FACTORS AFFECTING THE ACTIVITY OF GUANYLATE CYCLASE IN LYSATES OF HUMAN BLOOD PLATELETS

By

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ABSTRACT

The mechanism by which physiological stimuli increase cyclic GMP formation in platelets or in other cells is unknown. Agents that promote the formation of cyclic GMP in intact cells have in general not been found to stimulate the activity of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2.] in broken cell preparations. Therefore, possible mechanisms for the activation and control of guanylate cyclase activity in platelets were investigated in this thesis.

It was found that over 90% of the total guanylate cyclase activity is present in supernatant fractions of hypotonically lysed platelets. Platelet particulate fractions contained no guanylate cyclase activity that could not be accounted for by contaminating soluble enzyme, suggesting that physiological aggregating agents may increase cyclic GMP levels in intact platelets through the effects of intermediary factors. Because of the possiblity that soluble as well as particulate factors may be involved in the control of enzyme activity, whole platelet lysate was used in studies of the properties and activation of guanylate cyclase.

Under optimal ionic conditions (4.0 mM-$MnCl_2$), the specific activity of guanylate cyclase in fresh platelet lysates was about 10 nmol of cyclic GMP formed/20 min per mg of protein at 30°C, which is higher than that of any other mammalian cells or tissues studied. Activity was 15% of optimum with 10.0 mM-$MgCl_2$ and negligible with 4.0 mM-$CaCl_2$. Synergism between $MnCl_2$ and $MgCl_2$ or $CaCl_2$ was observed when $[MnCl_2] \leq [GTP]$; under

more physiological ionic conditions (Mg^{2+} present), micromolar concentrations of Ca^{2+} stimulated enzyme activity by about 50%.

Lower than optimal specific activities were obtained in assays containing large volumes of platelet lysate, owing to the presence of inhibitory factors that could be removed by ultrafiltration. Adenine nucleotides and glutathione accounted for less than 50% of the inhibitory activity. The combined effects of inhibitory factors and of suboptimal ionic conditions are likely to lower the guanylate cyclase activity in intact platelets to almost negligible values in the absence of activating factors.

Dithiothreitol (5.0 mM) and *N*-ethylmaleimide (0.1 mM) inhibited the activity of platelet lysate by about 70 and 50%, respectively. Preincubation of lysate for 60 min at 37°C increased guanylate cyclase activity on average by 225%. This effect could be blocked with dithiothreitol or *N*-ethylmaleimide, but dithiothreitol could not fully reverse activation once it had occurred. Oxidants such as 4,4'-dithiodipyridine (0.04 mM), diamide (0.4 mM) and *tert*-butylhydroperoxide (1.0 mM) increased enzyme activity on average, by 40, 87 and 165%, respectively. Neither diamide nor *tert*-butylhydroperoxide had an effect on enzyme that had been preincubated or treated with *N*-ethylmaleimide.

Sodium azide (10.0 mM) increased guanylate cyclase activity by an average of 335%; this effect was both time- and temperature-dependent. Activation by sodium azide was not prevented by dithiothreitol. Sodium nitroprusside (1.0 mM) increased enzyme activity by about 1000%; this effect could be blocked by preincubation or by *tert*-butylhydroperoxide, but not by either dithiothreitol or *N*-ethylmaleimide.

Lubrol PX (1.0%, w/v) increased guanylate cyclase activity on average by 256%; whereas Triton X-100 was much less effective. Addition of Lubrol PX to preincubated-, *tert*-butylhydroperoxide-, sodium azide- or sodium nitroprusside-treated lysate resulted in approximately the same level of activity as observed when added to untreated lysate. Although neither dithiothreitol nor *N*-ethylmaleimide could prevent activation by Lubrol PX, activity was markedly inhibited.

Arachidonate (1.0 mM) increased guanylate cyclase activity on average by 245% and oleate (1.0 mM) by 206%; whereas palmitate was almost inactive. Pretreatment of lysate with indomethacin did not inhibit this effect of arachidonate. Oleate and arachidonate caused marked stimulation of guanylate cyclase in preincubated lysate, but inhibited enzyme activity in Lubrol PX-treated lysate. With the exception of arachidonate, aggregating agents that increase the concentration of cyclic GMP in platelets did not increase the guanylate cyclase activity of whole platelet lysate, thereby supporting the view that these agents increase cyclic GMP levels through the effects of intermediary factors.

In contrast to enzyme activated by preincubation, *tert*-butylhydroperoxide, Lubrol PX or fatty acids, activation by sodium azide or by sodium nitroprusside resulted in a marked increase in the effectiveness of Mg^{2+} as sole bivalent cation.

The findings described in this thesis suggest that the *in vitro* modulation of platelet guanylate cyclase activity may be a function of the redox state of sulfhydryl groups on the enzyme itself and/or on an associated regulatory component. At present, it is not known whether any of the activated and inhibited states of the enzyme induced *in vitro* exist in intact platelets or have a physiological parallel.

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To my husband, Timothy, who encouraged and supported me when no one else could...or would.

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Chapter 1

Introduction

1.1. The role of calcium and cyclic nucleotides in the regulation of platelet function

A wide variety of physiological stimuli can induce platelet activity, including collagen fibers, thrombin, ADP, 5-hydroxytryptamine, epinephrine, the prostaglandin endoperoxides PGG_2 and PGH_2, and thromboxane A_2 (Mustard & Packham, 1970; Samuelsson *et al.*, 1976). In general, platelets respond to such agents in the following way. Within seconds "rapid shape change" takes place in which the normal disc-shaped platelet is transformed into a spherical form with pseudopodia (Born, 1970). In the case of soluble agents, the spherical structures interact with one another to form macroscopic aggregates, while insoluble agents (*e.g.* collagen fibers) are covered by a monolayer of adherent platelets. Platelet aggregation is calcium-dependent, and in the case of some agents (*e.g.* ADP) the presence of fibrinogen is necessary as well. Platelets can also undergo a release reaction during which prostaglandins and thromboxanes are synthesized (Smith *et al.*, 1973; Hamberg *et al.*, 1975; Samuelsson *et al.*, 1976) and the contents of intracellular storage granules are secreted (Holmsen *et al.*, 1969). Platelet aggregation and the release reaction interact in a complex manner in that the granule constituents released include the potent aggregating agent ADP, as well as 5-hydroxytryptamine and catecholamines, which amplify the effect of the original signal, while aggregation potentiates secretion (Packham *et al.*, 1973). In addition to the contents of storage granules, the prostaglandin endoperoxides PGG_2 and PGH_2, and thromboxane A_2 are secreted from platelets during the release reaction (Hamberg *et al.*, 1974; 1975; Smith *et al.*, 1974; Moncada & Vane, 1978) and

although they are produced in very small quantities and are extremely short lived, they also serve to amplify the response of the platelet to a given stimulatory agent (Samuelsson *et al.*, 1976). Thus, platelet adhesion, aggregation and the release reaction are essential components in the formation of an effective hemostatic plug or in the development of an arterial thrombus (Mustard *et al.*, 1974).

On the basis of evidence from many laboratories, it is presently believed that the prime intracellular messenger in the regulation of platelet function is the Ca^{2+} ion. It has been demonstrated in studies using bivalent cation ionophores (*e.g.* A 23187), which facilitate the movement of Ca^{2+} ions across lipid membranes, and Ca^{2+} ion antagonists (*e.g.* tetracaine), that the induction of platelet responses depends on the release of Ca^{2+} ions into the cytosol from intracellular binding sites (Feinman & Detwiler, 1974; Feinstein *et al.*, 1976; Charo *et al.*, 1976; LeBreton *et al.*, Massini & Lüscher, 1976), including the dense tubular system, which may be analogous to the sarcoplasmic reticulum of muscle (White, 1972). Thus, increased concentrations of Ca^{2+} ions in the cytosol appear to induce the biochemical changes associated with aggregation or the release reaction such as activation of actomyosin ATPase, depolymerization of microtubules, activation of glycogenolysis and phospholipase A_2, and increased phosphorylation of specific proteins (Haslam *et al.*, 1978a).

In addition to stimulatory agonists, platelets also respond to inhibitory agonists such as PGI_2, PGD_2, PGE_1, adenosine and β-adrenergic agonists, with reversal or inhibition of aggregation and inhibition of the release reaction (Haslam *et al.*, 1978a). At present, there is a great deal of evidence indicating that these inhibitory agonists exert their effects

by activating adenylate cyclase and thereby increasing platelet cyclic AMP concentrations (Haslam, 1975, 1978; Haslam *et al.*, 1978*a*,*b*; Gorman *et al.*, 1977). Recent studies (Haslam & Lynham, 1976; Haslam *et al.*, 1978*a*; Käser-Glanzmann, 1977) have provided evidence in support of the view (White *et al.*, 1974; Haslam, 1975) that cyclic AMP acts primarily by stimulating the active transport of Ca^{2+} ions out of the cytosol, thereby inhibiting Ca^{2+}-dependent reactions. Thus, platelets form a bidirectional control system, as defined by Berridge (1975) in that the dominant second messenger stimulating cellular activity in one direction is the Ca^{2+} ion, while the stimulation of the cell in the opposite direction depends on a decrease in the concentration of Ca^{2+} ions brought about by the negative feedback effect of cyclic AMP.

