophoresis sample buffer containing 3% (w/v) SDS, 0.0025% (w/v) Bromophenol Blue, 62 mM TRIS-HCl, pH 6.8, 6% (w/v) glycerol and 5% (w/v) mercaptoethanol.

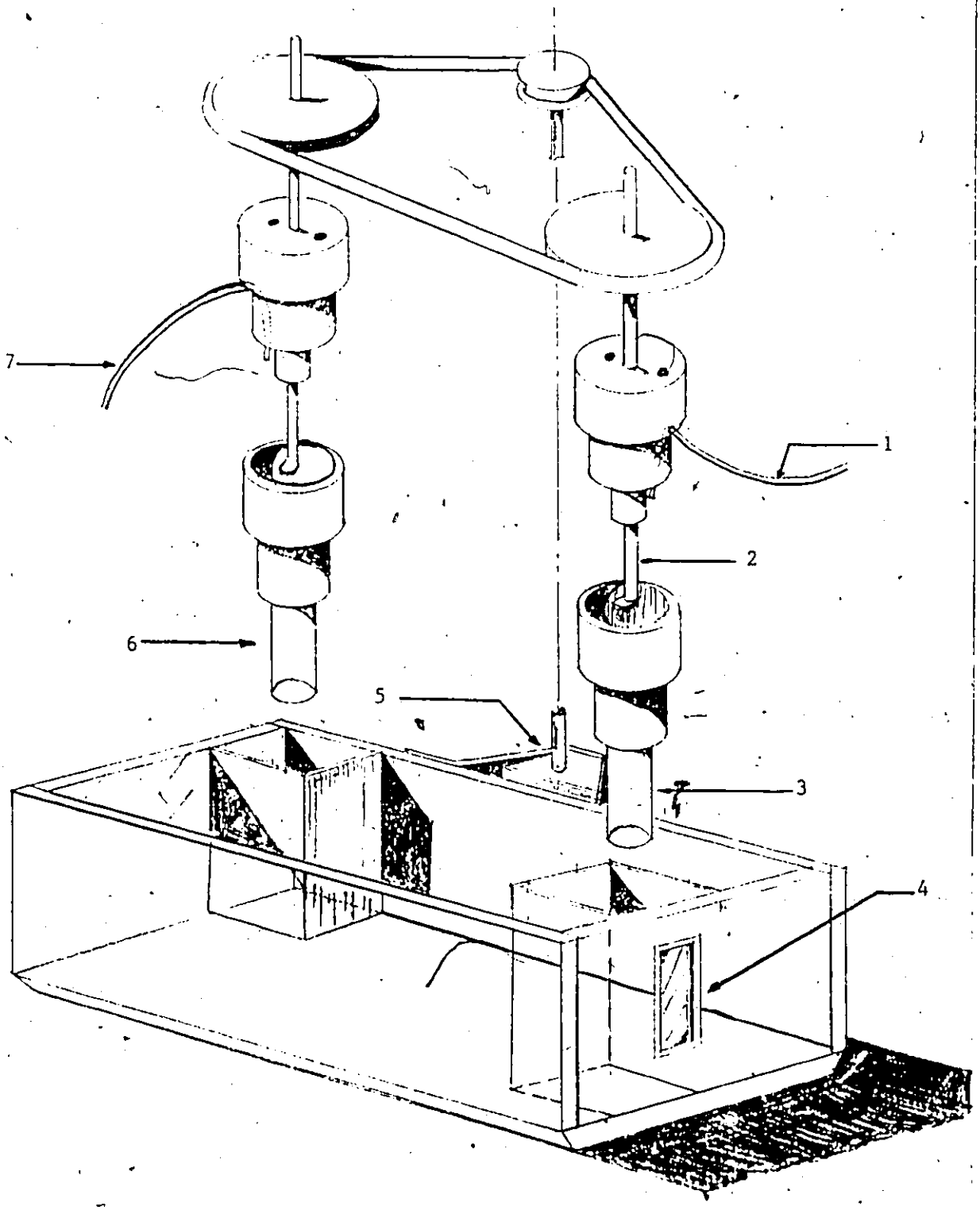
Figure 3

Schematic Diagram of Cuvette Holder

1. Tubing containing N_2 to flush cuvettes
2. Paddle stirrer
3. Irradiated cuvette
4. Quartz window
5. Stir motor
6. Non-irradiated cuvette
7. Tubing same as #1

Note: Cuvettes were actually square with 1 cm pathlength

Figure 3



These samples were heated at 80°C for 3 min. Care was taken to maintain pH at 6.8 by the addition of 5-10 µl of 1 M NaOH to neutralize any trapped trichloroacetic acid in the pellet. Protein determinations of these samples were done by fluorescence (see 2.12.2).

2.8 *Isolation of Cyclic [^{3}H] AMP*

Cyclic AMP was isolated by a modification (Haslam and Lynham, 1972) of the method of Krishna et al. (1968). To columns of length 165 mm and inner diameter 8 mm, 1.5 ml of Bio-Rad resin (AG50W-X8, 200-400 mesh) was added. All resin (700 gm batches) had been previously washed with 4 l H_2O; 4 l 0.5N NaOH; 4 l H_2O; 6 l 2N HCl; 8 l H_2O. Each assay sample was centrifuged for 4 min at 12,000 x g (Eppendorf centrifuge), placed on a separate column and eluted with 1 mM KH_2PO_4 buffer, pH 7.4. Cyclic AMP was eluted in the 6-18 ml fractions but only the 10-18 ml fractions were collected, giving a final recovery of 40-60%. The eluate was adsorbed twice with nascent $BaSO_4$ by adding 0.3 ml of 0.25 M $ZnSO_4$ plus an equivalent of $Ba(OH)_2$. After centrifugation (300 x g at 0°C), the supernatant was poured into scintillation vials and lyophilysed. The ^{3}H found in zero incubation time controls was subtracted from the cyclic [^{3}H]AMP found in the assays and corrections were made for the recovery of cyclic [^{14}C]AMP.

2.9 *Liquid Scintillation Counting*

^{3}H and ^{14}C were counted in a Beckman LS 230 scintillation counter in a mixture comprising 1 ml of aqueous sample and 7.5 ml of ACS (Aqueous Counting Scintillant, Amersham) fluid.

Counting efficiencies were approximately 16% and 50% for ^{3}H and ^{14}C respectively, under dual isotope conditions, and approximately 30% for ^{3}H alone. Results were corrected for background, channel crossover and variations in quenching. A Hewlett-Packard 9810 program was written which corrected for recovery and converted data to nmol of cyclic AMP/mg protein/10 min and reported the mean ± standard error of the mean for each triplicate.

2.10 *Adenosine Deaminase: Isolation and Assay*

Adenosine deaminase (adenosine aminohydrolase; E.C. 3.5.4.4) (Sigma, Type I) was freed of $(NH_4)_2SO_4$ by gel filtration. A 4.5 cm column of Sephadex G25 (fine) was prepared in a 3 mm (i.d.) tube. The Sephadex had been equilibrated in 150 mM TRIS, pH 7.4 for 2 h at 37°C or overnight at room temperature. The enzyme suspension was centrifuged for 2 min on Eppendorf centrifuge, the supernatant was removed and the solid enzyme pellet dissolved in 0.1 ml 150 mM TRIS, pH 7.4. The enzyme was added to the column and eluted with 150 mM TRIS, pH 7.4. Fractions (0.1 ml) were collected and a few µl were tested for the presence of protein and NH_3 with 10% trichloroacetic acid and Nessler's Reagent, respectively. Only samples negative for NH_3 but positive for protein were pooled and diluted for use.

The activity of the adenosine deaminase was checked prior to each experiment in which it was used, as it decreased with time even in the presence of 3.2 M $(NH_4)_2SO_4$. Enzyme (100 µl of a 1:10,000 dilution) was added to 45 µM adenosine in 150 mM TRIS (pH 7.4, 25°C). The rate of decrease in absorbance at 265 nm that

occurs when adenosine is converted to inosine was used to follow the reaction (Perkin-Elmer spectrophotometer). At pH 7.5, the ΔE^{m}_{265} between adenosine and inosine is 8,100. From this, the enzyme activity was calculated.

2.11 *SDS/Polyacrylamide-Gel Electrophoresis*

Samples to be used for electrophoresis were dissolved in 100-200 µl of sample buffer containing 3% (w/v) SDS, 0.0025% (w/v) Bromophenol Blue, 52 mM TRIS-HCl, pH 6.8, 6% (w/v) glycerol and 5% (w/v) mercaptoethanol. These samples were heated at 80°C for 3 min. Care was taken to maintain pH at 6.8. Precise volumes were electrophoresed on slab gels. Separating gels (approximately 19 cm long) contained 10% (w/v) acrylamide, 0.8% (w/v) NN'-methylenebisacrylamide, 0.1% (w/v) SDS, 0.025% (v/v) NNN'N'-tetramethylethylenediamine, 0.075% (w/v) ammonium sulphate, 0.1% (w/v) glycerol and 0.375 M TRIS-HCl, pH 8.8. Stacking gels (approximately 2 cm long) contained 5% (w/v) acrylamide, 0.8% (w/v) NN'-methylenebisacrylamide, 0.1% (w/v) SDS, 0.038% (v/v) NNN'N'-tetramethylethylenediamine; 0.3% (w/v) ammonium persulphate, 0.05% (w/v) glycerol and 0.125 M TRIS-HCl, pH 6.8.

The electrode buffer contained 0.1% (w/v) SDS, 0.192 M glycine and 25 mM TRIS base. Protein samples were electrophoresed at room temperature for 1 h at 150 mV and then at 175 mV for 7 h.

Gels were stained overnight in a solution of 0.1% (w/v) Coomassie Brilliant Blue R in methanol/acetic acid/water (5:1:5 by vol), destained for 4 h in methanol/acetic acid/water (5:1:5 by

vol), and then further destained in 10% (v/v) acetic acid for about 1 day. Gels were then soaked in water to remove acetic acid and then placed in 1 M sodium salicylate for 30-45 min to facilitate fluorography (Chamberlain, 1979).

Gels were dried under vacuum on Whatmann 3 MM chromatographic paper on a Savant Model SGD-200 slab gel dryer and then exposed to Kodak X-omat R X-ray film. The X-ray film was preflashed (Laskey and Mills, 1975) to provide greater sensitivity for ^{3}H detection. Films were developed on a Kodak RPX-omat processor.

2.12.1 *Protein Determination: Folin-Lowry*

Protein was determined by the method of Lowry et al. (1951) using human serum albumin as a standard.

The amount of platelet particulate fraction added to each assay or photolysis experiment was determined by measurement of protein in the resuspension medium in the absence and presence of the particulate fraction. Measurements were also made of the percentage of soluble platelet protein released during the freeze-thaw procedure.

$$\text{i.e.} \quad \frac{\text{mg protein/ml of lysate supernatant}}{\text{mg protein/ml of freeze thawed lysate}} \times 100.$$

The percent release in these experiments mounted to 66 ± 2% (mean ± S.E.M.) (n=32).

2.12.2 *Protein Determination: Fluorescence*

In photoaffinity labeling experiments where bound ^{3}H was to be detected by electrophoresis/sodium salicylate fluorography, the

protein in each sample was determined by fluorescence. An Aminco-Bowman spectrophotofluorometer was used. A Folin-Lowry protein determination was not possible due to the presence of mercaptoethanol in the electrophoresis sample buffer. A standard curve of known amounts of rabbit platelet protein (acid precipitated, particulate fraction) as determined by Folin-Lowry was used. This one standard curve was used for all protein determinations by fluorescence. Day-to-day fluctuations in the lamp of the spectrofluorometer were corrected by the use of human serum albumin standard curves done on the day of the rabbit platelet protein standard curve and a human serum albumin standard curve was also done along with the photolabeled protein samples. Insufficient amounts of rabbit platelet standard protein were available.

A precise volume (10 μl) of rabbit platelet particulate protein dissolved in electrophoresis sample buffer containing 3% (w/v) SDS was diluted to 1 ml with H_2O. The spectrofluorometer was set to 100% transmission (1 fluorescence unit) with 10 μM quinine sulphate, 345 nm excitation, 452 nm emission with all slit widths set at 2 mm. Protein measurements were made with an excitation wavelength of 280 nm and emission wavelength of 340 nm.

2.13 *Statistics*

The results presented consist of two types:

(a) data from single experiments in which assays were done in triplicate including photoaffinity labeling experiments except where indicated,

(b) data from more than one experiment.

(a) The triplicate observations were averaged and the standard error of the mean (S.E.M.) calculated:

$$\text{S.E.M.} = \sqrt{\frac{\text{variance}}{n}} \quad \text{where variance} = \frac{X^2 i - n\bar{X}^2}{n - 1}, \; n = \text{sample size}$$

Xi = sample observation, $\bar{X}$ = average of sample observations. These S.E.M. values are indicative only of experimental or assay error.

Standard error of the difference (S.E.D.) between the test compound activity and the appropriate control activity were calculated:

$$\text{S.E.D.} = \sqrt{\left(\text{S.E.M.}_{\text{control}}\right)^2 + \left(\text{S.E.M.}_{\text{test compound}}\right)^2}$$

(b) Test compound results from several experiments were averaged, each data point being the mean effect within a single experiment expressed as a percentage of its appropriate control. The S.E.M. values <u>within</u> each experiment were ignored and a new S.E.M. calculated using the data from the combined experiments. These S.E.M. values are indicative of both biological and experimental variation.

CHAPTER 3

Results: Section 3.1

Factors Affecting the Activation of Platelet Adenylate Cyclase by 2-Azidoadenosine

RESULTS: SECTION 3:1

Factors Affecting the Activation of Platelet Adenylate Cyclase by 2-Azidoadenosine

3.1.1 *Effects of Adenosine Deaminase and $MgCl_2$ Concentration on the Activation of Adenylate Cyclase in Human Platelet Particulate Fraction by Adenosine or 2-Azidoadenosine*

The effect of adenosine on human platelet adenylate cyclase activity at 5.0 mM $MgCl_2$ was biphasic; concentrations below 10 μM caused a weak activation while at 100 μM inhibition was observed (Table 1). At 0.8 mM $MgCl_2$, activation of adenylate cyclase activity was observed with all concentrations of adenosine tested. This enhancement of activation or reduction of inhibition at a low Mg^{2+} concentration in human platelets has been previously noted (see 1.2.2).

In the presence of 5 mM $MgCl_2$, 2-azidoadenosine stimulated adenylate cyclase activity at all concentrations tested. In the presence of adenosine deaminase, the activation rose from 55% to 76% with 10 μM 2-azidoadenosine. Reduction of $MgCl_2$ concentration to 0.8 mM increased the activation of adenylate cyclase by 2-azidoadenosine. With 10 μM 2-azidoadenosine activation was 77%, an increase of 22% relative to that with the higher Mg^{2+} concentration. Adenosine deaminase decreased basal adenylate cyclase activity at both $MgCl_2$ concentrations. The adenylate cyclase activity in the presence of 2-azidoadenosine was the same in the presence and

absence of adenosine deaminase. In the presence of adenosine deaminase, activation of human platelet adenylate cyclase by 2-azidoadenosine reached 126% in the presence of 0.8 mM $MgCl_2$, the highest achieved with enzyme from human platelets.

3.1.2 *Effects of Adenosine and of 2-Azidoadenosine in the Presence and Absence of Adenosine Deaminase on Rabbit Platelet Adenylate Cyclase*

As shown in Table 2, the effect of adenosine on rabbit platelet adenylate cyclase activity was biphasic at both Mg^{2+} concentrations; in the 0.5-10 μM range, adenosine showed marked activation of the enzyme while at 100 μM the activation was weak. No inhibition of enzyme activity was observed at 100 μM at either Mg^{2+} concentrations in contrast to the effect on human platelet adenylate cyclase activity (Table 1).

2-Azidoadenosine activated rabbit platelet adenylate cyclase activity at all concentrations tested at both concentrations of Mg^{2+}. The activation was similar to the response elicited by adenosine at 10 μM and lower concentration. At 100 μM, 2-azidoadenosine still showed marked activation of rabbit platelet adenylate cyclase, so that a biphasic response was not observed.

Increasing Mg^{2+} concentrations increased control adenylate cyclase activity but decreased nucleoside activation. In contrast to Table 1, an increased Mg^{2+} concentration did not cause the appearance of net inhibition by 100 μM adenosine.