Although the regulation of platelet function presently appears to be adequately described by the opposing actions of Ca^{2+} ions and cyclic AMP, the concept that cyclic GMP may mediate effects antagonistic to those mediated by cyclic AMP (Goldberg *et al.*, 1973*a*, 1974) led to investigations of the role of cyclic GMP in platelet function. The initial studies on cyclic GMP in platelets (White *et al.*, 1973; Haslam & McClenaghan, 1974; Goldberg *et al.*, 1974; Chiang *et al.*, 1975) indicated that agents causing aggregation and the release reaction increased cyclic GMP levels, which suggested an additional second messenger role for cyclic GMP. Subsequent studies (Haslam, 1975; Haslam, 1978; Haslam *et al.*, 1978*a*,*b*) indicated that these increases correlated much more closely with the extent of platelet aggregation that with the induction of the release reaction. It was first found that ADP increased cyclic GMP levels in heparinized platelet-rich-plasma, in which this aggregating agent does not induce the release reaction,

suggesting that elevation of cyclic GMP was not a sufficient stimulus for release to occur (Haslam, 1975). Secondly, it was found that EDTA blocked both the aggregation and increase in cyclic GMP caused by collagen without markedly diminishing the release of 5-hydroxytryptamine (Haslam *et al.*, 1978*a*). Thirdly, when aggregation was prevented by failure to stir platelet-rich-plasma, ADP was shown to cause a change in platelet shape but no increase in cyclic GMP (Haslam *et al.*, 1978*a*). Together, these findings led to the suggestion (Haslam, 1978; Haslam *et al.*, 1978*a*) that contact between platelet membranes was required for increases in platelet cyclic GMP to occur and that these increases were more likely to be an effect rather than a potential cause of aggregation. At present, there is no convincing evidence in favour of a regulatory role for cyclic GMP in platelet function. Although it has been reported that exogenous cyclic GMP and derivatives can potentiate the platelet release reaction (Chiang *et al.*, 1976), this observation has not been confirmed by other workers (Claesson & Malmsten, 1977; Haslam, 1978). Similarly, the finding that sodium ascorbate, which has no known major effect on platelet function, markedly increases platelet cyclic GMP levels (Goldberg *et al.*, 1975; Schoepflin *et al.*, 1977), throws doubt on the possibility that cyclic GMP is involved in platelet activation. On the other hand, the recent finding that sodium nitroprusside, a smooth muscle relaxant which is known to inhibit platelet aggregation and the release reaction (Glusa *et al.*, 1974), can increase platelet cyclic GMP levels by up to 50-fold, even in the absence of stirring (Haslam *et al.*, 1978*a*,*b*) has led to the suggestion (Haslam *et al.*, 1978*a*,*b*) that cyclic GMP may be a feedback inhibitor of platelet responses rather than a mediator of platelet activation. However, it is apparent that further studies are

required to determine whether cyclic GMP is involved in the control of platelet function. Despite the uncertainty regarding the role of cyclic GMP in platelet function, it is both of interest and importance to investigate possible mechanisms by which physiological and pharmacological stimuli increase cyclic GMP formation in platelets.

1.2. Early studies on cyclic GMP and guanylate cyclase

Although information on guanylate cyclase has increased at a tremendous rate during the past few years, only the basic properties of this enzyme had been established when the present investigation was begun in January of 1974. Therefore, to provide a historical perspective to this thesis, the early studies on cyclic GMP and guanylate cyclase are reviewed in the following section.

The discovery of cyclic GMP as a natural constituent of animal tissues was first reported in 1963 (Ashman *et al.*, 1963); however, there was no clear indication of the biological importance of this cyclic nucleotide until seven years later when George *et al.* (1970) reported that acetylcholine-induced supression of cardiac contractility was associated with a rapid elevation of the concentration of cyclic GMP in myocardial tissue. Thus, in contrast to cyclic AMP, which was discovered in the course of E.W. Sutherland's work on the regulation by epinephrine of hepatic glycogen metabolism (Sutherland & Rall, 1958), the discovery of cyclic GMP preceded the identification of a possible metabolic function. The relatively slow progress that characterized the early investigations of the role of cyclic GMP in cellular processes has been attributed to analytical difficulties associated with its measurement as the levels of cyclic GMP in a given tissue are often substantially lower than the levels of cyclic AMP (Goldberg *et al.*, 1973*b*). However, the development of a highly sensitive and specific radioimmunoassay for the quantitation of minute amounts of cyclic GMP in biological material (Steiner *et al.*, 1972) contributed to the rapid expansion of investigations in this field during the past six

years.

1.2.1. Distribution of cyclic GMP

The natural occurrence of cyclic GMP was discovered as a result of a study (Ashman *et al.*, 1963) in which ^{32}P-labeled organic compounds present in rat urine following the administration of ^{32}P-inorganic phosphate were isolated and identified. Cyclic GMP was identified by comparing the physical, chemical and biochemical properties of a ^{32}P-labeled compound with authentic cyclic GMP. By 1969, cyclic GMP had been detected in a wide variety of mammalian tissues (Goldberg *et al.*, 1969) as well as in a number of different tissues from several phyla (Ishikawa *et al.*, 1969). The identification of cyclic GMP as a natural constituent of many animal tissues supported the suggestion of Hardman *et al.* (1966) that cyclic GMP, like cyclic AMP, may be a mediator of hormone action. Since 1969, cyclic GMP has been detected in all phyla of the animal kingdom examined as well as in prokaryotes (Goldberg *et al.*, 1973*b*). In most mammalian tissues, the concentrations of cyclic GMP found were generally in the range of 10 to 100 nmol/kg (wet weight) or about 1/10 to 1/15 the concentration of cyclic AMP (Goldberg *et al.*, 1973*b*).

1.2.2. Biological role of cyclic GMP

The objective of the initial investigations into the biological role of cyclic GMP was to determine whether agents that induced the accumulation of tissue cyclic AMP had any influence on cyclic GMP steady-state levels. However, it was found by Goldberg *et al.* (1969) that treatment of animals with epinephrine, glucagon or alloxan (to induce a diabetic state) had no detectable effect on hepatic cyclic GMP concentrations though each of these agents promoted the elevation of cyclic AMP concentrations.

These findings, together with similar evidence obtained in a number of other laboratories (Steiner *et al.*, 1972; Kuo *et al.*, 1972; Schultz *et al.*, 1972) strongly supported the conclusion drawn by Goldberg *et al.* (1969) that the formation of cyclic AMP and cyclic GMP were under separate hormonal and metabolic control and that specific cyclases were likely to be involved in their biosynthesis.

Studying the urinary excretion of cyclic AMP and cyclic GMP in rats in various hormonal states, Hardman *et al.* (1966, 1969) were the first to demonstrate that the levels of the two cyclic nucleotides may be controlled by different hormonal or other biological factors. Similarly, in other studies of urinary excretion of cyclic nucleotides, it was found that glucagon (Broadus *et al.*, 1970; Murad & Pak, 1972; Steiner *et al.*, 1972) or β-adrenergic agonists (Kaminsky *et al.*, 1970; Ball *et al.*, 1970, 1972; Murad & Pak, 1972), which increase the rate of cyclic AMP excretion, had little or no effect on the rate of excretion of cyclic GMP. Thus, subsequent investigations of the role of cyclic GMP were directed toward identifying a biologically active agent that could promote the cellular accumulation of cyclic GMP at the same time that a definable response could be demonstrated in cell function.

Cyclic GMP and cholinergic action. The results of a study reported by George *et al.* (1970) in which the effects of acetylcholine on myocardial contractility and cyclic GMP levels were examined, provided the first evidence suggesting that cholinergic action might be linked to an action of cyclic GMP. In these experiments, it was found that the depression of cardiac contractility induced by acetylcholine resulted in a coincident elevation of the concentration of cyclic GMP, whereas no change or a small

delayed decrease occurred in cyclic AMP levels. Atropine blocked the cholinergic effects on both cardiac function and elevation of cyclic GMP levels. It was also found that the stimulatory effect of isoproterenol on cardiac contractility (*i.e.* stimulation of both the inotropic and chronotropic responses), which is known to occur in conjunction with an elevation of cyclic AMP levels, was accompanied by a decrease in the concentration of cyclic GMP.

Soon after the initial discovery of a relationship between acetylcholine action and tissue cyclic GMP in rat myocardium, similar relationships were reported in a number of other mammalian tissues (Goldberg *et al.*, 1973*b*; Schultz *et al.*, 1972, 1973*a*; Lee *et al.*, 1972). In each case, the action produced by cholinergic stimulation was opposite to the effect promoted by cyclic AMP or by agents known to stimulate the accumulation of cyclic AMP (Goldberg *et al.*, 1974). It was concluded that only the muscarinic type of cholinergic action, and not the nicotinic type, was linked to cyclic GMP generation, since atropine blocked the functional response as well as the increase in cyclic GMP levels and because cholinergic effects of the nicotinic type (*e.g.* neuronal stimulation of skeletal muscle contraction) were not associated with tissue accumulation of cyclic GMP (Goldberg *et al.*, 1973*b*, 1974; Lee *et al.*, 1972).

Other hormone-induced changes in cyclic GMP levels. One characteristic common to cholinergic stimulation of the muscarinic type is that the effects produced on cellular function and metabolism are usually opposite to those induced by agents such as β-adrenergic agonists, which stimulate the generation of cyclic AMP. Thus, it is not surprising that agents such as 5-hydroxytryptamine and histamine, which stimulate intestinal

contractility, were also found to enhance tissue cyclic GMP accumulation (Schultz *et al.*, 1973*a*). Similar results have been reported by Goldberg *et al* . (1973*a*,*b*) who studied the effects of oxytocin, 5-hydroxytryptamine and prostaglandin $F_{2\alpha}$ on uterine tissue. Although there is an apparent relationship between smooth muscle contraction and elevated cyclic GMP levels, the role played by cyclic GMP in the regulation of excitation-contraction coupling in smooth muscle is still unclear (Diamond *et al.*, 1978).

The "Yin-Yang" hypothesis of biological control. On the basis of the observations summarized above, Goldberg *et al.* (1973*a*,*b*; 1974) concluded that there are many biological systems in which cyclic GMP and cyclic AMP appear to have opposing or antagonistic regulatory influences. According to the "Yin-Yang" or dualism hypothesis proposed by Goldberg *et al* . (1973*a*,*b*; 1974) the opposing actions of cyclic GMP and cyclic AMP are expressed in systems that are susceptible to both stimulatory and inhibitory controlling influences that may be antagonistic to one another (*i.e.* 'bidirectionally controlled' systems). Although this concept of the regulatory relationship between cyclic GMP and cyclic AMP has not been substantiated, it provided a valuable framework for investigations of the physiological role of cyclic GMP in a wide variety of cellular processes.

1.2.3. Guanylate cyclase

In spite of the failure of early studies to define the biological significance or function of cyclic GMP, it was believed that cyclic GMP would ultimately be found to have regulatory functions analogous to those of cyclic AMP. This assumption led to investigations of the basic features of cyclic GMP biosynthesis in cell-free systems. Although these studies

(Hardman & Sutherland, 1969; Ishikawa *et al.*, 1969) clearly showed that the formation of cyclic GMP from GTP is catalyzed by an enzyme system which is separate and distinct in several respects from adenylate cyclase, attempts to demonstrate *in vitro* activation of guanylate cyclase by hormones or other physiological agents that promote the accumulation of cyclic GMP in intact cells were unsuccessful. This was (and still is) a fundamental characteristic of the guanylate cyclase-cyclic GMP system that markedly differs from its cyclic AMP counterpart, as adenylate cyclase from disrupted cells can generally be activated by those agents that stimulate cellular cyclic AMP accumulation. In an effort to determine the mechanism(s) by which guanylate cyclase activity is modulated by physiological agents *in vivo*, the subcellular distribution, ionic requirements and kinetic properties of this enzyme were characterized.

Subcellular distribution. Unlike adenylate cyclase, which is almost always found solely in the particulate fraction, guanylate cyclase activity is found in both supernatant and particulate fractions of homogenates of most mammalian tissues (White *et al.*, 1969; White & Aurbach, 1969; Hardman & Sutherland, 1969; Ishikawa *et al.*, 1969; Schultz *et al.*, 1969; Kimura & Murad, 1974*a*, 1975*a*,*b*). The distribution of enzyme activity varies markedly from one tissue to another; for example, 80 to 90% of the total guanylate cyclase activity found in rat lung, spleen and liver homogenates was found in high speed supernatant fractions (Hardman & Sutherland, 1969); whereas in other tissues, the distribution between the soluble and particulate fractions was either intermediate (*e.g.* 70% particulate in rat heart) or almost entirely particulate (*e.g.* 90% particulate in rat intestine)(Hardman & Sutherland, 1969; Ishikawa *et al.*, 1969).

Furthermore, in some instances the subcellular distribution of guanylate cyclase activity of a given tissue was found to vary with the species examined (Kimura & Murad, 1975*b*). In contrast to the varying distribution of guanylate cyclase activity in the supernatant and particulate fractions of mammalian tissues, the enzyme activity present in certain lower phylogenetic forms, notably the sperm of the sea urchin, *Strongylocentrotus purpuratus* (Gray *et al.*, 1970) and the bacterium, *Bacillus licheniformis* (Clark & Bernlohr, 1972), appears to be entirely particulate. The varying patterns of distribution of mammalian guanylate cyclase have suggested that this enzyme may be associated with cellular membranes or other structures *in situ* but in certain tissues may be released from some sites during the homogenization or fractionation procedures (Hardman *et al.*, 1973; Goldberg *et al.*, 1973*b*). Insight into this problem was provided by studies in which synthetic non-ionic detergents such as Triton X-100 and Lubrol PX were added either to incubation mixtures or directly to tissue preparations prior to centrifugation. The apparent subcellular distribution of guanylate cyclase activity of many tissues was found to be markedly altered by non-ionic detergents. Ishikawa *et al.* (1969) first demonstrated that Triton X-100 could produce a 2.5-fold increase in guanylate cyclase activity from a particulate fraction of rat small intestine. Later, Hardman *et al.* (1972, 1973) found that non-ionic detergents could produce a seven- to 10-fold stimulation of particulate guanylate cyclase activity frm rat lung, liver or spleen, but only a two- to three-fold enhancement of the assayable activity in the 100 000*g* supernatant fraction. Although the mechanism(s) by which these non-ionic detergents stimulated guanylate cyclase activity was not known, these observations suggested that in some mammalian tissues the

proportion of particulate or membrane-associated catalytic activity was potentially much greater than had initially been assumed. Thus the guanylate cyclase activity unmasked by non-ionic detergents became equal to or greater than the activity found in tissues in which as much as 80 to 90% of the total enzyme activity appeared to be soluble in the absence of detergent. While some workers believed that these findings strongly supported the view that the appearance of enzyme activity in high speed supernatant fractions could be attributed to an *in vitro* artifact related to the sensitivity of the tissue to disruptive fractionation procedures (Goldberg *et al.*, 1973*b*), other workers suggested that the guanylate cyclase activities found in both soluble and particulate fractions may in fact represent two forms of the enzyme that differ in properties other than solubility (Hardman *et al.*, 1973). This latter hypothesis stimulated careful examination of the physical and kinetic properties of the soluble and particulate forms of guanylate cyclase in a number of different tissues. The results of these studies are discussed in Section 1.3.2.

Bivalent cation requirements. Another characteristic in which guanylate cyclase differs from adenylate cyclase is its marked dependence on Mn^{2+} ions for maximum activity. While the bivalent cation requirement of adenylate cyclase can be satisfied almost equally well by either Mg^{2+} or Mn^{2+}, guanylate cyclase was shown to be 10-fold more active in the presence of Mn^{2+} than with equimolar concentrations of Mg^{2+} (White & Aurbach, 1969; Hardman & Sutherland, 1969). The expression of maximum basal guanylate cyclase activity was also found to be critically dependent upon an optimal ratio of GTP to Mn^{2+} by both White & Aurbach (1969) and by Hardman & Sutherland (1969) who demonstrated that increasing concentrations of GTP

(>1.0 mM) markedly inhibited enzyme activity when assayed at fixed, low concentrations of Mn^{2+} (1.0 to 3.0 mM). Further studies by Hardman *et al.*, (1973) showed that Ca^{2+} (0.1 to 3.0 mM), though even less effective than Mg^{2+} in satisfying the bivalent cation requirement alone, could synergistically enhance guanylate cyclase activity several-fold when present together with Mn^{2+} and concentrations of GTP either equal to or greater than those of Mn^{2+}. However, when Mg^{2+} was used instead of Mn^{2+}, Ca^{2+} decreased enzyme activity. The ability of Ca^{2+} but not Mg^{2+} to act synergistically with Mn^{2+} in increasing guanylate cyclase activity was attributed to the displacement of Mn^{2+} from GTP by Ca^{2+} which thereby increased the availability of free Mn^{2+} for interaction at the presumed activator site. Although it was not known whether physiological levels of Ca^{2+} could exert a similar effect in intact cells, the apparent stimulation of guanylate cyclase activity in disrupted cell preparations suggested a possible role of Ca^{2+} in the regulation of this enzyme, thus supporting the view that there may be a causal relationship between cholinergic action, calcium translocation and changes in tissue cyclic GMP production (Schultz *et al.*, 1973*b*).

Inhibition of guanylate cyclase activity. A number of cellular metabolites including various nucleoside tri-, di- and monophosphates as well as oxaloacetate and phosphoenolpyruvate were found to inhibit guanylate cyclase activity from several mammalian tissues (White & Aurbach, 1969; Hardman & Sutherland, 1969; Ishikawa *et al.*, 1969). However, with the exception of ATP, the effective concentrations of these substances were from one to three orders of magnitude greater than those known to be present under physiological conditions. Thus, the marked inhibition of guanylate cyclase by concentrations of ATP normally found in intact cells suggested

that ATP could be a physiologically important regulator of guanylate cyclase activity.

Effects of hormones on guanylate cyclase activity. Initial attempts in a number of laboratories to demonstrate a specific and direct activation of guanylate cyclase by hormonal or other physiological agents known to increase cyclic GMP accumulation in intact cells were unsuccessful (Hardman & Sutherland, 1969; White & Aurbach, 1969; Böhme, 1970; Schultz *et al.*, 1972; Marks, 1973, Nakazawa & Sano, 1974). While several reports have described increased guanylate cyclase activity in preparations from liver (Thompson *et al.*, 1973*a*,*b*, 1974) and gallbladder (Amer & McKinney, 1973; Amer, 1974) with secretin and pancreozymin, respectively; results from a more recent study (Ichihara *et al.*, 1977) indicate that the apparent stimulatory effects of these agents may be attributable to bile salt contaminants present in the hormone preparations that are stimulatory to the soluble enzyme.

Possible mechanisms by which guanylate cyclase activity may be regulated in vivo by physiological agents. Determination of the precise subcellular distribution of guanylate cyclase is crucial to an understanding of the mechanism of activation of this enzyme by biological signals. Compartmentation of guanylate cyclase in the cell membrane would enable direct interaction of a stimulatory and/or inhibitory agent with a membrane-bound regulatory component of the enzyme in a manner analogous to the adenylate cyclase system. Alternatively, a cytoplasmic localization of guanylate cyclase would suggest that indirect mechanisms exist by which extracellular stimuli can activate the soluble enzyme. For example, interaction of the hormone with a receptor on the cell membrane would

then result in the generation or the transport of a third component (*e.g.* calcium), which could serve as an activator of cytoplasmic guanylate cyclase activity. Although an association of guanylate cyclase within the cell membrane would suggest a more direct link between hormone receptor and catalytic portions of the enzyme, the failure to demonstrate direct and specific hormonal effects on the activity of particulate guanylate cyclase suggests that an intermediary component may be required under these conditions as well (Goldberg *et al.*, 1973*b*).

1.3. *Present understanding of the subcellular distribution, physical and kinetic properties of guanylate cyclase*

From 1974 to 1978, the cyclic GMP field expanded exponentially; however, despite the vast number of articles that were published during this period, neither the biological importance nor the mechanism by which hormones modulate guanylate cyclase activity has yet been elucidated. Although recent evidence has provided some new insight into these problems, a great deal of work must be carried out if the cyclic GMP-guanylate cyclase system is to be understood as well as its cyclic AMP-adenylate cyclase counterpart.