The addition of adenosine deaminase reduced control adenylate cyclase activities but in the presence of 0.5 μM 2-azidoadenosine, the deaminase did not affect adenylate cyclase activity. This resulted in a larger percent activation of adenylate cyclase activity by 2-azidoadenosine in the presence of the deaminase. The combined effects of a low Mg^{2+} concentration and the addition of adenosine deaminase led to the highest activation observed by 2-azidoadenosine, a value 200% above control levels. This activation was almost double the maximum effect of 2-azidoadenosine observed with human platelet adenylate cyclase (Table 1).

3.1.3 *Attempts to Reduce the Control Activity of Rabbit Platelet Adenylate Cyclase with Various Agents*

As the addition of adenosine deaminase showed that adenosine was present in the assay, either added in the enzyme preparation or formed from the breakdown of exogenous ATP, additional agents were used in an attempt to reduce the control adenylate cyclase activity even further (Table 3). Indomethacin, a fatty acid cyclo-oxygenase inhibitor, was used to block production of any stimulatory prostaglandins and (-)-propranolol, a beta adrenergic receptor antagonist was used to block the effects of any endogenous epinephrine.

Of the three agents tested by themselves, only adenosine deaminase significantly reduced control adenylate cyclase activity ($2P < 0.05$). Various combinations of the agents did not reduce the control activity any more than adenosine deaminase. In the presence

of indomethacin and adenosine deaminase with or without (-)-propranolol the control activity was lower than with the deaminase alone, but this difference was not significant.

In the presence of 10 μM 2-azidoadenosine, the adenylate cyclase activity was not significantly different in the additional presence of any of the agents tested, alone or in combination. The percent increases in adenylate cyclase activity were largest when adenosine deaminase was present.

Table 1

Effects of Adenosine Deaminase and $MgCl_2$ Concentration on the Activation of Adenylate Cyclase in Human Platelet Particulate Fraction by Adenosine or 2-Azidoadenosine.

Mean values ± S.E.M. for the percentage changes in activity from a number(n) of experiments are given. In each of the experiments, the changes in adenylate cyclase activity in the presence of a nucleoside was compared to the control containing no added nucleoside. In the presence of adenosine deaminase, the change in adenylate cyclase activity by 2-azidoadenosine was compared to the control containing no added nucleoside but containg adenosine deaminase.

Table 1

Effects of Adenosine Deaminase and $MgCl_2$ Concentration on the Activation of Adenylate Cyclase in Human Platelet Particulate Fraction by Adenosine or 2-Azidoadenosine

Nucleoside	Nucleoside Concn. (μM)	$MgCl_2$ (mM)	Changes in Adenylate Cyclase Activity (%) Adenosine Deaminase* absent	present
Adenosine	0.5	5.0	+25 ± 3(n=4)	---
	2.0		+31 ± 2(n=4)	---
	10.0		+29 ± 2(n=4)	---
	100.0		-33 ± 1(n=4)	---
	0.5	0.8	+30 ± 7(n=4)	---
	2.0		+44 ± 3(n=4)	---
	10.0		+57 ± 4(n=4)	---
	100.0		+70 ± 9(n=4)	---
2-Azidoadenosine				
	0.5	5.0	+25 ± 2(n=5)	+38 ± 3(n=3)
	2.0		+32 ± 3(n=5)	+64 ± 4(n=3)
	10.0		+55 ± 8(n=5)	+76 ± 4(n=4)
	100.0		+40 ± 7(n=5)	+72 ± 7(n=4)
	0.5	0.8	+24 ± 5(n=5)	+36 ± 6(n=3)
	2.0		+42 ± 4(n=5)	+84 ± 10(n=4)
	10.0		+77 ± 9(n=5)	+113 ± 13(n=4)
	100.0		+79 ± 9(n=5)	+126 ± 11(n=3)

*Average adenosine deaminase activity was 13 units/ml in the assay mixture.

Table 2

Effect of Adenosine and of 2-Azidoadenosine in the presence and absence of Adenosine Deaminase on Rabbit Platelet Adenylate Cyclase

Adenylate cyclase activities are means ± SEM from three determinations on the same enzyme preparation. Changes in adenylate cyclase activity (difference between means ± SED) are expressed as percentages of the appropriate controls. The controls for stimulation by adenosine and 2-azidoadenosine, in the absence of adenosine deaminase, are indicated as † 0.8 mM $MgCl_2$ and ¶ 5.0 mM $MgCl_2$. These were also used in calculating the effect of adenosine deaminase on control adenylate cyclase activity. Controls used to calculate percent changes in the presence of adenosine deaminase are: § at 0.8 mM and * at 5.0 mM $MgCl_2$. The adenosine deaminase (AD) activity was 7.1 units/ml in the assay mixture.

Table 2

Nucleoside	Nucleoside concn. (μM)	$MgCl_2$ (mM)	Adenylate Cyclase Activity nmol cyclic AMP/mg protein/10 min −AD	+AD	% Change −AD	+AD
Control	—	0.8	0.223 ± 0.006†	0.176 ± 0.009 §	---	−21.1 ± 5.1
		5.0	0.562 ± 0.015¶	0.493 ± 0.020*	---	−12.1 ± 4.4
Adenosine	0.5	0.8	0.364 ± 0.027	---	+62.6 ± 12.4	---
		5.0	0.811 ± 0.048	---	+44.2 ± 9.0	---
	2.0	0.8	0.417 ± 0.028	---	+86.5 ± 12.7	---
		5.0	0.981 ± 0.022	---	+74.3 ± 4.7	---
	10.0	0.8	0.469 ± 0.009	---	+110.0 ± 5.0	---
		5.0	0.912 ± 0.017	---	+62.0 ± 3.9	---
	100.0	0.8	0.251 ± 0.008	---	+12.3 ± 4.8	---
		5.0	0.611 ± 0.014	---	+8.6 ± 3.6	---
2-Azido-Adenosine	0.5	0.8	0.409 ± 0.025	0.376 ± 0.007	+83.0 ± 11.6	+113.2 ± 6.5
		5.0	0.898 ± 0.035	0.795 ± 0.025	+59.4 ± 6.8	+61.3 ± 6.5
	2.0	0.8	0.463 ± 0.016	0.456 ± 0.010	+107.1 ± 7.7	+158.4 ± 7.8
		5.0	0.924 ± 0.022	0.986 ± 0.027	+64.1 ± 4.7	+100.0 ± 6.9
	10.0	0.8	0.488 ± 0.005	0.487 ± 0.017	+118.4 ± 3.6	+176.3 ± 11.2
		5.0	0.948 ± 0.034	1.008 ± 0.013	+68.5 ± 6.6	+104.5 ± 4.9
	100.0	0.8	0.526 ± 0.017	0.524 ± 0.021	+135.3 ± 8.0	+197.1 ± 12.8
		5.0	1.008 ± 0.027	0.986 ± 0.024	+79.0 ± 5.4	+100.0 ± 6.4

Table 3

Attempts to Reduce the Control Activity of Rabbit Platelet Adenylate Cyclase with Various Agents

Adenylate cyclase activities are means ± SEM from three determinations on the same platelet preparation. Indomethacin was added at a final concentration of 20 μM prior to freezing the platelets and was also present in the resuspension mixture; (-)-propranolol and adenosine deaminase were added prior to the incubation. All assays were in the presence of 5.0 mM $MgCl_2$. Changes in adenylate cyclase activity (difference betwwen means ± SED) are expressed as percentages of the appropriate controls. In the absence of 2-azidoadenosine, adenylate cyclase activities are compared to the controls containing no added compounds. In the presence of 2-azidoadenosine, each adenylate cyclase activity was compared to the corresponding activity produced by the same combination of agents in the absence of the nucleoside.

Table 3

Compounds Added	Adenylate Cyclase Activity nmol cyclic AMP/mg protein/10 min	% Change
In the Absence of 10 μM 2-Azidoadenosine		
None	0.275 ± 0.006	—
Adenosine Deaminase (8.5 units/ml) (AD)	0.239 ± 0.007	−13.3 ± 2.6
10 μM (−) Propranolol (Pro)	0.286 ± 0.003	+3.6 ± 1.2
20 μM Indomethacin (Indo)	0.282 ± 0.007	+2.5 ± 3.3
AD + Pro	0.223 ± 0.005	−15.5 ± 1.9
AD + Indo	0.212 ± 0.006	−22.9 ± 3.1
Pro + Indo	0.261 ± 0.011	−0.5 ± 4.5
AD + Pro + Indo	0.212 ± 0.001	−22.9 ± 2.2
In the Presence of 10 μM 2-Azidoadenosine		
None	0.441 ± 0.013	+59.7 ± 4.7
AD	0.423 ± 0.006	+77.1 ± 3.7
Pro	0.436 ± 0.014	+52.5 ± 5.0
Indo	0.433 ± 0.009	+54.5 ± 4.1
AD + Pro	0.429 ± 0.009	+85.1 ± 4.2
AD + Indo	0.446 ± 0.012	+110.6 ± 6.2
Pro + Indo	0.455 ± 0.013	+74.6 ± 6.5
AD + Pro + Indo	0.432 ± 0.009	+103.2 ± 4.2

Results: Section 3.2

Photolysis of 2-Azidoadenosine

RESULTS: SECTION 3.2

3.2 *Photolysis of 2-Azidoadenosine*

3.2.1 *Energy Output of Mercury Lamp*

The Bausch and Lomb monochromator, as previously described (section 2.7) was equipped with a high pressure mercury lamp. The energy output of the lamp, passing through the monochromator, was measured using a radiometer. Using the completely dark irradiation chamber as the zero, the energy output of the lamp at various wavelengths (10 nm bandpass) is shown in Figure 4. The energy output shows a peak of 5.9×10^3 ergs/cm^2.s at 313 nm. At 270 and 320 nm, the energy output was 4.2 and 81.5% of the peak.

3.2.2 *Absorption Spectra of 2-Azidoadenosine Under Various Conditions.*

The absorption spectrum of 2-azidoadenosine (Figure 5) showed a peak at 270 nm and two shoulders at 308 nm and 321 nm. This absorption spectrum was very similar to that described by Temple et al. (1966) and Cusack and Born (1977). Irradiation at 313 nm was selected because it was the peak lamp output, some absorption by the nucleoside was possible at this wavelength and because this wavelength would avoid membrane damage. A 10 nm bandpass kept most of the radiation above 300 nm (see section 1.4).

The effect of 313 nm light on the 2-azidoadenosine absorption spectrum is shown in Figure 6. Thirty minutes of irradiation

caused complete photolysis since further alteration in the absorbance profile were not obtained with increasing lengths of UV exposure. The increase in absorbance at 246 nm was related to the time of photolysis of 2-azidoadenosine. This total absorbance at this wavelength (246 nm) was plotted against time (Figure 7). From this figure, an irradiation of 10 min was determined also to be an effective length of time necessary to produce extensive photolysis (> 80%).

In preliminary studies a 5 min irradiation of 2-azidoadenosine led to a reduction in absorption at the 270 nm peak yet at 308 nm little or no change occurred. In the experiment shown in Figure 8, an incubation period of 30 min followed a 5 min irradiation. During this period the absorption peak at 270 nm increased while the shoulder at 308 nm decreased. These changes suggest movement towards an equilibrium.

2-Azidoadenosine has two tautomers (see 1.4), the azide and tetrazole, with the azide derivative usually being the more photolabile (Bayley and Knowles, 1977). The absorption spectrum of 2-azidoadenosine will be the sum of its tautomers and in order to determine the photolabile species the individual spectra must be separated.

Temple et al. (1966) had shown that under acid conditions the azide is the major tautomer present, whereas basic conditions favour tetrazole formation. Figure 9 shows the effect of pH on the 2-azidoadenosine absorption spectrum. At pH 2.3, the spectrum is unchanged in several appearances, but, the 270 nm peak shows an increased absorbance and the two shoulders are less prominent

(cf. Figure 5). Under basic conditions two absorption peaks were observed; the 270 nm peak was diminished and shifted to 278 nm whereas the two shoulders had merged to form a peak at 313 nm. An isobestic point at 293 nm in Figure 9 is also seen in Figure 8.

Figure 9 indicates the azide has a large absorbance at 270 nm and the tetrazole has a predominate absorbance at 308 nm. Figure 8 indicated the photolabile species had a predominate absorbance at 270 nm with little or no change at 308 nm immediately after irradiation. Figure 9 suggested the photolabile species was the azide while the tetrazole was the unchanged tautomer. The 30 min incubation period allowed the azide:tetrazole equilibrium to re-establish since azide had been removed by photolysis. Figure 8 reveals that the lost azide was replaced by tetrazole indicated by the increase in the 270 nm (azide) and the decrease in absorbance at 308 nm (tetrazole).

The kinetics of the azide:tetrazole equilibrium were studied by following the change in absorption spectrum after rapid neutralization of acidic or basic solutions of 2-azidoadenosine (Figure 10A). The change in absorbance was measured at 320 nm to obtain the greatest difference and the logarithm of the change of absorbance was plotted against time (Figure 10B). The graph shows that the azide: tetrazole interconversion follows first order kinetics with a half-life of 15.3 min at 22°C.

Evidence that the nucleoside could undergo photolysis with platelet protein present is provided in Figure 11. Platelet protein has a high absorbance below 300 nm, whereas at 313 nm the absorption of platelet protein is less than the absorption of 50 μM 2-azidoadenosine. Evidence that photolysis has occurred is shown in the loss of absorption of irradiated 2-azidoadenosine compared to non-irradiated nucleoside. The irradiated nucleoside underwent the shift in azide:tetrazole ratio when the irradiated sample was allowed to equilibrate.

3.2.3 *Effect of Dithiothreitol on 2-Azidoadenosine*

Carpenter et al. (1978) showed that dithiothreitol (DTT) could reduce aryl azides to the corresponding aryl amines and by implication might therefore reduce 2-azidoadenosine to 2-aminoadenosine. Since DTT was present in both the adenylate cyclase assays (30°C for 11 minutes; 10 min assay plus 1 min temperature equilibration) and in the incubation medium during photolysis (4°C for 30 min), Carpenter's reaction was investigated. Table 4 shows DTT was lost spontaneously, probably due to oxidation, but that 2-azidoadenosine did not enhance this loss of DTT. In the presence of 313 nm radiation alone, DTT did not break down but when 2-azidoadenosine was also present, DTT was consumed. This loss of DTT corresponds to a 71.8% loss of 2-azidoadenosine. This is probably due to DTT scavenging the nitrene radical since DTT is a well known scavenger (Bayley and Knowles, 1977).

3.2.4 *Azide or Tetrazole: Which Tautomer Interacts with the Adenosine Receptor?*

In order to determine the tautomer of 2-azidoadenosine that interacts with the adenosine receptor, the nucleoside was incubated in base for 2 h and then neutralized. Table 5 shows the effect of the shifting equilibrium upon the activation on platelet particulate fraction adenylate cyclase. Two minutes after neutralization both neutral and base incubated nucleoside produced the same activation of adenylate cyclase. Longer times after neutralization reveal no further change. Pre-incubation in acid (not shown) also produced the same amount of activation. This data suggests both tautomers of 2-azidoadenosine, the photolabile azide and the tetrazole, interact with the adenosine receptor of adenylate cyclase.