1.3.1. *Subcellular distribution*

Guanylate cyclase activity has now been detected in a wide variety of tissues from organisms including bacteria (Sun *et al.*, 1974; Macchia *et al.*, 1975; Silverman, 1975), mollusks (Higgens, 1974; Sulakhe *et al.*, 1976), insects (Filburn & Wyatt, 1976; Catalan *et al.*, 1976; Fallon & Wyatt, 1977), fish and birds (Sulakhe *et al.*, 1976). In mammals, guanylate cyclase activity has been found in all tissues studied with the exception of sperm (Gray *et al.*, 1976). Although guanylate cyclase activity has not yet been identified in plants, it is likely to be present as cyclic GMP has been detected in plant tissue (Haddox *et al.*, 1974).

As discussed earlier, the apparent intracellular distribution of guanylate cyclase activity among soluble and particulate fractions of broken cell preparations was found to be strongly tissue-dependent. In an effort to determine a role for cyclic GMP in various tissues, the

subcellular distribution of guanylate cyclase has been examined more closely in recent studies. These studies have clearly shown that within a particular tissue there also appears to be a highly selective distribution of guanylate cyclase activity in biologically discrete structures or cell types. For example, the particulate activity in rat liver is specifically associated with plasma membrane, endoplasmic reticulum and Golgi vesicles (Kimura & Murad, 1975a,c). Particulate guanylate cyclase activity has also been identified in sarcoplasmic reticulum of heart (White, 1975), mitochondria of brain (Nakasawa & Sano, 1974) and nuclear preparations of liver (Earp *et al.*, 1977) and uterus (Siegel *et al.*, 1976).

The mammalian retina is an excellent example of the selective distribution of guanylate cyclase activity in specialized subcellular structures. Initially, very high levels of guanylate cyclase activity were found in whole retina and in rod outer segments, which are the photoreceptor organelles for scotopic vision (Goridis *et al.*, 1973; Pannbacker, 1973; Bensinger *et al.*, 1974). In a subsequent study, Virmaux *et al.* (1976) demonstrated that the high retinal guanylate cyclase activity was intrinsic to rod outer segments and that enzyme activity associated with inner retinal structures was extremely low in comparison. Other workers (Raveed *et al.*, 1976) later found that the guanylate cyclase activity of the rod outer segment was actually associated with the ciliary structure. Together, these findings supported the view (Virmaux, 1976) that cyclic GMP was the cyclic nucleotide involved in the regulation of photoreceptor function.

In the brain, it was found that guanylate cyclase activity was selectively enriched in the soluble compartment of the synaptosomes (Goridis

& Morgan, 1973; Nakazawa *et al.*,1976; Deguchi *et al.*, 1976) thereby resembling the subcellular distribution of neuronal soluble enzyme markers such as glutamate decarboxylase and tyrosine hydroxylase. Investigation of guanylate cyclase in pure brain cell cultures revealed that cyclase activity was predominantly located in neurones, whereas no activity was detectable in either glial or meningial cells (Goridis *et al.*, 1974). The apparent preferential neuronal localization of brain guanylate cyclase is therefore consistent with a possible role for cyclic GMP in synaptic transmission (Ferrendelli *et al.*, 1970; 1972; Kuo *et al.*, 1972).

Of the three regions of the kidney (cortex, outer medulla, inner medulla), guanylate cyclase activity was highest in the soluble fraction of the cortex (Craven & DeRubertis, 1976). However, subcellular fractionation of the cortex revealed that guanylate cyclase activity was preferentially enriched in particulate fractions of pure glomeruli, where its specific activity was about 45-fold higher that assayed in the soluble fraction of the tubular fragments (Helwig *et al.*, 1975). Thus, although the glomeruli constitute less than 8% of the total mass of the kidney cortex, more than 60% of the activity present in the homogenate was associated with this structure (Helwig *et al.*, 1975). Although cyclic GMP has been identified as a natural constituent of kidney tissue, no functional role has yet been attributed to kidney cyclic GMP. However, the presence of guanylate cyclase in the glomeruli suggests that perhaps part of the cyclic GMP excreted in urine might be synthesized by the kidney (Helwig *et al.*, 1975).

In the small intestine, it was found that guanylate cyclase activity markedly increased from the depth of the crypt to the tip of the villus, and that a major portion of enzyme activity in the villus cell resides in

the microvillus structure of the intestinal brush border, while the remaining part appears to be localized primarily in the basal-lateral plasma membranes of the epithelial cell (DeJonge, 1975*a*; Quill & Weiser, 1975). Although the villus to crypt gradient of guanylate cyclase suggests that cyclic GMP may have a specialized role in the differentiated villus cell, the function of this cyclic nucleotide in the small intestine is entirely unknown (DeJonge, 1975*b*).

The highly active guanylate cyclase of invertebrate sperm appears to be a totally particulate enzyme (Gray *et al.*, 1970; 1975). Further studies of the subcellular distribution of sea urchin guanylate cyclase activity suggested that the primary and perhaps only intracellular locale of this enzyme may be the flagellar plasma membrane (Gray & Drummond, 1976; Sano, 1976). While these observations suggest that cyclic GMP may participate in some physiological function of sea urchin sperm, its function clearly must be unique to invertebrate sperm because guanylate cyclase is totally lacking from sperm of higher vertebrate forms (Gray & Drummond, 1976).

1.3.2. Multiple forms of guanylate cyclase

Detailed investigations (Kimura & Murad, 1974*a*, 1975*a*,*b*,*c*,*d*; Chrisman *et al.*, 1975) of the kinetic and physical properties of guanylate cyclase present in high speed supernatant and particulate fractions of rat heart, liver and lung revealed several apparent differences in both kinetic properties and molecular sizes that depended on subcellular rather than tissue source. Similar findings have subsequently been reported in a wide variety of tissues including kidney (Craven & DeRubertis, 1976), brain (Nakazawa *et al.*, 1976), intestine (DeJonge, 1975*a*,*b*), uterus (Siegel

et al., 1976) and parotid (Durham, 1976). In addition, the soluble and particulate activities in liver appear to have different mechanisms regulating their synthesis (Kimura & Murad, 1975*a*,*e*). This conclusion was drawn from changes in soluble and particulate activities in regenerating and fetal rat liver studies (Kimura & Murad, 1975*a*,*e*). Furthermore, proliferating tissue, such as regenerating liver, fetal liver, transplantable hepatomas and renal tumours, typically exhibit an altered distribution of enzyme activity with greater amounts associated with the particulate fraction (Criss *et al.*, 1976*a*,*b*; Goridis & Reutter, 1975; Kimura & Murad, 1975*a*). Recently, Blosser & Appel (1978) reported that the specific activity of soluble guanylate cyclase from mouse dystrophic muscle was 2.5-fold greater than that of normal muscle, while the particulate enzyme showed little change; however, denervation increased the specific activity of both forms by approximately two-fold. Selective alteration of soluble guanylate cyclase has also been shown in rat testes following surgical crytorchidism (Spruill *et al.*, 1977).

1.3.3. Physical properties of soluble and particulate guanylate cyclase

Based on gel filtration data, molecular weights ranging from 30 000 to 900 000 have been estimated for the soluble and particulate forms of guanylate cyclase in a number of different tissues. Chrisman *et al.* (1975) reported that soluble guanylate cyclase present in a 32 000*g* supernatant fraction of rat lung homogenate had an approximate molecular weight of 450 000, whereas the molecular weight of the Triton X-100-dispersed particulate fraction was estimated to be from 700 000 to 900 000. In the rat renal medulla (Neer & Sukiennik, 1975), the molecular weight of

the soluble enzyme was estimated at about 150 000 in the presence or absence of detergent (Lubrol PX), while that of the detergent-solublized particulate form was 300 000. Garbers (1976) found that the apparent molecular weight of purified, Lubrol PX-dispersed, particulate guanylate cyclase from sea urchin sperm was 182 000, which was identical to that of the unpurified enzyme. The molecular weight of purified, soluble guanylate cyclase from *Escherichia coli* was estimated to be about 30 000 (Macchia *et al.*, 1975). Furthermore, sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis indicated that this purified soluble enzyme consisted of a single polypeptide chain. In another microorganism, *Caulobacter crescentus*, the molecular weight of the partially purified soluble enzyme was found to be about 140 000 (Sun *et al.*, 1974). Thus, guanylate cyclase of *C. crescentus* is apparently much larger than that of *E. coli*. Recently, Asano & Hidaka (1977) described a method for the purification of soluble guanylate cyclase from human blood platelets. The apparent molecular weight of this enzyme preparation was estimated to be about 180 000.

In order to elucidate the nature of the interaction of guanylate cyclase with non-ionic detergents, Neer & Sukiennik (1975) studied several physical parameters of both the soluble and particulate forms of guanylate cyclase from rat renal medulla. On the basis of data obtained from sucrose density gradient centrifugation and gel filtration in H_2O and D_2O, the enzyme from the soluble cell fraction was estimated to have the following properties: A Stokes radius of 54 Å, partial specific volume of 0.75 ml/g, f/f_o value of 1.4, axial ratio (prolate ellipsoid), of 7, and an $s_{20,\omega}$ value of 6.35S. Treatment of the soluble fraction with 0.1% Lubrol PX resulted in increased activity, an increase in the Stokes radius to 62 Å, a

decrease in the $s_{20,\omega}$ value to 5.5S, and no change in either the mass or the partial specific volume. From these observations it was concluded that the detergent-promoted activation is the result of the binding of a small number of detergent molecules to specific hydrophobic sites, which causes a conformational change to a more asymmetric protein, rather than the result of surfactant action or the binding of large amounts of detergent to the enzyme. These workers also found that the physical properties of guanylate cyclase solubilized from the particulate cell fraction with 1.0%-Lubrol PX exhibited markedly different properties from those of guanylate cyclase in the soluble cell fraction. This form of the enzyme appeared to be a large, heterogenous aggregate (molecular weight of 300 000) with a value of $S_{20,\omega}$ of about 10S and a Stokes radius of 65 Å. Together, the findings of Chrisman *et al.* (1975) and of Neer & Sukiennik (1975) support the view that two, easily distinguishable forms of guanylate cyclase exist in the rat lung and renal medulla, respectively.

1.3.4. Kinetic properties of soluble and particulate guanylate cyclase

The biosynthesis of cyclic GMP has been shown to proceed according to the following reaction:

$$GTP \xrightarrow{Metal^{2+}} \text{cyclic GMP} + PPi$$

in which the α-phosphate of GTP is incorporated into the cyclic nucleotide (Hardman *et al.*, 1971). Both of the reaction products have been isolated and identified by Garbers *et al.* (1975) with partially purified soluble guanylate cyclase from rat lung. As mentioned earlier, the metal·GTP complex is believed to serve as the substrate, with Mn^{2+} being the preferred cation.

In addition to differences in molecular size, soluble and particulate guanylate cyclases from a number of different tissues (*e.g.* lung, liver, heart, brain, kidney, uterus) exhibit several marked differences with respect to their kinetic characteristics (*e.g.* Kimura & Murad, 1974*a*, 1975*a*,*b*,*c*,*d*; Chrisman *et al.*, 1975; Nakazawa *et al.*, 1976; Craven & DeRubertis, 1976; Siegel *et al.*, 1976). Soluble guanylate cyclase exhibits classical Michaelis-Menten behaviour with respect to GTP concentration with apparent K_m values for Mn·GTP ranging from approximately 10 to 70μM. In contrast, higher substrate concentrations were required by the particulate enzyme (*i.e.* K_m or $S_{0.5}$ values of 50 to 300 μM) which exhibited apparent cooperative behaviour with Hill coefficients for metal-nucleotide binding sites of 1.4 to 1.7 indicating two or more interactive sites for Mn·GTP (Kimura & Murad, 1975*a*). While both the soluble and particulate guanylate cyclases clearly require Mn^{2+} in excess of Mn·GTP for maximum activity, the particulate form generally exhibits less of a dependence than the soluble form in a wide variety of tissues. For example, optimal Mn^{2+} to GTP ratios of 4:1 have been reported for the heart (Kimura & Murad, 1974*a*) and parotid (Durham, 1976) soluble enzymes; whereas, ratios of 2:1 and 1.5:1 have been reported for the particulate form of the enzyme from the same tissues, respectively. Chrisman *et al.* (1975) have suggested that excess Mn^{2+} may serve as an activator of guanylate cyclase by binding at a specific cation site. Thus, in the presence of Mn^{2+} at saturating substrate concentration, other bivalent cations such as Mg^{2+} or Ca^{2+} may stimulate guanylate cyclase activity either by interacting directly with the cation activator site or by displacing Mn^{2+} from GTP which would then become available to bind at the activator site. Soluble guanylate cyclase

from kidney (Böhme *et al.*, 1970), epidermis (Marks, 1973), brain (Nakazawa & Sano, 1974), lung (Chrisman *et al.*, 19750, heart (Kimura & Murad, 1974*a*), parotid (Durham, 1976) and platelets (Adams & Haslam, 1978) is activated by millimolar concentrations of Ca^{2+} in the presence of Mn^{2+} at saturating substrate concentrations; whereas under the same conditions, the particulate enzyme from heart (Kimura & Murad, 1974*a*; Sulakhe, 1976), lung (Chrisman *et al.*, 1975), liver (Kimura & Murad, 1974*a*), and parotid (Durham, 1976) is markedly inhibited.

As mentioned earlier, ATP has been shown to be a potent inhibitor of guanylate cyclase activity in a number of different tissues (Limbird & Lefkowitz, 1975; Kimura & Murad, 1974*a*, 1975*a*,*c*; Thompson *et al.*, 1973*a*; Durham, 1976; DeJongé, 1975; Criss *et al.*, 1976; Deviller *et al.*, 1975; Adams & Haslam, 1978). This effect appears to be the result of competition with GTP at the substrate site. K_i values of 0.011 mM, 0.4 mM and 1.0 mM have been reported for the soluble enzyme from liver (Thompson *et al.*, 1973*a*) and for the heart soluble and particulate enzymes (Kimura & Murad, 1974*a*), respectively. In general, the soluble form is more sensitive to inhibition than the particulate. The almost complete inhibition of the soluble enzyme by physiological concentrations of ATP has led Kimura & Murad (1975*a*) to suggest that under normal conditions in the cell, little of the soluble guanylate cyclase activity would be expressed unless factors exist that can overcome the inhibitory effects of ATP.

Although the soluble and particulate forms of guanylate cyclase have different properties and appear to have differences in their regulation, it is not possible at present to determine whether or not these differences are due to different proteins, the environment of the enzyme in

assays or other phenomena. Recent studies have shown that soluble and particulate activities are antigenically different in that antibody prepared to purified particulate sea urchin sperm guanylate cyclase (Garbers, 1976) inhibits mammalian particulate but not soluble activity (Garbers, 1978). While these studies imply that soluble and particulate activities are structurally different, additional studies with purified preparations are required in order to determine what similarities and differences actually exist in these proteins and in their regulation. The finding that purified particulate guanylate cyclase from sea urchin sperm displays classical Michaelis-Menten kinetics with respect to GTP compared to the cooperativity of crude preparations (Garbers, 1976) is of interest as it suggests that the two forms of the enzyme may be capable of interconversion.

1.4. Activation of guanylate cyclase in vitro

A key process that remains to be determined is the mechanism by which cyclic GMP-linked signals modulate guanylate cyclase activity. The apparent hormonal insensitivity of guanylate cyclase in cell-free systems suggests that the regulation of this enzyme is accomplished indirectly and/or that a greater degree of cell integrity is necessary to transmit regulatory signals to guanylate cyclase. Although there is no definitive evidence for an indirect mechanism of activation, reports that guanylate cyclase is stimulated by Ca^{2+} ions (Kimura & Murad, 1974*a*; Chrisman *et al.*, 1975; Katagiri *et al.*, 1976; Wallach & Pastan, 1976*a*), lysophosphatides (Shier *et al.*, 1976), phospholipids (Limbird & Lefkowitz, 1975), fatty acids (Wallach & Pastan, 1976*b*; Barber, 1976; Glass *et al.*, 1977*a*; Adams & Haslam, 1978), fatty acid hydroperoxides (Glass *et al.*, 1977*a*; Hidaka & Asano, 1977 ; Goldberg *et al.*, 1978), free radicals (Mittal & Murad, 1977*a*, *b*; Murad *et al.*, 1978) or other substances with oxidizing potential such as dehydroascorbic acid (Goldberg & Haddox, 1977; Haddox *et al.*, 1978; Goldberg *et al.*, 1978) suggest the possibility of such a mechanism.

1.4.1. Effects of hormones

Although cholinergic agents have been reported to stimulate guanylate cyclase activity in a variety of tissues including heart (White *et al.*, 1973; St. Louis & Sulakhe, 1976), gall bladder (Amer, 1974) and islets of Langerhans (Howell & Montague, 1974), these findings have not been confirmed in other laboratories (Stoner, 1974; Limbird & Lefkowitz, 1975). Furthermore, these agents failed to stimulate guanylate cyclase

activity in other cholinergically-responsive tissues such as brain (Nakazawa & Sano, 1974) and renal cortex (Helwig *et al.*, 1975; DeRubertis & Craven, 1976). Other hormones and membrane-active agents that have been found to have no significant effect on guanylate cyclase activity include: serotonin, glucagon, histamine, vasopressin, parathyroid hormone, pentagastrin, hypertensin, bradykinin, thyroxin, somatotropin, prostaglandins A_1, E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$ and concanavalin A (Goldberg & Haddox, 1977).

1.4.2. Effects of physiological concentrations of calcium

The suggestion that calcium may function as an intracellular regulator of guanylate cyclase was based on a report by Schultz *et al.* (1973a) that cholinergically-induced accumulation of cyclic GMP in smooth muscle was dependent upon the presence of calcium in the medium. Since then, it has been shown in a number of systems that extracellular calcium is an essential requirement for the hormonal activation of guanylate cyclase in intact cells (Ferrendelli *et al.*, 1973; Van Sande *et al.*, 1975; Clyman *et al.*, 1973; Fain & Butcher, 1976; Pointer *et al.*, 1976; Berridge, 1975). Furthermore, observations that the basal level of cyclic GMP can be raised by increasing the extracellular calcium concentration (Schultz *et al.*, 1973a) and that treatment of cells with the calcium ionophore, A 23187, in the presence of calcium also rapidly increases cyclic GMP levels (Van Sande *et al.*, 1975; Fain & Butcher, 1976; Pointer, 1976) support the view that Ca^{2+} is a possible regulator of cyclic GMP synthesis (Schultz & Hardman, 1975; Berridge, 1975).

As discussed in Section 1.3.4., calcium has been reported to increase the activity of soluble guanylate cyclase, and under the same conditions inhibit the activity of the particulate enzyme (Kimura & Murad,

1974*a*; Chrisman *et al.*, 1975). These effects of calcium were only evident with millimolar concentrations of Ca^{2+} when Mn^{2+} was the major cation in the assay. The stimulatory effects of Ca^{2+} did not appear to be due to the free cation, but rather to the Ca·GTP complex which served as a substrate and/or allosteric effector of the enzyme (Chrisman *et al.*, 1975; Garbers *et al.*, 1974). In view of the fact that the concentration of free Ca^{2+} in the cytosol is in the micromolar range in activated cells (Rasmussen, 1970), and that the amount of Mn^{2+} in cells is about two orders of magnitude less than that of Mg^{2+} (Thiers & Vallee, 1957; Cotzias, 1962), these effects of Ca^{2+} and Mn^{2+} on guanylate cyclase activity are unlikely to be relevant to the *in vivo* regulation of cyclic GMP metabolism.