FIGURE 4

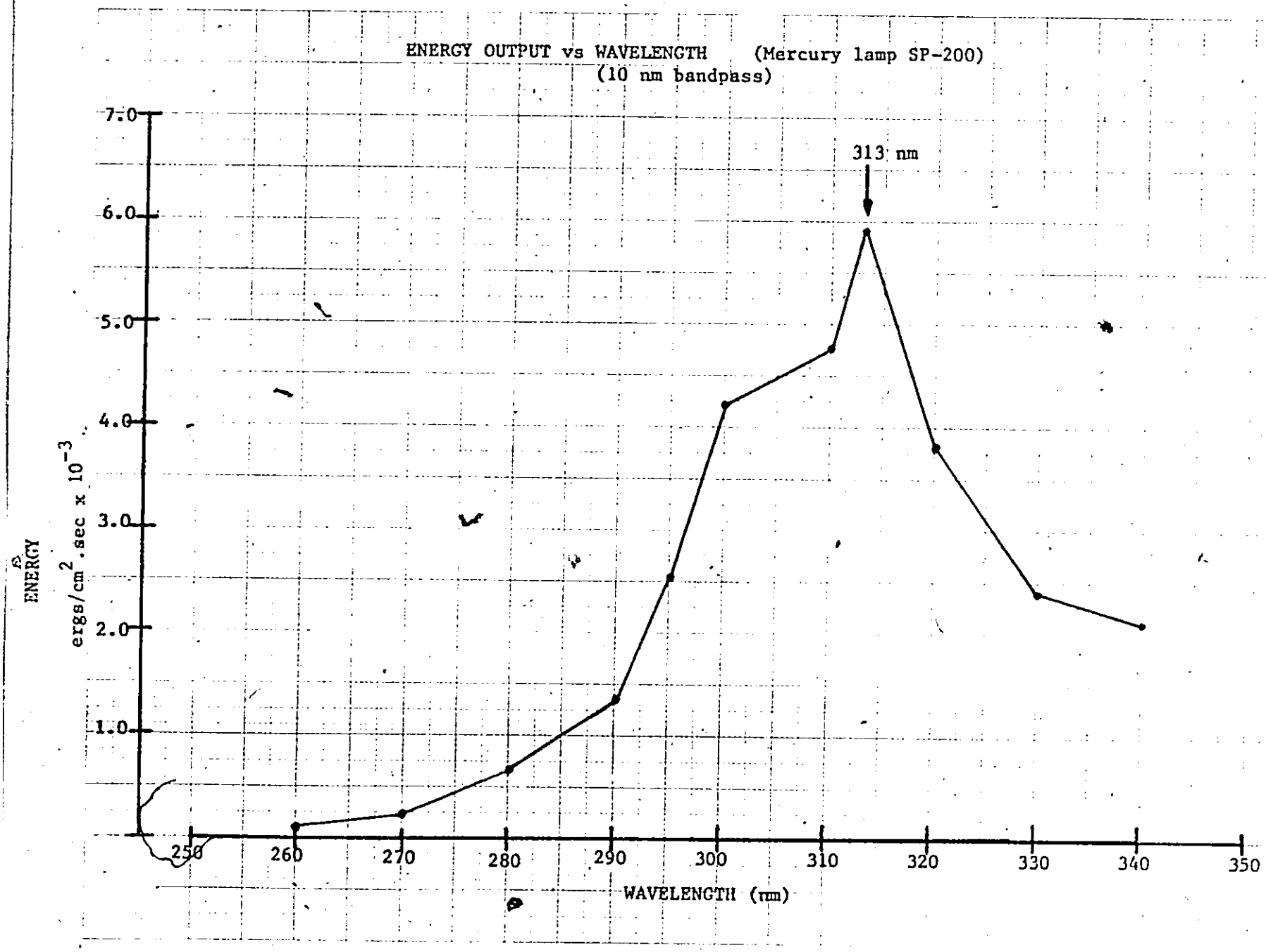

ENERGY OUTPUT vs WAVELENGTH (Mercury lamp SP-200)
(10 nm bandpass)
313 nm
ENERGY
ergs/cm^2.sec x 10^{-3}
7.0
6.0
5.0
4.0
3.0
2.0
1.0
250
260
270
280
290
300
310
320
330
340
350
WAVELENGTH (nm)

FIGURE 5

Absorption Spectrum of 2-Azidoadenosine in 150 mM TRIS-HCl (pH 7.4)

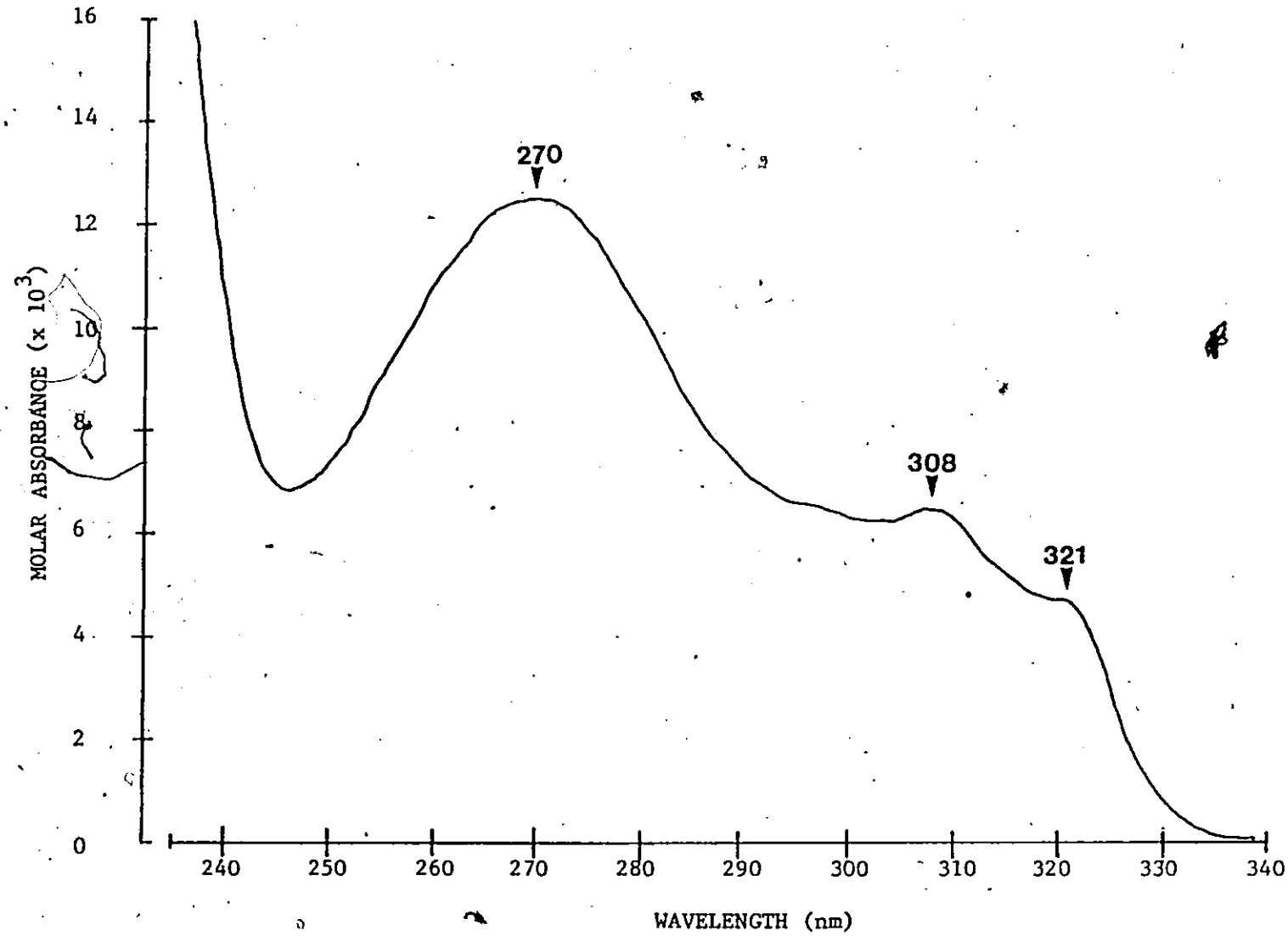


FIGURE 6

Effect of Irradiation at 313 nm on the Absorption Spectrum of 2-Azidoadenosine

A single sample of 10 μM 2-azidoadenosine in 150 mM TRIS-HCl (pH 7.4) was photolysed in a quartz cuvette for the indicated times. After each period of irradiation an absorption spectrum was obtained using a Cary 118 spectrophotometer.

FIGURE 6

Effect of 313 nm Radiation on 2-Azidoadenosine

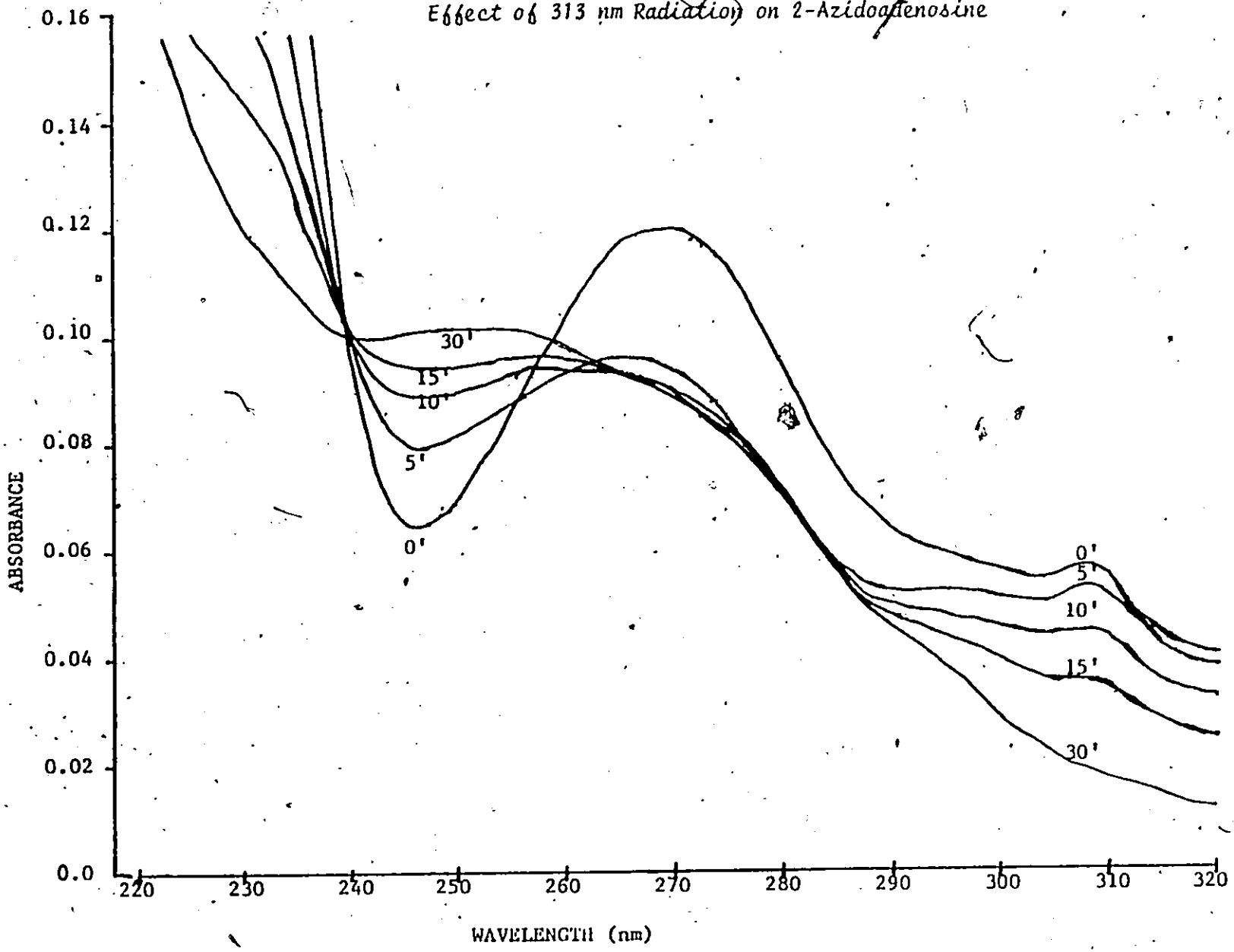


Figure 7

Increase in Absorbance at 246 nm during Photolysis: Comparison of Different Radiation Wavelengths and Replacement of Mercury Lamp

The increase in absorbance at 246 nm from U.V. spectra during the photolysis of 10 μM 2-azidoadenosine in 150 mM TRIS-HCl (pH 7.4) was plotted against length of exposure to U.V. radiation. Irradiation wavelengths of 313 nm (●,◆ done on two different mercury HP-200 lamps) and 300 nm were used, both with a 10 nm bandpass.

Figure 7

Increase in Absorbance at 246 nm during Photolysis

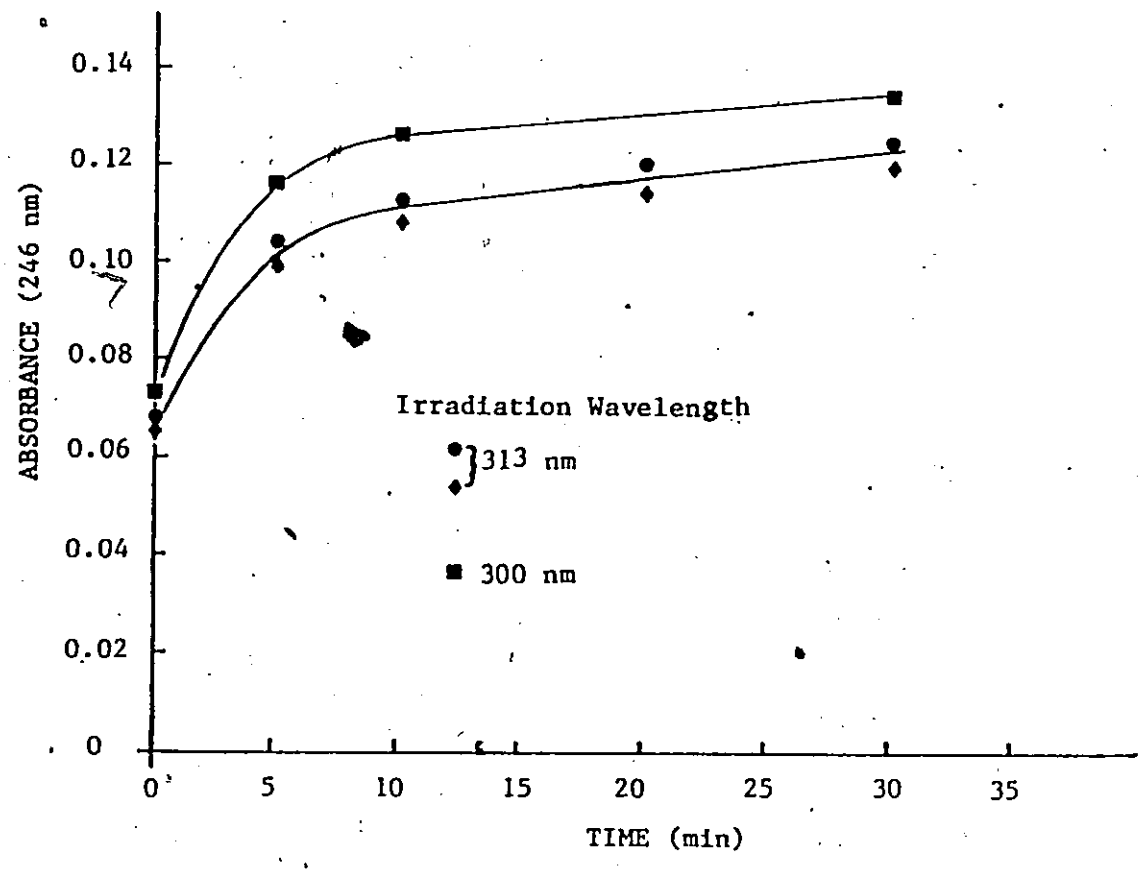


Figure 8

Disturbance of Azide:Tetrazole Equilibrium Due to Photolysis

2-Azidoadenosine (10 μM) in 150 mM TRIS-HCl, pH 7.4, was irradiated for 5 min. The azide: tetrazole mixture was allowed to approach equilibrium for 30 min at 22 °C. Spectra were recorded as follows:

a) 0 min:10 μM 2-azidoadenosine

b) 5 min:2-azidoadenosine after irradiation

b') 35 min:2-azidoadenosine after irradiation plus 30 min at 22 °C

FIGURE 8

Disturbance of Azide:Tetrazole Equilibrium Due to Photolysis

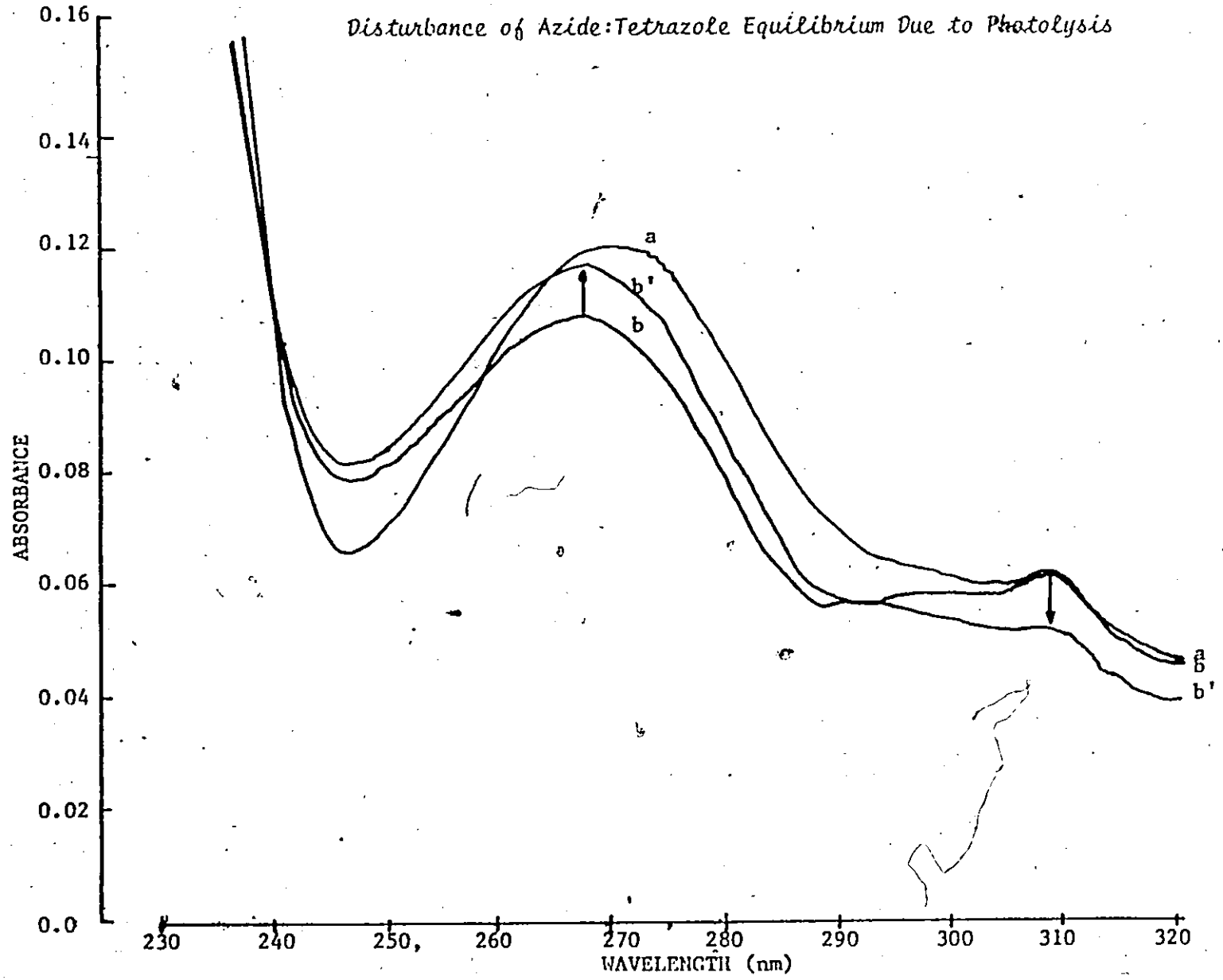


FIGURE 9

Effect of pH on the Absorption Spectrum of 2-Azidoadensoine

2-Azidoadenosine (10 μM) was incubated in either 0.05 M HCl or 0.05 M NaOH for 2 h. Absorption spectra were obtained using a Cary 118 spectrophotometer.

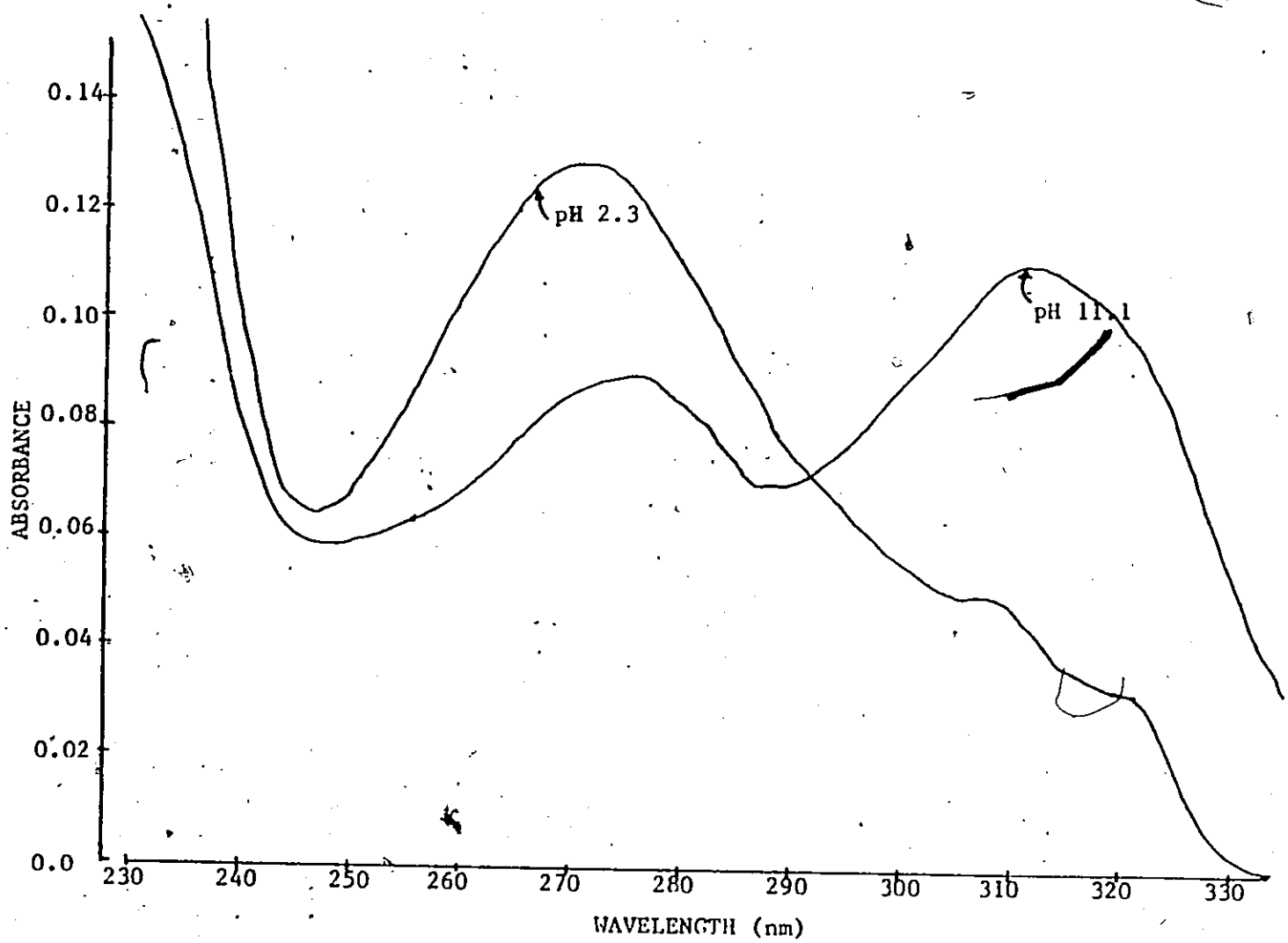


FIGURE 10

Kinetics of Azide:Tetrazole Tautomerization

2-Azidoadenosine (0.625 mM) was incubated for at least 2 h in either 0.05 M HCl or 0.05 M NaOH (final volume 0.192 ml). After this time had elapsed the nucleoside was neutralized with 2.8 ml of 0.6 M TRIS-HCl buffer (final pH 7.4, 22°C). The change in absorpance at 320 nm was measured using a Perkin-Elmer 124 double beam spectrophotometer.

A, rate of absorbance change for tetrazole and azide

B, semi-log plot of difference from final absorbance of 0.25. The half-life of the equilibration was 15.3 min at 22°C.

FIGURE 10

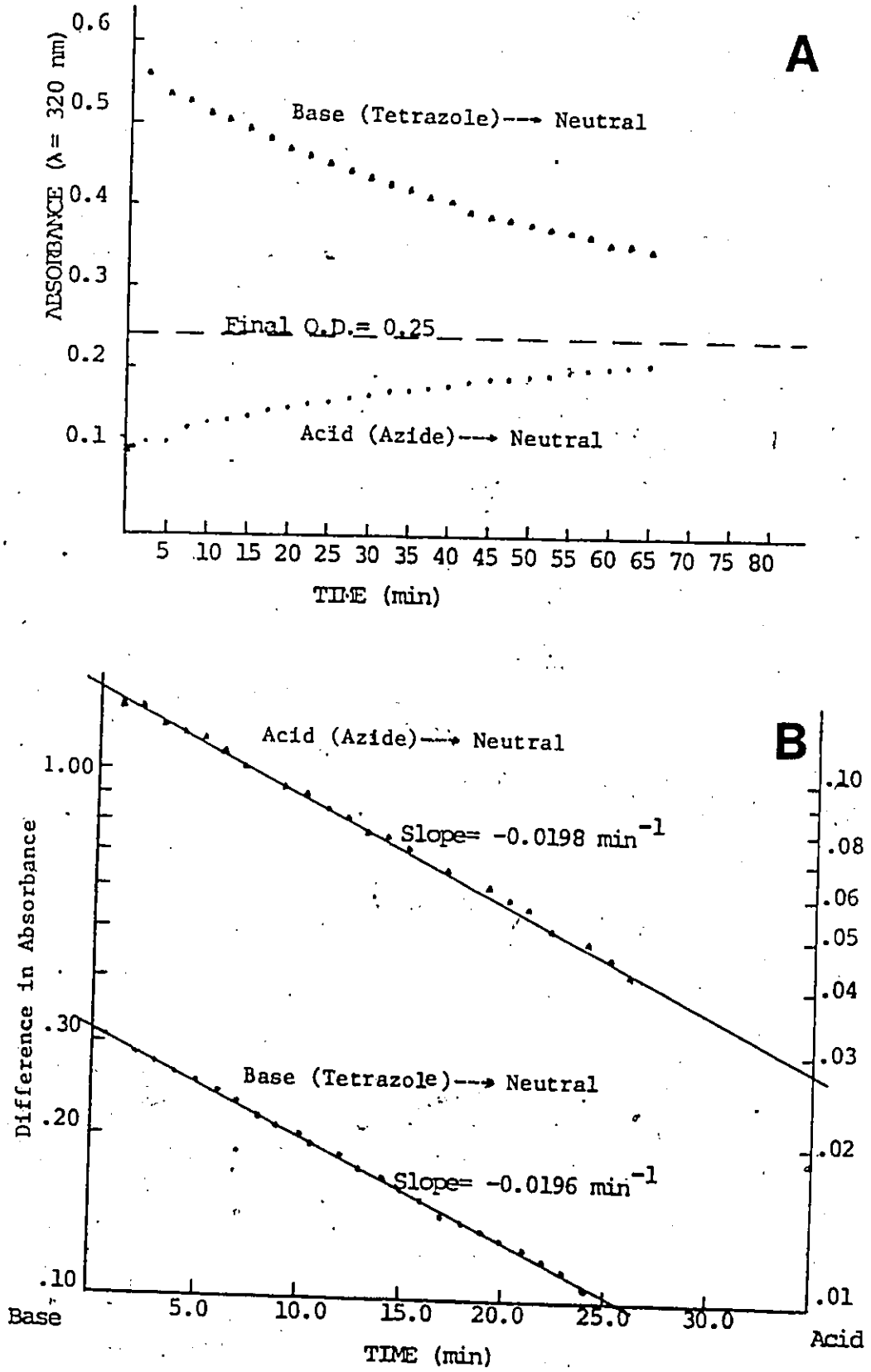

A
ABSORBANCE (λ = 320 nm)
0.6
0.5
0.4
0.3
0.2
0.1
Base (Tetrazole)---→ Neutral
Final O.D.= 0.25
Acid (Azide)---→ Neutral
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
TIME (min)
B
Acid (Azide)---→ Neutral
Slope= -0.0198 min-1
Base (Tetrazole)---→ Neutral
Slope= -0.0196 min-1
Difference in Absorbance
1.00
.30
.20
.10
Base
.10
.08
.06
.05
.04
.03
.02
.01
Acid
5.0 10.0 15.0 20.0 25.0 30.0
TIME (min)

FIGURE 11

Evidence that Photolysis occurs in the Incubation Mixture

About 150 mg (wet weight) of freeze-thawed rabbit platelets were resuspended in a mixture containing 75 mM TRIS-HCl (pH 7.4), 0.8 mM $MgCl_2$, 0.4 mM DTT, 0.4 mM ~~Na~~ MgEGTA and 0.1% bovine serum albumin in the presence(A,B) and absence(C,D) of 50 μM 2-azidoadenosine. The lysed platelets were either irradiated for 30 min (A,C) or kept in the dark (B,D). The lysates were then centrifuged at 48,000 x g (20 min, 5°C) and the supernatants removed for recording an absorption spectrum on a Cary 118 spectrophotometer.

Absorption Spectrum	Conditions
A	Lysate irradiated with 50 μM 2-azidoadenosine for 30 min
A'	Repeat of absorption spectrum A after a 35 min incubation in the dark at 22°C
B	Lysate with 50 μM 2-azidoadenosine non-irradiated
B'	Repeat of absorption spectrum B after a 44 min incubation in the dark at 22°C
C	Lysate irradiated without nucleoside
D	Lysate kept in the dark without nucleoside

FIGURE 11

Evidence that Photolysis occurs in the Incubation Mixture

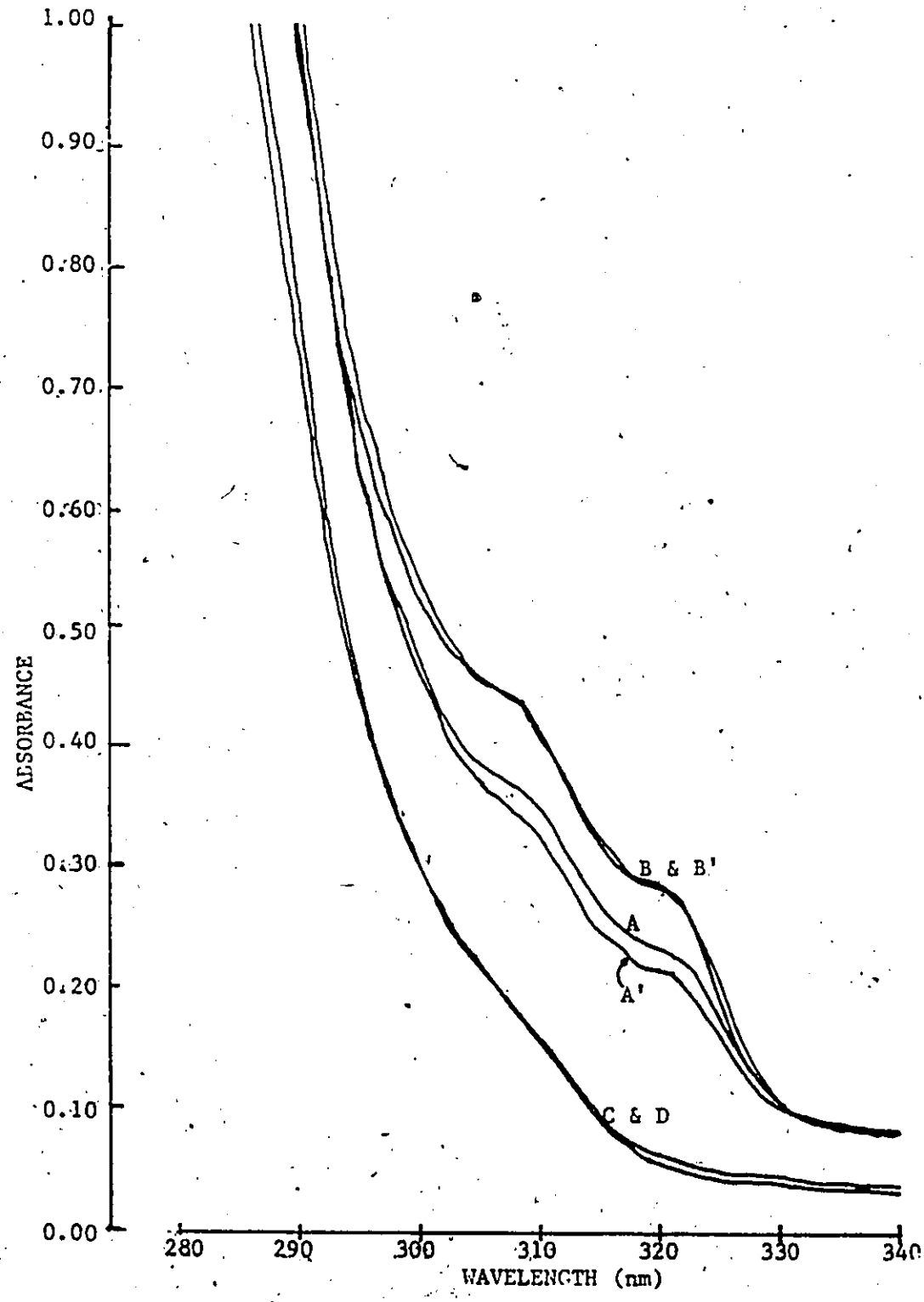


TABLE 4

Effect of Dithiothreitol (DTT) on 2-Azidoadenosine

Incubation Conditions	Nucleoside (100 μM)	nmol DTT consumed/0.2 ml	2P
13 min at 30°C (nonirradiated)	2-aminoadenosine	1.8 ± 0.2	---
	2-azidoadenosine	1.4 ± 0.4	NS
30 min at 4°C			
nonirradiated	2-aminoadenosine	3.9 ± 0.2	---
	2-azidoadenosine	3.5 ± 0.4	NS
irradiated at 313 nm	2-aminoadenosine	3.6 ± 0.2	---
	2-azidoadenosine	14.4 ± 0.4	<0.001

Results are means ± SEM from triplicate determinations. 2-Azidoadenosine (100 μM) or 2-aminoadenosine (100 μM) were incubated with 220 μM DTT in 150 mM TRIS-HCl, pH 7.4, under various conditions indicated. The reaction was stopped by adding 200 μl of the incubation mixture to 500 nmol Ellman's reagent(5,5'-dithiobis(2-nitrobenzoic acid)) in 2.8 ml of 150 mM TRIS-HCl (pH 7.4, final vol 2.85 ml). The absorbance was read at 412 nm in a Perkin-Elmer 124 double beam spectrophotometer. The E_M^{412} of Ellman's reagent is 13,600 at pH 8.0. The zero time control contained 44.0 ± 0.2 nmol of DTT/0.2 ml. The significance tests (Student's t-test) are with respect to control containing 2-aminoadenosine under each incubation condition.