In an attempt to explain calcium related increases in tissue cyclic GMP levels, Wallach & Pastan (1976*a*) studied the effects of physiological concentrations of this cation on the activity of particulate guanylate cyclase from cultured fibroblasts. These workers showed that with Mg^{2+} as the major bivalent cation, 3 μM Ca^{2+} resulted in a 25% stimulation of activity while 30 μM Ca^{2+} increased activity by about 100%. From these observations, Wallach & Pastan (1976*a*) concluded that the *in vitro* activity of guanylate cyclase with Mg^{2+} represents its behaviour *in vivo* and that Ca^{2+} regulates cyclic GMP levels in cells by directly stimulating guanylate cyclase activity. Thus, the alteration in cyclic GMP metabolism associated with hormonal stimulation would be a secondary response to primary changes in cytoplasmic calcium distribution (Schultz & Hardman, 1975; Berridge, 1975).

1.4.3. Surfactant effects of non-ionic detergents and lipids

As previously mentioned, synthetic non-ionic detergents such as Triton X-100 and Lubrol PX have been shown to stimulate the activity of

both the soluble and particulate forms of guanylate cyclase from a number of different tissues (Ishikawa *et al.*, 1969; Hardman *et al.*, 1971; Kimura & Murad, 1974*a*, 1975*a*,*c*; Neer & Sukiennik, 1975; Chrisman *et al.*, 1975; Deguchi *et al.*, 1976; Adams & Haslam, 1978). Although the marked activation of particulate guanylate cyclase by non-ionic detergents might simply be due to the release of a bound form of the enzyme from particulate material thereby enhancing the interaction of the enzyme with substrate and cofactors, the smaller stimulatory effect on the activity of the supernatant enzyme cannot be explained by this mechanism (Kimura & Murad, 1975*a*). However, there are a number of other possible ways in which detergents could activate the nonsedimentable guanylate cyclase; for example, it could form micelles into which the enzyme inserts and then becomes activated by the hydrophobic, membrane-like environment, or a hydrophobic environment might be provided by binding of a large amount of detergent to the surface of the molecule. Alternatively, the detergent could activate by binding at a few specific sites causing a conformational change in the enzyme (Neer & Sukiennik, 1975; Helenius & Simons, 1975). This latter possibility is strongly supported by the findings of Neer & Sukiennik (1975).

The general similarity of the surfactant properties of non-ionic detergent, lysophosphatides and sodium salts of long chain fatty acids (Helenius & Simons, 1975) prompted several workers to investigate the effects of these naturally occurring detergents on guanylate cyclase activity. In 1975, White & Lad briefly reported that both soluble and insoluble guanylate cyclase activities in rat lung were stimulated by lysolecithin. This finding, combined with other observations that lysolecithin stimulated membrane-associated sialyltransferase (Shier & Trotter,

1976) and galactosyltransferase activities (Kirschbaum & Bossman, 1973) in a manner similar to Triton X-100, led Shier *et al.* (1976) and Zurier *et al.* (1976) to study the effects of lysolecithin on guanylate cyclase from cultured fibroblast and neuroblastoma cells, respectively. In the former study, Shier *et al.* (1976) found that lysolecithins from various plant and animal sources markedly enhanced the activity of the particulate enzyme; whereas other phospholipids such as lecithin, lysophosphatidylethanolamine and lysodimethylphosphatidylethanolamine had no effect. These results indicated that the observed stimulatory effect was not specifically related to the substructure of lysolecithin, but rather to its surfactant properties (Helenius & Simons, 1975). Zwiller *et al.* (1976) similarly found that lysolecithin but not other phospholipids could stimulate the activity of the soluble as well as the membrane-associated guanylate cyclase. Although these observations are consistent with the reported stimulation of guanylate cyclase from several tissues by phospholipases A or C (Kimura & Murad, 1974*a*; White & Lad, 1975; Fujimoto & Okabayashi, 1975; Shier *et al.*, 1976; Zwiller *et al.*, 1976; Sulakhe *et al.*, 1976), the role of lysophosphatides in the modulation of guanylate cyclase activity *in vivo* is uncertain.

In addition to the effects of lysolecithin and phospholipases A and C, phosphatidylserine (Limbird & Lefkowitz, 1976) and a number of unsaturated fatty acids (Wallach & Pastan, 1976*b*; Barber, 1976; Asakawa *et al.*, 1976; Glass *et al.*, 1977*a*; Adams & Haslam, 1978) have been reported to stimulate the activity of guanylate cyclase from several tissues. Wallach & Pastan (1976*b*) found that the saturated, short chain (12 to 16) fatty acids as well as several longer, unsaturated fatty acids stongly stimulated particulate guanylate cyclase activity in cultured fibroblasts.

Since the effectiveness of the fatty acids appeared to correlate with their ability to interact as amphiphiles with protein or lipid, the authors concluded that a specific fatty acid-binding site on the enzyme was unlikely. However, the observation that optimal concentrations of Lubrol PX potentiated the fatty acid effect indicated that non-ionic detergents and fatty acids activated guanylate cyclase by different mechanisms. In these experiments, the concentration of fatty acid required for optimal stimulation was about 0.3 to 0.6 mM. Similar concentrations of oleic, linoleic, linolenic and arachidonic acids were required to stimulate the particulate enzyme from isolated fat cells by seven- to 10-fold (Asakawa *et al.*, 1976). Although a surfactant-protein interaction may constitute one general mechanism by which guanylate cyclase activity can be modulated, it is unlikely that the relatively high concentrations of surfactant required to stimulate enzyme activity *in vitro* would be found *in vivo*.

1.4.4. Oxidative and reductive modulation of guanylate cyclase

There is increasing evidence that cellular events involving oxidation and reduction may represent a general mechanism for the regulation of guanylate cyclase activity and the metabolism of cyclic GMP (Haddox *et al.*, 1976). This concept has developed from observations that a wide variety of agents and conditions activate guanylate cyclase by processes that appear to involve alterations in the redox state of the enzyme and the formation of reactive free radicals.

Spontaneous activation. The spontaneous time- and temperature-dependent activation of guanylate cyclase was first described by Böhme *et al.* (1974) in a brief report of the properties of the soluble enzyme from

human platelet homogenates. In addition, it was shown that activation could be blocked by pretreatment of the enzyme preparation with the reducing agent, dithiothreitol. These observations have since been confirmed by Glass *et al.* (1977*a*) and extended by Adams & Haslam (1978), Goldberg *et al.* (1978) and Haddox *et al.* (1978). Similar characteristics of spontaneous activation have been reported for the soluble enzyme from a number of other tissues, including rat lung (Chrisman *et al.*, 1975; White & Lad, 1975; White *et al.*, 1976), mouse and guinea pig splenic cells (Haddox *et al.*, 1976, 1978; Goldberg *et al.*, 1978) and rat uterus (Kraska *et al.*, 1977). The most extensive investigation of the processes involved in spontaneous activation of guanylate cyclase has been carried out by White *et al.* (1976) with soluble enzyme from rat lung homogenates. These workers found that the two- to three-fold increase in guanylate cyclase activity produced by preincubation of the enzyme for 30 minutes at 30°C required oxygen (*i.e.* no effect was observed in an atmosphere of nitrogen) and copper, and may also involve the intermediate generation of H_2O_2. Although this phenomenon was completely inhibited by thiol-reducing agents such as 2-mercaptoethanol, dithiothreitol and glutathione, addition of 2-mercaptoethanol to a preincubated enzyme preparation could not reverse the activation. White *et al.* (1976) hypothesized that guanylate cyclase was ultimately activated by H_2O_2, which was believed to be generated non-enzymically as a result of the interaction of oxyhemoglobin with a proton donor such as ascorbic acid, both of which are present in lung homogenates. While the identity of the activating species cannot be specifically determined from this study, the evidence does suggest that spontaneous activation during preincubation may involve the oxidation of enzyme sulfhydryl groups.

Activation by oxidants. In 1976, Haddox *et al.* (1976) reported that sodium periodate increased the concentration of cyclic GMP in mouse and guinea pig splenic cells by two- to 10-fold in the absence of extracellular calcium; whereas the reducing agent cysteine, either alone or together with sodium periodate, significantly lowered the concentration of this cyclic nucleotide. In subsequent studies (Haddox *et al.*, 1977, 1978), these workers found that while ascorbic acid also markedly increased the steady-state levels of cyclic GMP in intact splenic cells, it had no effect on the activity of guanylate cyclase after cell disruption. On the other hand, dehydroascorbic acid enhanced intracellular cyclic GMP concentrations as well as the activity of both the soluble and particulate forms of guanylate cyclase from guinea pig splenic cells (Goldberg *et al.*, 1978). Moreover, the relatively stable dehydroascorbic acid-induced elevation of cyclic GMP in intact cells or activation of guanylate cyclase in broken cell preparations, which persisted after removal of the oxidant, could be reversed upon reduction with dithiothreitol; while the inhibition induced by dithiothreitol could also be reversed by the subsequent addition of the oxidant. On the basis of these findings, Haddox *et al.* (1976, 1977, 1978) proposed that intracellular oxidative and reductive events may indirectly modulate guanylate cyclase activity.