Table 5

Determination of the Active Tautomer of 2-Azidoadenosine by Activation of Rabbit Platelet Adenylate Cyclase at 0.8 mM $MgCl_2$.

2-Azidoadenosine (20 µl of 125 µM) was incubated for 2 h at 22 °C in 80 µl of 0.04M NaOH. Neutralization was achieved by the addition of 80 µl of 0.04M HCl plus 1.8 ml of 150 mM TRIS-HCl, pH 7.4, at 22 °C. At various times after neutralization a 20 µl sample was assayed for its ability to activate adenylate cyclase. A neutral control, done at the same time, was obtained by mixing NaOH and HCl plus buffer prior to the addition of the nucleoside, followed by 2 h incubation. The final concentrations of the extra additions to the adenylate cyclase assays were 0.1 µM 2-azidoadenosine, 0.3 mM NaCl and 10.8 mM TRIS-HCl. Adenylate cyclase activities are means ± SEM from 3 determinations on the same enzyme preparation. Percentage changes in adenylate cyclase activity are means ± SED.

Pre-incubation Condition	Time after Neutralization (min)	Adenylate Cyclase Activity (nmol cyclic AMP/mg protein/5 min) Control	2-Azidoadenosine (0.1 µM)	Changes in Adenylate Cyclase Activity (%)
Neutral	2	0.106 ± 0.002	0.155 ± 0.005	+46 ± 5
	10	0.120 ± 0.004	0.172 ± 0.007	+43 ± 7
	20	0.116 ± 0.008	0.174 ± 0.004	+50 ± 8
	45	0.144 ± 0.002	0.190 ± 0.010	+32 ± 7
Basic	2	0.120 ± 0.007(n=2)	0.168 ± 0.001	+40 ± 5
	10	0.118 ± 0.002	0.177 ± 0.002	+50 ± 5
	20	0.116 ± 0.001	0.176 ± 0.006	+52 ± 5
	45	0.124 ± 0.004	0.185 ± 0.006	+49 ± 6

Results: 3.3

Attempts to Demonstrate Photoaffinity Labeling with Non-Radioactive 2-Azidoadenosine

3.3 *Attempts to Demonstrate Photoaffinity Labeling with Non-Radioactive 2-Azidoadenosine*

In these photoaffinity labeling experiments, the effect of photolysis of 2-azidoadenosine on particulate fraction adenylate cyclase activity was studied.

In Figure 12, the results of an experiment using 10 μM 2-azidoadenosine that was irradiated for 30 min are presented. After two additional cycles of centrifugation and resuspension, platelet particulate fraction still exhibited adenylate cyclase activity and was sensitive to activation by 2-azidoadenosine. The effects of irradiation and/or pre-exposure to 10 μM 2-azidoadenosine caused some activation of adenylate cyclase activity. The platelet particulate fraction (2) that was pre-exposed to the nucleoside without irradiation had a 0.080 ± 0.011 nmol cyclic AMP/10 min/mg protein (17 ± 6%) increase in adenylate cyclase activity above control particulate fraction 1; irradiation of particulate fraction 3 caused a 0.178 ± 0.012 nmol cyclic AMP/10 min/mg protein (52 ± 7%) increase in adenylate cyclase activity compared to fraction 1; the effects of both pre-exposure to 2-azidoadenosine and irradiation for 30 min on particulate fraction 4 -- the "photoaffinity labeled" receptors -- caused an increase in adenylate cyclase activity of 0.253 ± 0.006 nmol cyclic AMP/10 min/mg protein (63 ± 6%) above fraction 1. The effects of pre-exposure and irradiation were

additive (17% + 52% = 69%), so that no evidence for activation by photoaffinity labeling was obtained.

The presence of adenosine deaminase (Figure 12B) lowered all adenylate cyclase activities measured in the absence of 2-azidoadenosine but, as noted previously, had little effect on adenylate cyclase activities measured when 2-azidoadenosine was present in the assay mixture. Nucleoside added during the enzyme assay activated all four particulate fraction adenylate cyclases in a concentration dependent manner. This effect was superimposed on any activation caused by the photolysis conditions.

Although pre-exposure to 2-azidoadenosine and UV radiation both increased platelet adenylate cyclase activity, only exposure to UV radiation affected the activation of adenylate cyclase by 2-azidoadenosine added to the assays (Table 6). After irradiation, this activation was almost half the activation observed in the non-irradiated fractions. The presence of adenosine deaminase increased nucleoside activation in all cases but the relative effect of UV radiation was not reduced. This suggested that the breakdown of endogenous nucleotides to adenosine, as a result of irradiation, was not the reason for the enhancement of basal adenylate cyclase activity and depression of enzyme activation by 2-azidoadenosine.

No evidence was obtained for the inactivation of adenosine receptors by photoaffinity labeling, in that there was no inhibition of the activation of adenylate cyclase by 2-azidoadenosine added to the enzyme assay attributable to irradiation in the presence of 2-azidoadenosine rather than irradiation alone.

To reduce or eliminate the activation of adenylate cyclase by UV radiation and pre-exposure to 2-azidoadenosine, the photolysis conditions were changed to 10 min irradiation in the presence of 2 μM 2-azidoadenosine. The effects of these changes are shown in Figure 13. A 10 min exposure to 313 nm radiation did not significantly increase adenylate cyclase activity above the non-irradiated control. Pre-exposure to 2 μM 2-azidoadenosine did increase adenylate cyclase activity when 2-azidoadenosine was added to the assays but had a smaller effect on basal adenylate cyclase activity.

Similar evidence of a decreased activation by 2-azidoadenosine added to assays of irradiated enzyme was obtained when percentage activation was calculated (Table 7). However, irradiation in the presence of 2-azidoadenosine, the "photoaffinity labeled" fraction, revealed no difference from the effect of pre-exposure to 2-azidoadenosine alone (Table 7). This confirms that photoaffinity labeling cannot be detected using unlabeled 2-azidoadenosine.

Figure 12

The Effect of Irradiation of Platelet Particulate Fraction in the presence and absence of 2-Azidoadenosine on Platelet Adenylate Cyclase Activity: 10 μM 2-azidoadenosine, 30 min

Platelet particulate fraction was prepared for photolysis (see 2.7) and divided into four preparations: 1) ○,● , non-irradiated particulate fraction incubated for 30 min at 0 °C 2) ◇,◆, non-irradiated particulate fraction incubated with 10 μM 2-azidoadenosine for 30 min at 0 °C, 3) ▽,▼, particulate fraction irradiated for 30 min at 0 °C and 4) □,■, particulate fraction irradiated for 30 min in the presence of 10 μM 2-azidoadenosine at 0 °C. Each of the four preparations were then washed twice by centrifugation and resuspension (see 2.7) and its adenylate cyclase activity measured at 0.8 mM $MgCl_2$ in the absence and presence of 2-azidoadenosine (0.5, 10 μM) both without (A) and with (B) adenosine deaminase (14.7 units/ml in the assay mixture).

Figure 12

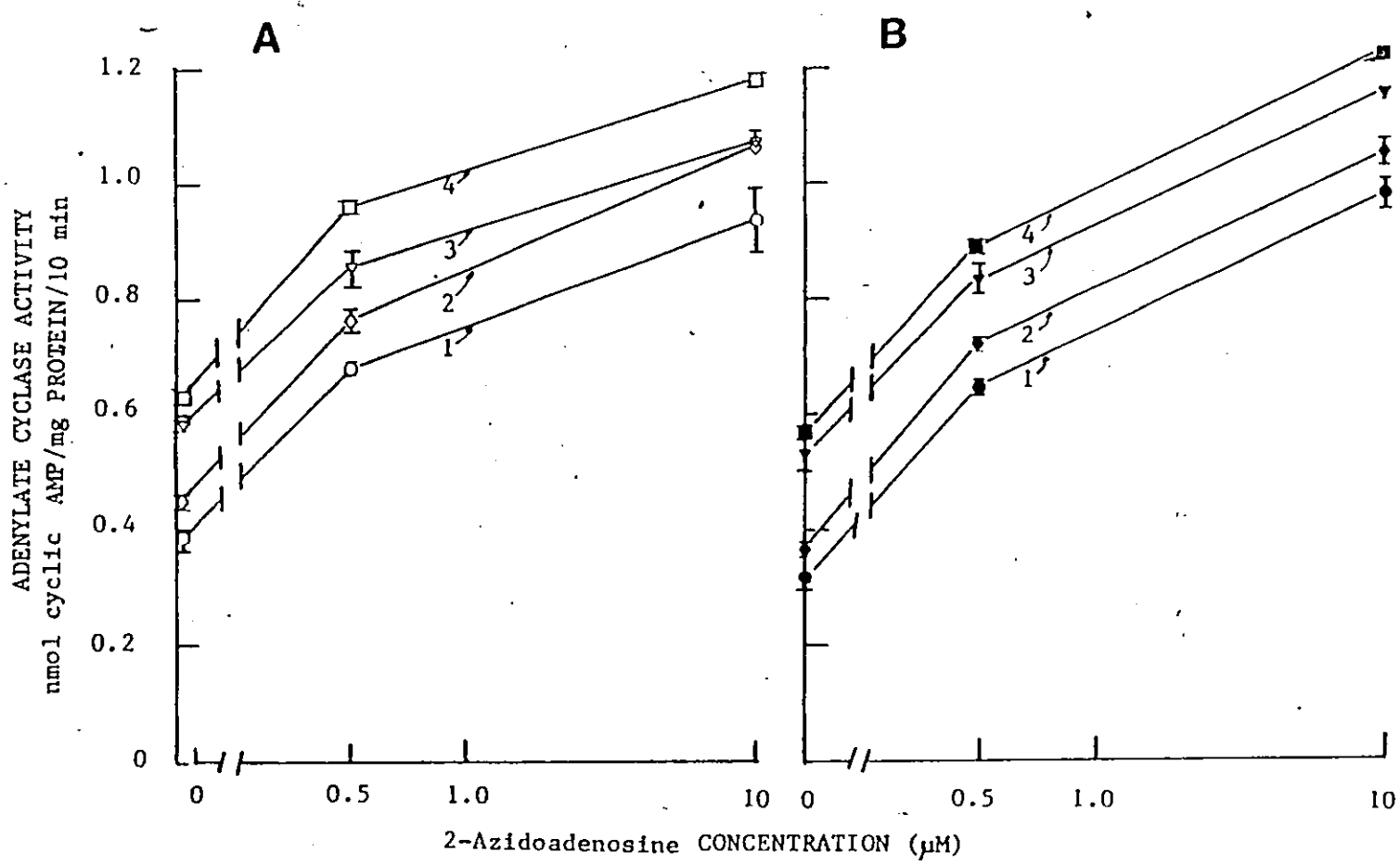

A
B
ADENYLATE CYCLASE ACTIVITY
nmol cyclic AMP/mg PROTEIN/10 min
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
10
2-Azidoadenosine CONCENTRATION (μM)
1
2
3
4

Figure 13

The Effect of Irradiation of Platelet Particulate Fraction in the presence and absence of 2-Azidoadenosine on Platelet Adenylate Cyclase Activity: 2 μM 2-azidoadenosine, 10 min

Platelet particulate fraction was prepared for photolysis (see 2.7) and divided into four preparations: 1) ○, ●, non-irradiated particulate fraction incubated for 10 min at 0 °C, 2) ◇, ◆, non-irradiated particulate fraction incubated with 2 μM 2-azidoadenosine for 10 min at 0 °C, 3) ▽, ▼, particulate fraction irradiated for 10 min at 0 °C and 4) □, ■, particulate fraction irradiated for 10 min in the presence of 2 μM 2-azidoadenosine at 0 °C. Each of the four preparation were then washed twice by centrifugation and resuspension (see 2.7) and its adenylate cyclase activity measured at 0.8 mM $MgCl_2$ in the absence and presence of 2-azidoadenosine (0.5, 10 μM) both without (A) and with (B) adenosine deaminase (5.2 units/ml of the assay mixture).