Activation by fatty acid peroxides. The report (Glass *et al.*, 1977) that prostaglandin endoperoxide PGG_2 can serve as an activator of platelet guanylate cyclase led to the investigation of the effects of other fatty acid peroxides on enzyme activity. Hidaka & Asano (1977*a*,*b*) believe that the stimulatory effect of arachidonate on platelet guanylate cyclase activity depends on its conversion into the hydroperoxy derivative by

lipoxygenase present in the enzyme preparation. Recently, Goldberg *et al.* (1978) reported the results of an extensive investigation of the effects of naturally occurring fatty acid hydroperoxides and prostaglandin endoperoxides on the activity of soluble guanylate cyclase from guinea pig splenic cells. This study was carried out to determine whether the stimulatory effects of these agents were a function of their oxidizing potential. These workers found that micromolar concentrations of PGG_2 and PGH_2 increased guanylate cyclase activity by three- to four-fold in an oxygen or argon atmosphere, which suggested that the stimulatory effect of these prostaglandin endoperoxides was not dependent on the presence of molecular oxygen. It was also shown that dithiothreitol or glutathione not only prevented, but also reversed activation by the endoperoxy- and/or hydroperoxy-containing fatty acids studied; while low concentrations of the sulfhydryl-reactive reagent, *N*-ethylmaleimide, blocked activation. From these results, Goldberg *et al.* (1978) concluded that these "hydrophilic" oxidants activate guanylate cyclase by promoting a sulfhydryl-disulfide interconversion at specific hydrophobic regulatory sites.

Activation by superoxide dismutase and hydroxyl radical. Mittal & Murad (1977*b*) reported that superoxide dismutase markedly enhanced the activity of partially purified soluble guanylate cyclase from rat liver. The presence of superoxide ion in the enzyme preparation combined with observations that activation could be prevented by inhibitors of superoxide dismutase such as KCN or thiols, by catalase, which removes H_2O_2 generated by superoxide dismutase, or by scavengers of hydroxyl radicals, suggested that the formation of both superoxide ion and H_2O_2 were required for activation. On the basis of these findings, Mittal & Murad (1977*b*) proposed

that hydroxyl radicals, which are formed by the Haber-Weiss reaction (Haber & Weiss, 1934) from superoxide ion and H_2O_2, are ultimately responsible for superoxide dismutase activation of guanylate cyclase. This hypothesis is also supported by the observation of White *et al.* (1976) that low concentrations of H_2O_2 in the presence of KCN could activate guanylate cyclase in lung preparations. Although it is not known whether spontaneous activation is due to an effect of hydroxyl radicals generated during preincubation, Mittal & Murad (1977*b*) have suggested that the formation of superoxide ion, hydrogen peroxide and hydroxyl radical is likely to be the mechanism for physiological and hormonal regulation of guanylate cyclase and cyclic GMP metabolism.

Activation by nitric oxide. Kimura *et al.* (1975*b*) first reported that sodium azide (NaN_3), a potent metabolic inhibitor and strong nucleophilic agent, can increase guanylate cyclase activity in many but not all tissue preparations. The presence of activating and inhibiting factors was demonstrated by mixing azide-responsive and non-responsive preparations (Kimura *et al.*, 1975*b*; Mittal *et al.*, 1975, 1977, 1978). Some of the inhibitory materials have been identified as hemoglobin and myoglobin (Miki *et al.*, 1977*a*; Mittal *et al.*, 1977; Murad *et al.*, 1978). The "azide activator factor" has been characterized and is probably catalase (Miki *et al.*, 1977*a*; Mittal *et al.*, 1977); however, a variety of enzymes such as peroxidase, cytochrome b_2 and cytochrome c can substitute for the activating factor (Mittal *et al.*, 1977, 1978; Murad *et al.*, 1978). These proteins convert azide to nitric oxide, which can activate most preparations of guanylate cyclase that have been tested (Mittal & Murad, 1977*b*; Murad *et al.*, 1978). In addition to sodium azide, a number of other strongly nucleophilic

compounds can activate guanylate cyclase, such as $NaNO_2$, hydroxylamine, phenylhydrazine, nitroglycerin and sodium nitroprusside (Kimura *et al.*, 1975; Katsuki *et al.*, 1977; Mittal & Murad, 1977*b*). Other workers have reported that the carcinogens; nitrosoguanidine (DeRubertis & Craven, 1976, 1977), nitrosoureas (Vesely *et al.*, 1977) and hydrazine (Vesely & Levey, 1977; Craven & DeRubertis, 1977) have similar effects on guanylate cyclase activity. Since these materials can also be converted to nitric oxide under the appropriate redox and/or enzymatic conditions, Murad *et al.* (1978) have recently hypothesized that the stimulatory effects of these agents are indirectly due to activation of guanylate cyclase by nitric oxide.

1.5. *Objectives*

The primary objective of this thesis was to investigate mechanisms that could be involved in the physiological activation and control of guanylate cyclase activity in platelets. The studies described in this thesis were therefore directed towards:

(a) Establishing the linearity of guanylate cyclase activity with respect to protein concentration and period of incubation.

(b) Characterizing the subcellular distribution and bivalent cation requirements of guanylate cyclase in platelets.

(c) Determining whether aggregating agents that increase the concentration of cyclic GMP in intact platelets have an effect on guanylate cyclase activity in broken cell preparations.

(d) Identifying and studying the effects and interactions of a wide range of agents that stimulate or inhibit enzyme activity in broken cell preparations in order to throw light on mechanisms that could be responsible for the modulation of guanylate cyclase activity in intact platelets.

Chapter 2

Experimental

2.1. *Materials*

2.1.1. *Radioactive compounds*

[8-^{3}H]GTP (10 Ci/mol), cyclic [8-^{14}C]GMP (60 mCi/mmol) and cyclic [8-^{3}H]GMP were obtained from Amersham Corporation, Oakville, Ontario. [8-^{3}H]GTP was purified by t.l.c. in two dimensions on cellulose, by using the solvents described by Haslam & McCleneghan (1974).

2.1.2. *Proteins and enzymes*

Unless otherwise mentioned, all proteins and enzymes were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.: *i.e.* protein standard solution [5% (w/v) human albumin and 3% (w/v) human globulin], crystalline bovine serum albumin, essentially fatty acid-free bovine serum albumin (prepared from crystalline bovine serum albumin), creatine phosphokinase (150 units/mg of protein), cyclic nucleotide phosphodiesterase (0.27 unit/mg of protein), phosphoglycerokinase (2100 units/mg of protein), glyceraldehyde-3-phosphate dehydrogenase (55 units/mg of protein), lactate dehydrogenase (430 units/mg of protein), pyruvate kinase (645 units/mg of protein), myokinase (2370 units/mg of protein). Apyrase (EC 3.6.1.5.) was prepared by the method of Molnar & Lorand (1961) and was a gift from Dr. J.F. Mustard, McMaster University, Hamilton, Ontario. This preparation hydrolysed 1.7 μmol of ATP/min per mg of protein.

2.1.3. *Nucleotides*

All nucleotides were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.: *i.e.* cyclic GMP, GMP, GDP, GTP, cyclic AMP, AMP, ADP, ATP, NADH.

2.1.4. Non-ionic detergents

Lubrol PX and Triton X-100 were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

2.1.5. Fatty acids

Arachidonic, oleic and palmitic acids were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Arachidonic acid was purified as described by Davies *et al.* (1976). All fatty acids were dissolved in ethanol, neutralized with Na_2CO_3 and diluted with water to a final concentration of 20 mM containing 10% (v/v) ethanol. In experiments with fatty acids, the final concentration of ethanol in assays was adjusted to 0.5%, which had no effect on guanylate cyclase activity.

2.1.6. Pharmacological agents

Unless otherwise noted, most agents were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.: *i.e.* indomethacin, 5-hydroxytryptamine, *l*-epinephrine, sodium azide, sodium nitroprusside, dithiothreitol, glutathione, oxidized glutathione, cysteine, *N*-ethylmaleimide, diamide, 4,4'-dithiodipyridine, 5,5'-dithiobis-(2-nitrobenzoic acid), ethylenediamine tetraacetic acid, ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*'-tetraacetic acid. 3-Isobutyl-1-methylxanthine was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A., and *tert*-butylhydroperoxide (purity of 70%, v/v) from Koch-Light Laboratories Limited, Colnbrook, Bucks, England.

2.1.7. Buffers

Trizma (tris) base, triethanolamine hydrochloride and *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

2.1.8. Chromatographic materials

Neutral alumina (WN-3) was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Anion exchange resin (AG 1-X2, 200-400 mesh, Cl^- form) was obtained from Bio-Rad Laboratories, Richmond, California, U.S.A. and cellulose powder for t.l.c. (MN 300 HR) from Brinkman Instruments (Canada), Toronto, Ontario.

2.1.9. Ultrafiltration apparatus

Ultrafiltration membrane cones (Centriflo, 224-UF-50) were purchased from Amicon Company, Lexington, Massachusetts, U.S.A.

2.1.10. Liquid scintillation 'cocktail'

Quantafluor liquid scintillation cocktail was obtained from Mallinckrodt, St. Louis, Missouri, U.S.A.

All other reagents were of analytical grade.

2.2. *Methods*

2.2.1. Preparation of suspensions of washed platelets

Venous blood was obtained from healthy volunteer donors who had not taken any medication for the preceding 10 days. From 150 to 300 ml of blood was collected into a siliconized flask containing acid-citrate-dextrose anticoagulant (85 mM-trisodium citrate, 71.4 mM-citric acid, 2% (w/v) dextrose)(Aster & Jandl, 1964) to give a final concentration of 16% (v/v).

Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 250*g* for 10 min at 4°C. This step was repeated in order to increase the yield of PRP. The supernatant PRP was transferred to siliconized conical tubes (12 ml) and centrifuged at 1020*g* for 20 min at 4°C in an angle head. The supernatant platelet poor plasma (PPP) was aspirated and the red blood cells removed from the platelet pellet with a Pasteur pipette. The platelet pellets were resuspended in acid-citrate-dextrose washing solution (13 mM-sodium citrate, 5 mM-dextrose, 135 mM-NaCl; adjusted to pH 6.5 with HCl)(Haslam & Lynham, 1972) and centrifuged at 365*g* for 20 min, at 4°C. The weight of the platelet pellet was measured and the washed platelets were resuspended in 150 mM-Tris, adjusted to pH 7.4 with HCl, to give 50 to 100 mg wet weight of platelets/ml (approximately 3.5 to 7.0 mg of protein/ml). Approximately 500 mg wet weight of platelets were obtained from 250 ml of blood. Platelet suspensions were stored at 0°C for up to one hour before use.