Figure 13

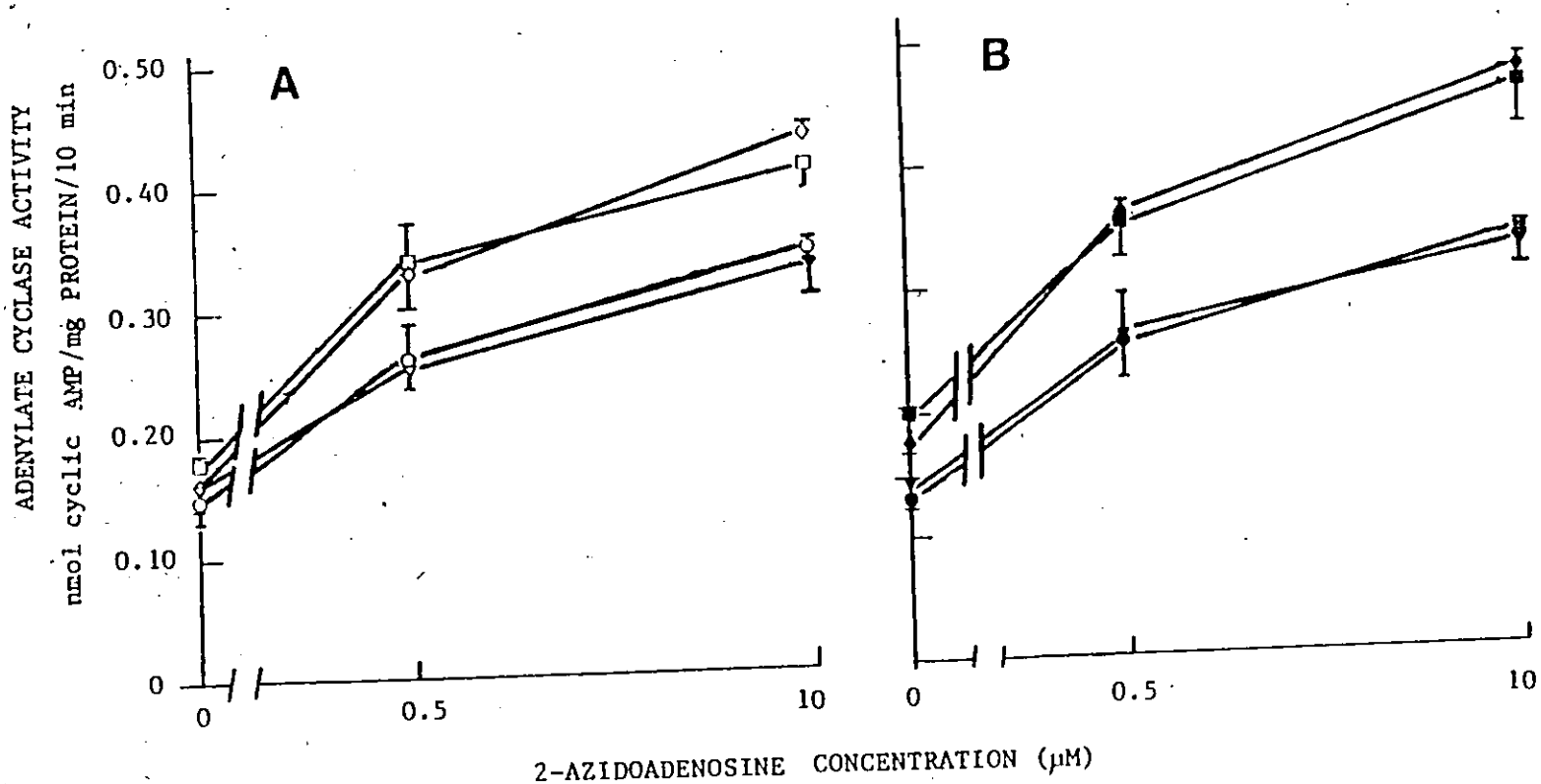

A
B
ADENYLATE CYCLASE ACTIVITY
nmol cyclic AMP/mg PROTEIN/10 min
0.50
0.40
0.30
0.20
0.10
0
0
0.5
10
0
0.5
10
2-AZIDOADENOSINE CONCENTRATION (μM)

Table 6

*Effects of Different Photolysis Conditions on the Activation of Adenylate Cyclase in Rabbit Platelet Particulate Fraction by 2-Azidoadenosine**

(10 μM 2-Azidoadenosine, 30 min Irradiation)

Photolysis Conditions	Adenosine Deaminase (units/ml)	Changes in Adenylate Cyclase Activity (%) 2-Azidoadenosine (μM) 0.5	10.0
Dark for 30 min	0	+77 ± 5	+143 ± 13
	14.7	+104 ± 7	+208 ± 9
Irradiated for 30 min	0	+47 ± 4	+81 ± 4
	14.7	+57 ± 7	+114 ± 6
Dark for 30 min + 10 μM 2-azidoadenosine	0	+70 ± 4	+135 ± 3
	14.7	+95 ± 3	+191 ± 6
Irradiated for 30 min + 10 μM 2-azidoadenosine	0	+53 ± 2	+88 ± 2
	14.7	+58 ± 1	+116 ± 2

*Data from Figure 12. Assays were carried out in triplicate as described in Figure 12. In the absence of adenosine deaminase, percent changes (caused by 2-azidoadenosine-activation of adenylate cyclase) are calculated with respect to enzyme activity containing no added 2-azidoadenosine to the assay mixture for each different photolysis condition. Similiarly in the presence of adenosine deaminase percent changes are calculated with respect to adenylate cyclase activity containing adenosine deaminase but no added 2-azidoadenosine to the assay mixture for each different photolysis condition. All values are given as the means ±SED

Table 7

*Effects of Different Photolysis Conditions on the Activation of Adenylate Cyclase in Rabbit Platelet Particulate Fraction by 2-Azidoadenosine**

(2 μM 2-Azidoadenosine, 10 min Irradiation)

Photolysis Conditions	Adenosine Deaminase (units/ml)	Changes in Adenylate Cyclase Activity (%) — 2-Azidoadenosine Concn. (μM) 0.5	10.0
Dark for 10 min	0	+74 ± 20	+127 ± 12
	5.2	+96 ± 24	+160 ± 10
Irradiated for 10 min	0	+57 ± 14	+102 ± 26(n=5)
	5.2	+80 ± 26	+130 ± 17(n=5)
Dark for 10 min + 2 μM 2-Azidoadenosine	0	+108 ± 22	+171 ± 7
	5.2	+103 ± 9	+165 ± 9
Irradiated for 10 min + 2 μM 2-Azidoadenosine	0	+94 ± 18	+130 ± 14
	5.2	+78 ± 13	+129 ± 20

*Data from Figure 13. Assays were carried out in triplicate as described in Figure 13. In the absence of adenosine deaminase, precent changes (caused by 2-azidoadenosine-activation of adenylate cyclase) are calculated with respect to enzyme activity containing no added 2-azidoadenosine to the assay mixture for each different photolysis condition. Similiarly in the presence of adenosine deaminase percent changes are calculated with respect to adenylate cyclase activity containing adenosine deaminase but no added 2-azidoadenosine to the assay mixture for each different photolysis condition. All values are given as the means ± SED.

Results: Section 3.4

Labeling of Rabbit Platelet Particulate Fraction during Photolysis of 2-Azido[^{3}H]adenosine

3.4 Labeling of Rabbit Platelet Particulate Fraction during Photolysis of 2-Azido[^{3}H]adenosine

3.4.1 Effects of Different Incubation Conditions on the Incorporation of ^{3}H

The amount of ^{3}H incorporated into the rabbit platelet particulate fraction during irradiation in the presence of increasing concentrations of 2-azido[^{3}H]adenosine increased linearly up to at least 0.20 μM 2-azido[^{3}H]adenosine (Figure 14). Some evidence of saturation of binding sites was obtained with higher concentrations. In most subsequent experiments 0.20-0.25 μM 2-azido[^{3}H]adenosine was used. The ^{3}H incorporated in the non-irradiated controls remained constant regardless of pre-incubation times. A control experiment in which 0.10 μM 2-azido[^{3}H]adenosine was irradiated for 10 min prior to the addition of platelet particulate fraction, followed by a 10 min incubation in the dark, lead to binding of 0.16 pmole 2-azido[^{3}H]adenosine/mg protein. This amount of [^{3}H] nucleoside binding was very similar to that found in non-irradiated samples.

Increasing the pre-incubation time with the [^{3}H] nucleoside appeared to reduce the amount of labeling per mg protein slightly (Figure 15). Pre-incubation with unlabeled 2-azidoadenosine (25 μM) reduced labeling by an average 14 ± 4% (mean ± S.E.M.). This suggests a large component of the labeling was non-specific.

In order to allow the receptor-ligand equilibrium to be freely established, a 30 min pre-incubation period was selected for future experiments.

The effect of increasing the irradiation period is shown in Figure 16. The amount of ^3H bound/mg protein increased with irradiation times up to at least 30 min. However, after 10 min of irradiation, 80% of the ^3H was already bound. The shape of Figure 16 resembles Figure 8, which showed the change in the UV absorption spectrum at 246 nm during photolysis of 2-azidoadenosine.

3.4.2 *Effects of Different Ligands on the Incorporation of ^3H*

Table 8 indicates the labeling was not all non-specific in that unlabeled 2-azidoadenosine (as also shown in Figures 15 and 16) and theophylline caused some inhibition of the radiation-dependent incorporation of ^3H into the platelet particulate fraction. This experiment was carried out with 0.1 μM 2-azido[^3H]-adenosine at which concentration the dark reaction amounted to 25% of the incorporation of ^3H due to irradiation. The ^3H bound in the dark was independent of added ligand. In subsequent experiments, the concentration of [^3H] nucleoside was maintained above 0.2 μM to keep the dark reaction to less than 10% of the control value.

Tables 9-11 show the effects of different ligands on the labeling. Using adenosine analogs that interact with the adenosine receptor linked to adenylate cyclase, unlabeled 2-azido-adenosine (62.5 μM) produced a 20-24% decrease in labeling and

2-chloroadenosine produced a 17-18% inhibition. Theophylline, an antagonist of these receptors (see 1.2.3), inhibited labeling by 34%. However, adenosine itself and N^6-cyclohexyladenosine produced no decrease in labeling. 2',5'-Dideoxyadenosine, which interacts with the "P" inhibitory site (Haslam et al., 1978b), decreased photolabeling by 28%. Coformycin, an adenosine deaminase inhibitor (Prémont et al., 1977), reduced labeling by 10%. Dipyridamole, an inhibitor of both adenosine transport and cyclic AMP phosphodiesterase (Mills and Smith, 1971; Subbarao et al., 1977) reduced labeling 29%.

3.4.3 *Further Studies on the ^3H Incorporation into Platelet Particulate Fraction During Photolysis of 2-Azido[^3H]adenosine*

2-Azidoadenosine is a known competitive inhibitor of adenosine deaminase but in the presence of UV radiation irreversibly inactivates the enzyme (Cusack and Born, 1976). Photoaffinity-labeled adenosine deaminase was therefore used to determine whether sodium salicylate fluorography is sufficiently sensitive to detect 2-azido[^3H]adenosine bound to platelet particulate fraction. 2-Azido[^3H]adenosine (0.5 μM) was incubated with 300 μg of adenosine deaminase in 150 mM TRIS-HCl, pH 7.4. After irradiation for 10 min at 313 nm the protein was acid-precipitated, washed and dissolved in electrophoresis sample buffer; it contained 2.5 x 10^3 dpm ^3H/μg. After polyacylamide gel electrophoresis of a sample containing 2 x 10^4 dpm ^3H and sodium salicylate fluorography (4.3 days), the X-ray film had an absorbance of 0.04. This was less than expected

(0.1) from the results of Laskey and Mills (1975). Although the latter workers used DMSO/POPOP fluorography, Chamberlain (1979) has found no difference between DMSO/POPOP and sodium salicylate in their ability to facilitate detection of ^3H by fluorography. The possibility that only part of the ^3H was protein-bound was therefore investigated by gel filtration of the sample on Sephadex G25 (Figure 17A). Two peaks of radioactivity with only one corresponding to the protein were found. The actual counts bound to protein were 1.1×10^3 dpm/μg, 45% of the original value. This accounts for the low density of the fluorogram.

The unbound ^3H could appear in the samples of solubilized protein by several mechanisms: 1.) by ^3H exchange within sample buffer; 2.) by trapping of non-covalently bound ^3H within the acid precipitated protein pellet; or 3.) by covalent bond hydrolysis by some added reagent. Investigation of ^3H exchange at pH 8.8 (maximum pH during electrophoresis) for 24 h (Figure 17B) revealed less than 10% of the protein-bound ^3H was lost. Since samples were normally stored frozen and at pH 7, exchange of ^3H from the 8-position on the covalently linked adenosine residue must be negligible. Polyacrylamide gel electrophoresis of photolabeled adenosine deaminase was repeated and the gels were exposed to preflashed film for 26 days. Table 12 indicates the sensitivity of detection of ^3H by sodium salicylate fluorography. Calculation of the time required to detect a polypeptide containing one third of the total ^3H incorporation into 100 μg of platelet particulate

fraction (approximately 200 dpm) indicated that an exposure of 200 days would be required even after film preflash. This exposure would produce a film absorbance of approximately 0.1; an absorbance of 0.05, just near detectable limits, would require an exposure of 100 days.

Labeled platelet particulate fraction in 3% SDS was subjected to gel filtration of Sephadex G-25 to determine if some of the ^3H was not protein-bound (Table 13). By the washing procedure of two trichloroacetic acid precipitations followed by dissolving the pellet in SDS, the amount of protein-bound ^3H represented the same fraction of the total ^3H (approximately 55%) regardless of the added competing ligand. These results are consistent with the hypothesis that the bound ^3H was susceptible to removal after treatment of the protein with acid and SDS. These possibilities were investigated by omitting acid precipitation of the platelet particulate fraction. The photolysis samples were washed twice then dissolved in 3% SDS. Each sample was gel filtered on Sephedex G-25 and the protein-bound ^3H and the unbound ^3H separated(Table 14). The protein-bound fraction isolated by gel filtration remained protein-bound when (a) left in 3% SDS at 22°C for 20 h, (b) acidified with 10% trichloroacetic acid at 22°C for 20 h and then neutralized. The ^3H found in each photolysis sample, regardless of the washing procedure, must represent a covalently bound compound plus a tightly but non-covalently bound fraction.

Because only part of the ^3H was covalently bound, the non-irradiated and pre-irradiated controls were repeated using the gel filtration procedure (Table 14). Although the results again showed that covalent labeling of platelet protein was radiation dependent,

it was found that pre-irradiated controls contained much less non-covalently bound ^3H than samples irradiated in the presence of particulate fraction. There was no significant difference in the covalently bound ^3H in the two cases. Thus covalent labeling of platelet protein during photolysis of 2-azido[^3H]adenosine represents affinity labeling by a photolysis product rather than true photoaffinity labeling.

Figure 14

Effect of Increasing Concentrations of 2-Azido[^{3}H]adenosine on Incorporation of ^{3}H into Platelet Particulate Fraction

Samples of platelet particulate fraction were resuspended in 75 mM TRIS-HCl, pH 7.4, 0.4 mM DTT, 0.1% bovine serum albumin and 0.4 mM NaMgEGTA. Samples were incubated in the dark at 0 ^{o}C for 20 min after the addition of 2-azido[^{3}H]adenosine (9 Ci/mmol). Samples were flushed with N_2 and stirred 5 min prior to irradiation at 313 nm for 30 min at 0 ^{o}C. Non-irradiated samples were present in the photolysis chamber but were protected from light. After irradiation samples were diluted 5-fold with 75 mM TRIS-HCl, pH 7.4, containing 0.4 mM NaMgEGTA and centrifuged 48,000 x g, 0 ^{o}C for 20 min. The platelet particulate fraction was resuspended in 75 mM TRIS-HCl, pH 7.4, containing 0.4 mM NaMgEGTA then acid precipitated and acid washed (10% trichloroacetic acid final). These samples were dissolved in electrophoresis sample buffer containing 3% (w/v) SDS, 0.0025% (w/v) Bromophenol Blue, 62 mM TRIS-HCl, pH 6.8, 6% (w/v) glycerol and 5% (w/v) mercaptoethanol and the ^{3}H counted. Further details are given in 2.7 and 2.11. The results represent single determinations from 4 different experiments with the non-irradiated ^{3}H incorporation subtacted.

Figure 16

Effect of Increasing Concentrations of 2-Azido[^{3}H]adenosine on Incorporation of ^{3}H into Platelet Particulate Fraction

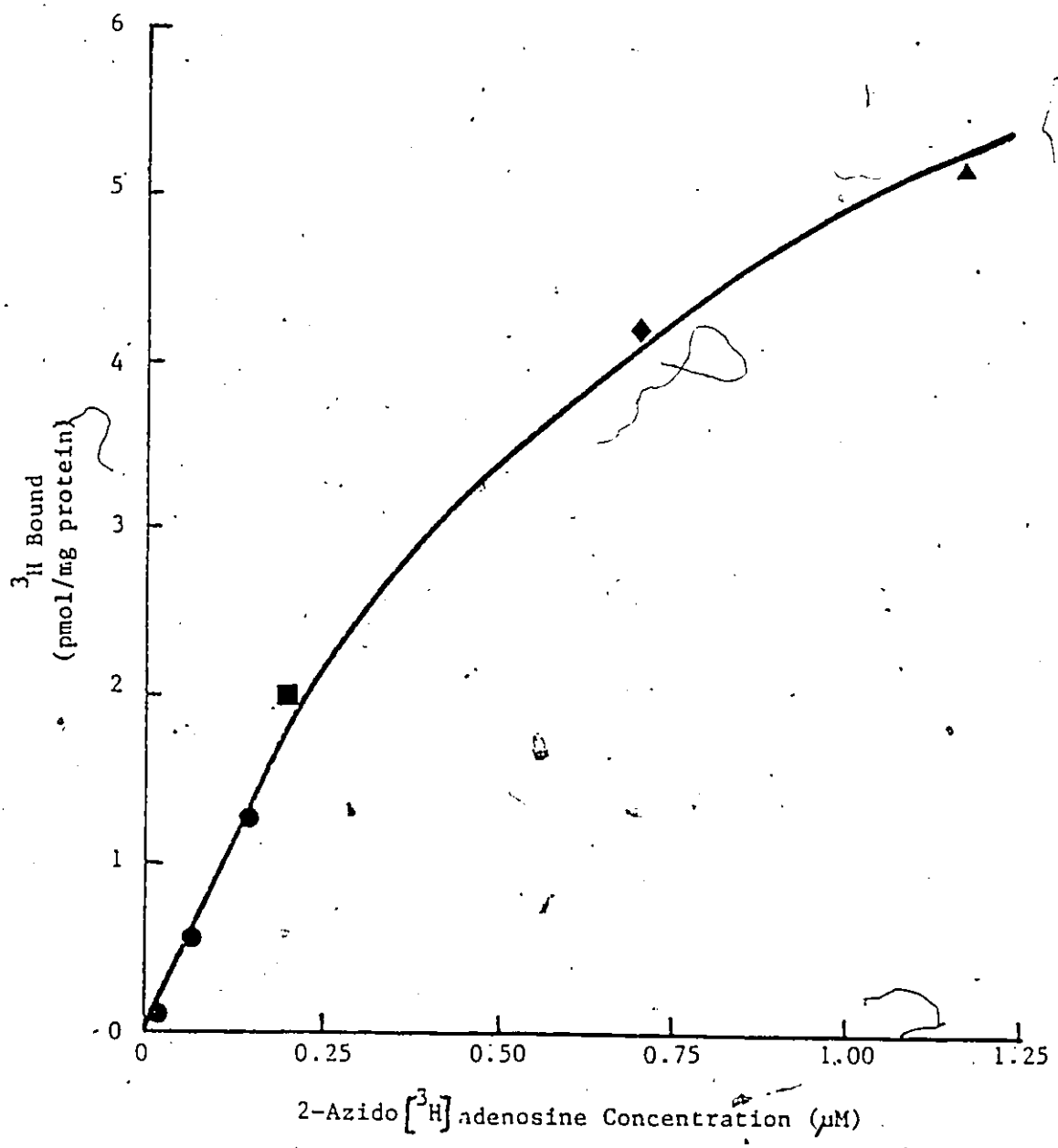


Figure 15

Effect of Length of Pre-Incubation Time upon 2-Azido[^{3}H]adenosine Incorporation into Platelet Particulate Fraction

The results represent single values obtained from the same particulate fraction. Platelet particulate fraction was resuspended, irradiated and processed as usual(Figure 14) except for the following: the samples (0.98 mg protein/1 ml sample) were incubated in the dark at 0 °C with 0.20 μM 2-azido[^{3}H]adenosine (9 Ci/mmol) for various times and then irradiated for 10 min at 313 nm, 0 °C. Samples containing unlabeled 2-azidoadenosine were incubated first with unlabeled compound and then for the same amount of time with the further addition of [^{3}H] nucleoside (0.20 μM). Non-irradiated samples, pre-incubated with 0.20 μM 2-azido[^{3}H]adenosine for 30 min and 100 min, incorporated 0.29 and 0.30 pmol of 2-azido[^{3}H]adenosine/mg protein, respectively. (0.20 μM 2-azido[^{3}H] adenosine (◆); 25 μM 2-azidoadenosine + 0.20 μM 2-azido[^{3}H] adenosine (▼))

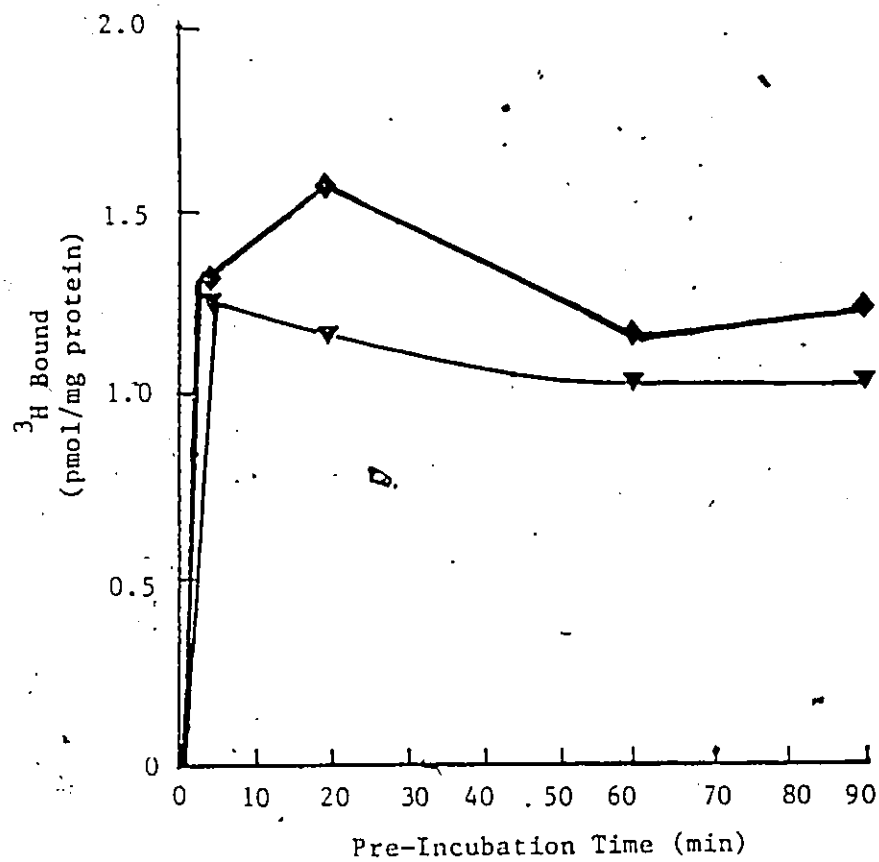


Figure 16

Effect of Length of Exposure to 313 nm Radiation upon 2-Azido [^{3}H] adenosine Incorporation into Platelet Particulate Fraction

The results represent single values obtained during the same experiment as in Figure 15. Platelet particulate fraction was resuspended, irradiated and processed as in Figure 14 except the particulate fraction was incubated in the dark for 20 min with 0.20 μM 2-azido [^{3}H] adenosine or for 20 min with 25 μM 2-azidoadenosine (unlabeled) followed by a further 20 min incubation with 0.20 μM 2-azido [^{3}H] adenosine. Samples were irradiated at 313 nm after flushing with N_2 for 5 min. Non-irradiated ^{3}H incorporation is given in Figure 15.

(0.20 μM 2-azido [^{3}H] adenosine alone (◆); 25 μM 2-azidoadenosine + 0.20 μM 2-azido [^{3}H] adenosine (▼))

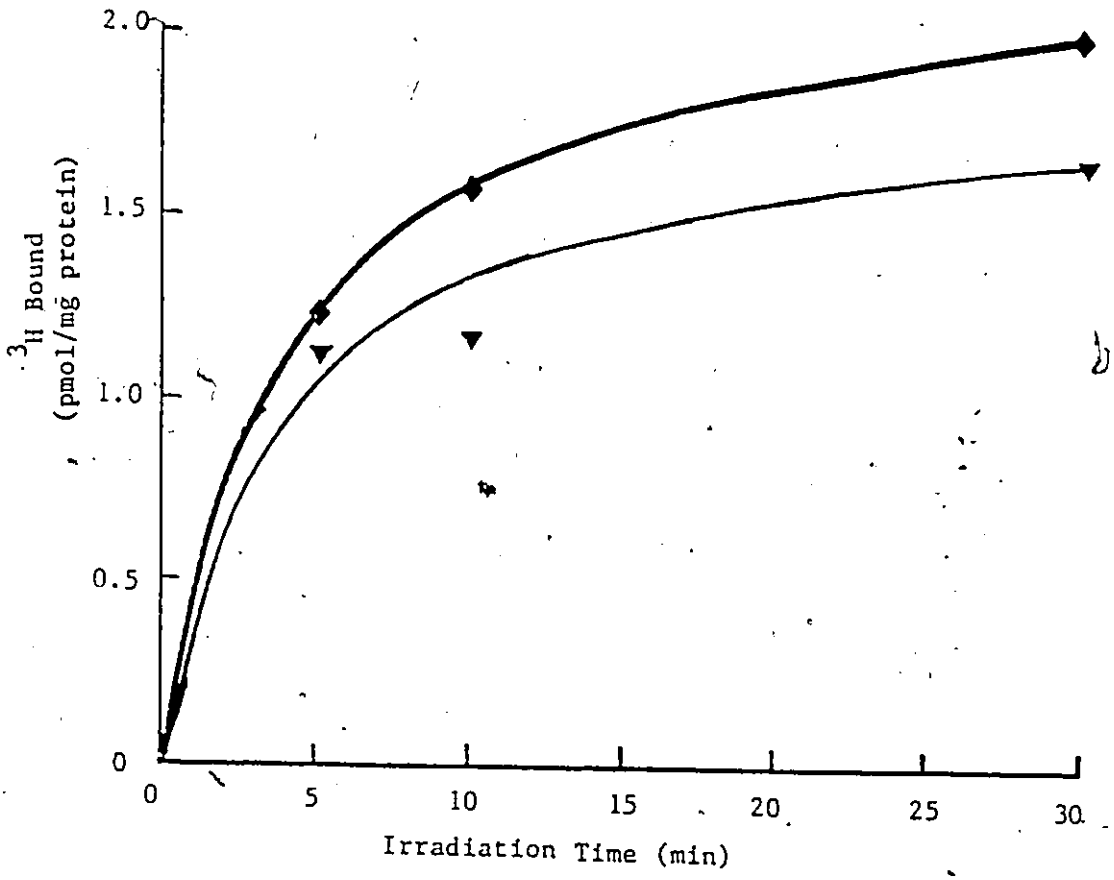


Table 8

Effect of Various Ligands on the Labeling of Platelet Particulate Fraction by 2-Azido[^{3}H]adenosine (0.10 μM)

Competing Ligand	$h\nu$	^{3}H Bound (pmol /mg protein)	% Change
None	+	0.40 ± 0.01	---
	−	0.11 ± 0.01	
2-Azido-adenosine (25 μM)	+	0.36 ± 0.01	−10 ± 3[a]
	−	0.11 ± 0.01	
Theophylline (4 mM)	+	0.33 ± 0.02	−18 ± 6[a]
	−	0.09 ± 0.02	

Samples of platelet particulate fraction were resuspended, irradiated and processed as described in Figure 14 except irradiation at 313 nm was for 10 min and the acid-precipitated pellets were dissolved in 0.6 M TRIS-HCl, pH 7.4, containing 3% SDS. Values are the means ± SEM from three determinations on the same particulate fraction. Percent changes in pmol ^{3}H bound/mg protein with respect to controls containing no competing ligand are given as means ± SED. The amount of platelet protein added per 1 ml sample was 1.18 mg. The percent of added ^{3}H bound in the controls was 0.47%;

$$\% \ ^3\text{H bound} = \frac{\text{pmol } ^3\text{H/mg protein x mg protein added/sample}}{\text{pmol 2-azido [}^3\text{H]adenosine added/sample}} \times 100$$

Significant changes (Student's t-test) are as indicated: [a] $2P > 0.05$

Table 9

Effect of Various Ligands on the Labeling of Platelet Particulate Fraction by 2-Azido[^{3}H]adenosine (0.25 μM)

Irradiations were carried out in triplicate as described in the legend to Table 8. Values are the means ± SEM from three determinations on the same particulate fraction. Percent changes in pmol ^{3}H bound/mg protein with respect to controls containing no added ligand are given as means ± SED. The amount of platelet protein added per 1 ml sample was 1.07 mg. The percent ^{3}H bound in the controls was 0.44%. Significant changes (Student's t-test) are indicated: [a] $2P > 0.025$, [b] $2P > 0.01$

Competing Ligand	hν	^{3}H Bound (pmol /mg protein)	% Change
None	+	1.04 ± 0.01	---
	-	0.05 ± 0.01	
2-Azido-adenosine (62.5 μM)	+	0.79 ± 0.07	-24 ± 7[a]
	-	0.05 ± 0.01	
Theophylline (4 mM)	+	0.69 ± 0.07	-34 ± 7[b]
	-	0.05 ± 0.01	

Table 10

Effect of Various Ligands on the Labeling of Platelet Particulate Fraction by 2-Azido[^{3}H]adenosine (0.21 μM)

Irradiations were carried out as described in the legend to Table 8. Values are the means ± SEM from three determinations on the same particulate fraction. Percent changes in pmol ^{3}H bound/mg protein with respect to controls containing no added ligand are given as means ± SED. The amount of platelet protein added per 1 ml sample was 0.97 mg. The percent ^{3}H bound in the controls was 0.41%. Significant changes (Student's t-test) are indicated: [a] 2P>0.05 [b] 2P>0.005

Competing Ligand	^{3}H Bound (pmol /mg protein)	% Change
None (Irradiated)	0.89 ± 0.03	---
(Dark)	0.04 ± 0.01	
Adenosine (62.5 μM)	0.82 ± 0.04	-7 ± 5
2-Azidoadenosine (62.5 μM)	0.71 ± 0.04	-20 ± 6[a]
2-Chloroadenosine (62.5 μM)	0.73 ± 0.04	-18 ± 5[a]
Theophylline (4 mM)	0.58 ± 0.02	-34 ± 4[b]

Table 11

Effect of Various Ligands on the Labeling of Platelet Particulate Fraction by 2-Azido[^{3}H]adenosine (0.21 μM)

Irradiations were carried out as described in the legend to Table 8. Values are the means ± SEM from three determinations on the same particulate fraction. Percent changes in pmol ^{3}H bound/mg protein with respect to controls containing no added ligand are given as means ± SED. The amount of platelet protein added per 1 ml sample was 0.76 mg. The percent ^{3}H bound in the controls was 0.48%. Significant changes (Student's t-test) are indicated: [a] 2P > 0.1, [b] 2P > 0.05, [c] 2P > 0.005, [d] 2P > 0.001.

Competing Ligand	^{3}H Bound (pmol /mg protein)	% Change
None	1.33 ± 0.03	---
2-Chloroadenosine (62.5 μM)	1.11 ± 0.06	-17 ± 5[b]
Coformycin (10 μM)	1.19 ± 0.04	-10 ± 4[a]
Dipyridamole (5 μM)	0.94 ± 0.03	-29 ± 3[d]
2',5'-Dideoxy-adenosine (62.5 μM)	0.96 ± 0.04	-28 ± 4[c]
N^6-Cyclohexyl-adenosine (62.5 μM)	1.27 ± 0.04	-4 ± 4

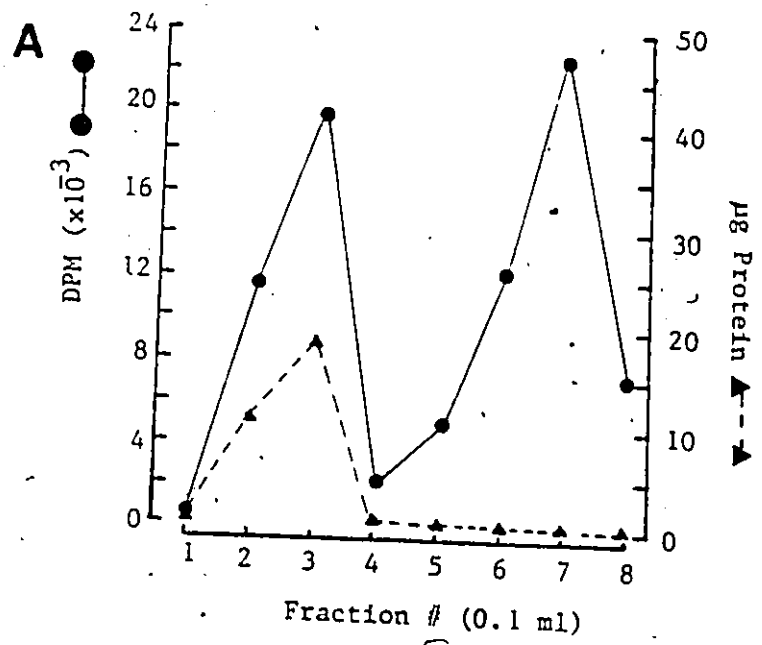
Figure 17

Gel Filtration of Photolabeled Adenosine Deaminase

A Photolabeled adenosine deaminase, dissolved in electrophoresis sample buffer (see 2.11), was eluted from a Sephadex G-25 column (4.6 x 0.6 cm) and was eluted with 1% SDS in 0.375 TRIS-HCl, pH 8.8. Aliquots (10 μl) of each 0.1 ml fraction were counted for ^{3}H and the protein was determined by fluoresence.

B Fraction 3 (80 μl remaining) of the above column was placed at 37 ^{o}C . After 4 h and 24 h, a 20 μl sample was eluted from the same column as above with 1% SDS in 0.375 TRIS-HCl, pH 8.8.

Figure 17


A
DPM (x10^{-3})
24
20
16
12
8
4
0
50
40
30
20
10
0
µg Protein
1 2 3 4 5 6 7 8
Fraction # (0.1 ml)

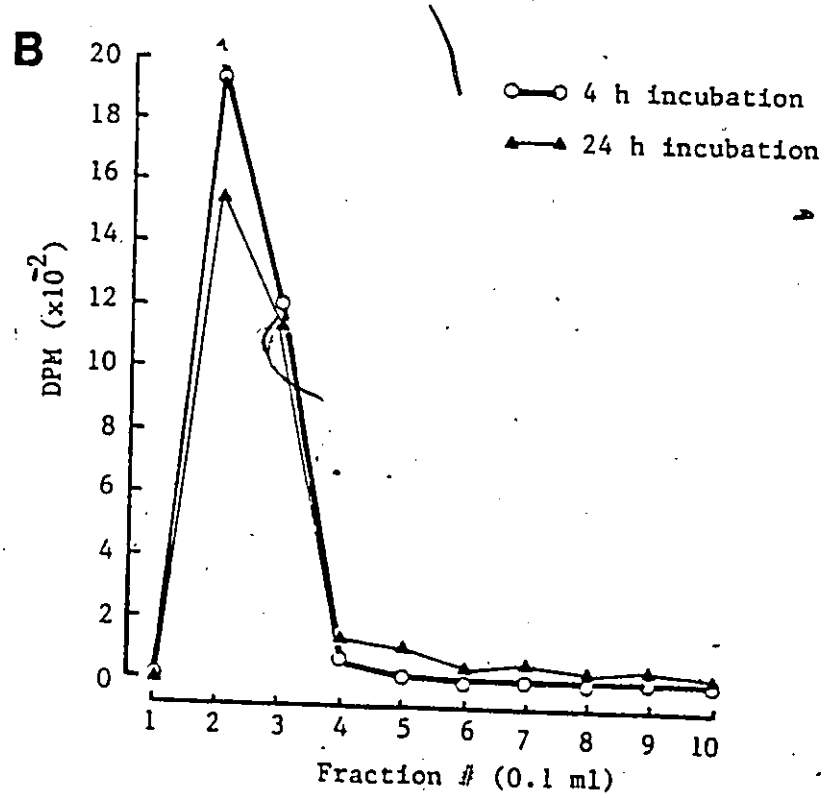

B
4 h incubation
24 h incubation
DPM (x10^{-2})
20
18
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10
Fraction # (0.1 ml)

Table 12

Sodium Salicylate Fluorography of Photolabeled Adenosine Deaminase with 2-Azido [^{3}H] adenosine

Adenosine deaminase (300 μg), freed from $(NH_4)_2SO_4$ as described in 2.10, was combined with 0.5 μM 2-azido ^{3}H·adenosine (10^7 dpm) in 150 mM TRIS-HCl, pH 7.4 and incubated in the dark for 30 min at 0 °C. The mixture was irradiated for 10 min at 313 nm and the protein was precipitated with trichloroacetic acid (10% final). After washing, the pellet was dissolved in electrophoresis sample buffer; 3.2% of the total ^{3}H was bound. The sample was subjected to electrophoresis, fixed and, prior to drying, the gel was soaked in 1 M sodium salicylate for 30 min. The gel was exposed to preflashed Kodak X-Omat R X-ray film for 26 days at -50 °C. The absorbance of the film was determined with a Joyce-Lobel microdensitometer. For further details see 2.10 and 2.11.

dpm/band	Absorbance/band
1620	0.11
3240	0.21
8100	0.67
16200	1.34

Table 13

Gel Filtration of ^3H Labeled Platelet Particulate Fraction

These results were obtained from single samples from the experiment shown in Table 11. These were eluted from Sephedex G-25 columns (4.6 x 0.8 cm, 0.8 ml volume) with 3% SDS in 0.6 M TRIS-HCl, pH 7.4. A similiar elution pattern to Figure 17A was obtained. Fractions 1-5 contained all the added protein and all of the ^3H was recovered in fractions 1-16. 2-Azido[^3H]adenosine, eluted after the protein, was used to identify the fractions likely to contain unbound ^3H.

Competing Ligand	Fraction 1-5 (dpm)	Fraction 6-16 (dpm)	dpm Protein Bound / Total dpm (%)
None	863	767	53
2-Chloroadenosine	778	651	54
Dipyridamole	733	596	55
N^6-Cyclohexyl-adenosine	914	704	56

Table 14

Gel Filtration of Labeled Platelet Particulate Fraction (0.25 μM 2-Azido [^{3}H]adenosine)

These results were obtained from platelet particulate fraction prepared and irradiated as described in Figure 14. Acid precipitation was omitted and another dilution with 75 mM TRIS-HCl, pH 7.4, containing 0.4 mM NaMgEGTA and centrifugation at 48,000x g, 0°C for 20 min repeated. The pellet was dissolved into 3% SDS containing 150 mM TRIS-HCl, pH 7.4. Columns and gel filtrations were done as in Table 13. Elution of the platelet protein (50 μl) was with the same SDS/buffer used to dissolve the platelet protein. Pre-irradiation was for 10 min on 2-azido[^{3}H]adenosine alone and then incubated with platelet particulate fraction for 45 min. Values are the means ± SEM from three determinations on the same particulate fraction. Percent inhibition in pmol ^{3}H bound/mg protein in dipyridamole sample with respect to control irradiated in the presence of particulate fraction was 32 ± 4% (mean ± SED). The amount of platelet protein per 1 ml was 0.74 mg. The percent ^{3}H bound in the control was 0.088%.

Incubation Condition	^{3}H DPM/mg protein (x 10^{-3})	^{3}H DPM Reversibly Bound/50 μl (x 10^{-3})	^{3}H Bound (pmol/mg protein)
Control	7.89 ± 0.14	1.97 ± 0.16	0.30 ± 0.01
Dipyridamole (5 μM)	5.30 ± 0.24	1.24 ± 0.03	0.20 ± 0.01
Dark	1.02 ± 0.01	1.05 ± 0.08	0.04 ± 0.01
Pre-Irradiated	7.26 ± 0.30	0.78 ± 0.01	0.27 ± 0.01

CHAPTER 4

Discussion

DISCUSSION

4.1 *Factors Affecting the Activation of Platelet Adenylate Cyclase By 2-Azidoadenosine*

The results showed that, using human platelets, 2-azidoadenosine could activate particulate fraction adenylate cyclase by up to 120% in the presence of adenosine deaminase and 0.8 mM $MgCl_2$. The activation of human platelet adenylate cyclase by 2-azidoadenosine has previously been reported (Haslam et al., 1978b; Haslam et al., 1979). With rabbit platelets, activation of adenylate cyclase by the adenosine analog was 200% above the control. Activation by adenosine itself was also greater in rabbit than in human (74% at 2 μM compared to 31% with human platelet adenylate cyclase). In contrast to human platelet material, net inhibition of rabbit platelet adenylate cyclase was not observed with 100 μM adenosine, probably because of the greater stimulatory effect of adenosine in the later species.

Londos and Wolff (1977) suggest that, in general, the potency of adenosine in activation is not affected by the Mg^{2+} concentration whereas the inhibitory potency is sensitive to divalent cation concentration. The present study using rabbit platelets leads to a somewhat different conclusion. A reduction in Mg^{2+} concentration increased activation by adenosine concentrations below 100 μM; with 100 μM adenosine the sum of the simultaneous inhibitory and stimulatory effects results in a small net stimulation that was the

same at both Mg^{2+} concentrations. This suggests an increase in potency of both actions of adenosine on reduction of the Mg^{2+} concentration. In the case of the rabbit platelet adenylate cyclase, the increased activation by adenosine was greater than the increase in inhibitory potency, when Mg^{2+} was reduced.

Reduction of Mg^{2+} concentration increased the activation of adenylate cyclase by 2-azidoadenosine at all nucleoside concentrations tested. Even with 0.5 μM 2-azidoadenosine, which must have little or no inhibitory effect, the increase in activation is statistically significant ($2P < 0.001$) when the Mg^{2+} concentration was reduced. This adds further evidence that the reduction of the Mg^{2+} concentration must increase the adenosine-dependent adenylate cyclase activation.

The addition of adenosine deaminase has been shown to increase the stimulation of adenylate cyclase by 2-azidoadenosine. This increase is probably due to removal of endogenous adenosine. Endogenous adenosine may result from breakdown of substrate ATP or be present in the enzyme preparation itself (Prémont et al., 1977). Endogenous adenosine has been eliminated not only by the use of adenosine deaminase but also by use of 2'-deoxy ATP as the substrate for adenylate cyclase. Low concentrations of 2'-deoxyadenosine have been shown to have no stimulatory and insignificant inhibitory activity in human platelets (Londos and Wolff, 1977; Haslam et al., 1978b). Addition of other agents, such as (-)-propranolol and indomethacin, either alone or together had no effect on control adenylate cyclase activity (Table 3).

In summary, an enhanced activation of platelet adenylate cyclase by 2-azidoadenosine has been achieved by reduction of Mg^{2+} concentration in the assay mixture, by addition of excess adenosine deaminase during the incubation and by a change of species from human to rabbit. Under these optimal conditions 2-azidoadenosine caused a 3-fold increase in platelet adenylate cyclase activity.

4.2 *Photolysis of 2-Azidoadenosine*

Knowles (1972) has described several criteria that should be met in the design of a useful photoaffinity reagent. In principle, many of these are satisfied by 2-azidoadenosine, which retains the biological activity of adenosine with respect to activation of adenylate cyclase. The analog can be readily synthesized under ordinary light conditions; 2-azidoadenosine is quite stable in aqueous buffer systems and undergoes conversion to a nitrene in the presence of UV radiation. Moreover, production of the reactive intermediate occurs under conditions that are unlikely to be harmful to platelet proteins.

2-Azidoadenosine does have one drawback as a photoaffinity label and that is the presence in aqueous neutral solutions of the compound of a mixture of tetrazole and azide. The tetrazole, although equipotent with the azide, is much less sensitive to photolysis than the azide. However, at pH 7.4, the tautomer ratio is 2:1 in favour of the azide (B.E. McCarry, personal communication, NMR spectra) allowing for a 66% chance of labeling a receptor with the azide tautomer.

4.3 *Attempts to Demonstrate Photoaffinity Labeling with Non-Radioactive 2-Azidoadenosine*

Photoaffinity labeling the adenosine receptor that interacts with adenylate cyclase could have a variety of effects upon adenylate cyclase activity. The photolabeled receptor could fail to interact with adenylate cyclase and this would appear as a normal control activity but with a reduced response to further adenosine-analog activation. Alternatively, the photolabeled receptor might cause a permanent activation of adenylate cyclase. Additional activation of this adenosine-dependent adenylate cyclase would be impossible, though other non-photolabeled adenosine receptors might still be available to activate adenylate cyclase. However, if sufficient receptors were labeled, an increased basal activity combined with a reduced activation by the nucleoside might be seen.

Figure 12 indicates that both irradiation of platelet particulate fraction and pre-exposure to 10 μM 2-azidoadenosine for 30 min caused activation of adenylate cyclase but no interactive effect attributable to irradiation in the presence of 2-azidoadenosine was detected. Thus, the increase in enzyme activity was the sum of the effects caused by UV radiation and pre-exposure to 2-azidoadenosine. The irradiated samples did show a reduced adenosine-dependent activation of adenylate cyclase but this was due to exposure to UV radiation alone. Reduction of UV exposure to 10 min did prevent the increase in adenylate cyclase activity suggesting ultraviolet radiation causes a time-dependent activation. It was apparent that

photoaffinity labeling could not be detected using non-radioactive 2-azidoadenosine. This implies that only a small proportion of the receptors, probably less than 20%, could have been labeled.

Other investigators have also reported that UV exposure causes cumulative and lasting effects on platelet function (Dickson et al., 1971; Lawler et al., 1979). The explanation for this effect was UV-induced cleavage of disulfide bonds (Dickson et al., 1971; Doery et al., 1973; Lawler et al., 1975; Lawler et al., 1979) which was thought to cause aggregation.

The reason pre-exposure to 2-azidoadenosine causes permanent activation is unknown at this time. The concentration of free 2-azidoadenosine remaining after washing the particulate fraction must be negligible (see Methods 2.7). It is also doubtful that the nucleoside is sufficiently tightly bound to survive prolonged washing. Half-maximal activation of the platelet adenylate cyclase required approximately 0.5 μM 2-azidoadenosine.

In conclusion, it would appear that photoaffinity labeling by 2-azidoadenosine cannot be detected by any change on the adenosine-dependent adenylate cyclase activity.

4.4 *Labeling of Rabbit Platelet Particulate Fraction during Photolysis of 2-Azido[^{3}H]adenosine*

In a previous study of adenosine binding proteins, Rosenblit and Levy (1977) photolabeled intact adipocytes with 8-azido[2-^{3}H]-adenosine. The specificity of this photolabeling reaction was not studied but radiation-dependent incorporation of ^{3}H into membrane

protein was demonstrated. Malbon et al. (1978) studied [^{3}H]adenosine binding to fat cell membranes. Some competing ligands were investigated and the adenosine binding sites were found to differ in their biological properties from those expected of the fat cell R-type adenosine receptor (see 1.2.3).

Recently Schwabe et al. (1979) presented data for [^{3}H]adenosine binding to rat brain membranes. Here 2-chloroadenosine was an inhibitor of adenosine binding but only at high concentrations (84 μM) and N^6-phenylisopropyladenosine exhibited only partial inhibition. The lack of stereospecificity of the N^6-adenosine analog, as both (-) and (+) isomers were equipotent, suggests that specific adenosine binding was not detected.

Newman et al. (1980) have reported adenosine binding sites associated with adenylate cyclase in the guinea pig cerebral cortex. Low concentrations of theophylline and 2-chloroadenosine (50 μM each) reduced [^{3}H]adenosine binding to 38% and 12% of control binding activity, however, no other adenosine analogs were reported to have been tested.

There have been no previous reports of binding of adenosine or analogs to platelet membranes. In this present study, a covalent radiation-dependent binding of ^{3}H , using 2-azido[^{3}H]adenosine, has been demonstrated. However, a photolysis product of 2-azido-[^{3}H]adenosine was shown to label platelet particulate fraction by affinity rather than by true photoaffinity labeling. Unlabeled 2-azidoadenosine, 2-chloroadenosine and theophylline did produce some inhibition of the labeling, suggesting that R-type adenosine

receptors could be participating in the binding detected. However, neither adenosine itself nor N^6-cyclohexyladenosine, an analog with R-type stimulatory activity but no P-type inhibitory activity (Haslam et al., 1978b), caused no significant inhibition of labeling. Unless an explanation can be found for these anomalous results, it mus be concluded that specific affinity labeling of R-type adenosine receptors was not detected.

In the platelet there are several other possible adenosine binding components other than the adenosine receptors associated with adenylate cyclase, such as the adenosine transport system, adenosine deaminase and adenosine kinase (Parks et al., 1975). That 2-azido[^{3}H]adenosine photolysis products may have labeled at least one and possibly two of these components is suggested by the inhibition of binding of dipyridamole and, to a lesser extent, coformycin. The effect of the former compound suggests the presence of adenosine transport proteins and, the effect of the latter, the presence of adenosine deaminase. Although 2',5'-dideoxyadenosine is best known as an inhibitor of adenylate cyclase activity at the P-site (Londos and Wolff, 1977; Haslam et al., 1978b), it is possible that it interacts with the adenosine transport system. Lips et al. (1980) showed both 2'-deoxyadenosine and 5'-deoxyadenosine to be powerful inhibitors of the high affinity adenosine transport system (K_m near 10 μM), though they did not test 2',5'-dideoxyadenosine itself. The latter workers suggested the presence of an intact purine ring and the 3' hydroxyl is typical for a substrate for the high affinity system. 2',5'-Dideoxyadenosine, 2-azidoadenosine, 2-chloroadenosine and theophylline (at 4 mM) could all possibly be such substrates of this system.

In order to determine the nature of the 2-azidoadenosine binding sites, further studies with various combinations of the ligands already tested, as well as other ligands, will be required.

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