Suspensions of washed platelets were also prepared from 4 to 5 day old platelet concentrates stored at 22°C, which were obtained from the

Canadian Red Cross. The platelet concentrates (50 ml of concentrate/1 unit of whole blood) were centrifuged at 250*g* for 10 min at 4°C to remove any remaining red blood cells. Washed platelet suspensions were prepared from the PRP as described above. Approximately 800 mg wet weight of platelets were obtained from one unit of platelet concentrate.

Only plastic ware or silicone-treated glassware was used to prepare the washed platelet suspensions.

2.2.2. Enzyme preparations

Platelet lysate was prepared by freezing the washed platelet suspension in a solid CO_2/acetone bath and then gently shaking the frozen platelets in a water bath at 37°C until just completely thawed. The freeze-thaw cycle was repeated once and the platelet lysate was stored at 0°C for up to two hours before use. In some cases, the platelet suspension was kept frozen at −50°C for up to five days. On the day of the experiment, the frozen suspension was thawed and the freeze-thaw cycle was repeated.

In some experiments, part of the lysate was preincubated for 60 min at 30°C or at 37°C and then stored at 0°C until use. In other experiments, lysate was ultrafiltered in Centriflo membrane cones (approximate molecular weight cut off of 50 000) that had been soaked in water for at least one hour. In the latter case, up to 2 ml of platelet lysate was centrifuged at 1000*g* for 60 min at 4°C. The volume of the ultrafiltrate was measured (70 to 90% of that of the lysate) and the concentrate was resuspended in an equivalent volume of 150 mM-Tris/HCl, pH 7.4 and stored at 0°C. Ultrafiltration resulted in a loss of about 20% of the lysate protein on the ultrafiltration membrane.

Supernatant and particulate fractions of platelet lysate were

usually obtained by centrifugation at 48 000*g* for 30 min at 4°C. In certain experiments, platelet lysate was centrifuged at 100 000*g* for two hours at 4°C. Supernatant and particulate fractions were stored at 0°C until use.

2.2.3. Guanylate cyclase assay

Assay mixtures (250 µl) contained (final concentrations): 1 mM-[8-^{3}H]GTP (2 mCi/mmol), 1 mM-3-isobutyl-1-methylxanthine, 4 mM-cyclic GMP, 1 mg of bovine serum albumin/ml, 4 mM-$MnCl_2$ and 60 mM-Tris/HCl, pH 7.4. Phosphocreatine (5 mM) and creatine phosphokinase (20 units/ml) were included unless otherwise indicated. Assays were started by the addition of 10 to 100 µl of enzyme preparation to freshly mixed reagents at 30°C and were stopped by the addition of 100 µl of a solution containing 0.001 µCi of cyclic [8-^{14}C]GMP (60 mCi/mmol) and 50 mM-EDTA (adjusted to pH 7.6 with $NaHCO_3$), followed by boiling for 3 minutes. The samples were finally transferred to an ice bath and 50 mM-Tris/HCl, pH 7.6, was added to give a total volume of 1 ml. Denatured protein was removed by centrifugation.

2.2.4. Isolation of cyclic [^{3}H]GMP

Cyclic [^{3}H]GMP was isolated by a modification of the method of White & Zenser (1971). Sample supernatants were applied to columns (internal diameter, 6 mm) containing 1 g of neutral alumina, which had been washed with 50 mM-Tris/HCl, pH 7.6; the first 2 ml from each column were discarded and the next 3 ml was collected into liquid-scintillation vials and freeze-dried. The residues were dissolved in 1 ml of water, mixed with 7.5 ml of Quantafluor, and counted for ^{3}H and ^{14}C radioactivity as described in Section 2.2.7.5.. Recovery of added cyclic [8-^{14}C]GMP in the fraction collected ranged from 40 to 70% (Table 2.2.4.1.). Zero-incubation-time blanks were included in each experiment and amounted to 100 to 300 d.p.m. of ^{3}H

(about 5% of the total d.p.m. of cyclic [^{3}H]GMP recovered during a typical assay)(Fig. 2.2.4.1. and Table 2.2.4.2.). This blank was subtracted before correction for recovery. Similar blank values were obtained from samples without enzyme which were not boiled (Table 2.2.4.2.), indicating that non-enzymic formation of cyclic [^{3}H]GMP during boiling (Kimura & Murad, 1974*b*) did not occur. The successful use of [8-^{3}H]GTP in this assay was dependent on prior purification of the substrate (Table 2.2.4.3.), and the removal by freeze-drying of small amounts of ^3H_2O formed during boiling of the samples (Table 2.2.4.2.).

2.2.5. Radiochemical purity of cyclic [^{3}H]GMP isolated from guanylate cyclase assays

Pooled eluates from identical samples applied to alumina columns were chromatographed on anion-exchange resin (Dowex AG 1-X2). Cyclic GMP was eluted as described by Haslam & McClenaghan (1974) and the eluate was freeze-dried. The residue was taken up in water, a sample was counted for ^{3}H and ^{14}C radioactivity and another sample was incubated for 60 minutes at 30°C in a reaction mixture (120 µl) containing 0.05 unit of cyclic nucleotide phosphodiesterase, 4 mg of bovine serum albumin/ml, 4 mM-$MgSO_4$, 0.25 mM-EDTA and 10 mM-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid (adjusted to pH 7.5 with NaOH). After the incubation, the reaction mixture was boiled and centrifuged, and the supernatant chromatographed on cellulose as described by Haslam & McClenaghan (1974). Product 5'-GMP was eluted with water and counted for ^{3}H and ^{14}C radioactivity. The ratio of ^{3}H to ^{14}C in this 5'-GMP was the same as in freeze-dried eluates from the columns containing either alumina or anion-exchange resin, indicating that the standard assay procedure isolated radiochemically pure cyclic [^{3}H]GMP

(Table 2.2.5.1.).

2.2.6. Measurement of cyclic GMP phosphodiesterase and guanosine triphosphatase activities present in the guanylate cyclase assays

The activity of cyclic GMP phosphodiesterase remaining under guanylate cyclase assay conditions was determined by substituting unlabeled GTP for [8-^{3}H]GTP and adding cyclic [8-^{3}H]GMP (0.025 µCi) to the assay mixture. Labeled cyclic GMP was isolated as usual and the percentage of cyclic [8-^{3}H]GMP broken down was shown to be less than 7% in 20 minute incubations with the highest enzyme concentrations used in this study (Table 2.2.6.1.).

To measure GTP breakdown, guanylate cyclase assays were carried out as usual, but the reactions were stopped by the addition of 100 µl of 50 mM-EDTA, pH 7.4, followed by boiling for 3 minutes. After centrifugation, 10 µl of the supernatant was mixed with 0.1 µmol each of GTP and of GDP and chromatographed on cellulose (t.l.c.) in two dimensions (Haslam & McClenaghan, 1974). The areas corresponding to GTP and GDP were eluted with 2 ml of water, the extinction values at 253 nm were measured and fractions of this material were counted for ^{3}H radioactivity. Guanosine triphosphatase activity was expressed as a percentage of the added [8-^{3}H]GTP broken down and amounted to about 1%/min with 300 µg of platelet protein in an assay tube (Table 2.2.6.2.). Therefore, in most experiments in which more protein was used or in which incubations were continued for longer than 20 minutes, phosphocreatine and creatine phosphokinase were included in the assay mixtures. Although the phosphocreatine/creatine phosphokinase GTP-regenerating system was found to prevent completely the breakdown of [8-^{3}H]GTP in guanylate cyclase assays (Fig. 2.2.6.1.), it had no significant effect on the accumulation of cyclic [^{3}H]GMP.

2.2.7. Other methods

2.2.7.1. Protein assay. Protein was assayed by the method of Lowry *et al.* (1951), by using a protein standard solution containing 5% (w/v) human albumin and 3% (w/v) human globulin. Tris/HCl (Ji, 1973) and other reagent blanks were subtracted. The concentration of protein in washed suspensions of platelets (100 mg wet weight of platelets/ml) was found to be 0.21 ± 0.01 mg of protein/10^8 platelets (mean ± S.E.M. of 5 determinations). Platelets were counted in a hemocytometer chamber by phase contrast microscopy (Harker, 1974). For every experiment, the concentration of protein in each enzyme preparation was assayed.

2.2.7.2. Lactate dehydrogenase assay. Lactate dehydrogenase activity in platelet fractions was determined by the method of Bergmeyer *et al.* (1965).

2.2.7.3. Adenine nucleotide assays. The concentration of ATP, ADP and AMP in enzyme preparations was assayed by the methods described by Adam (1965).

2.2.7.4. Assay of total and non-protein sulfhydryl groups. The concentration of total, protein-bound and non-protein sulfhydryl groups in washed platelet suspensions and enzyme preparations was estimated essentially as described by Sedlak & Lindsay (1968). This method is based on the method of Ellman (1958, 1959), who reported that 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) is reduced by sulfhydryl groups to produce 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of thiol. The nitromercaptobenzoic acid anion has an intense yellow colour that can be used to measure sulfhydryl groups spectrophotometrically.

All solutions used in the assays were prepared as

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described by Sedlak & Lindsay (1968). Reduced glutathione was used as a standard for both the total and non-protein thiol determinations. The absorbance (412 nm) produced by the reaction of DTNB with various concentrations of glutathione (0.001 to 0.1 mM; final thiol concentration in the colour reaction mixture) was linear. The experimentally determined molar extinction coefficient of reduced glutathione at 412 nm was 14.26 in both total and non-protein sulfhydryl procedures. The platelet suspensions or enzyme preparations to be assayed were stored for up to one week at -50°C until used.

Determination of total sulfhydryl groups. Aliquots of 60 µl of the platelet suspension or enzyme preparation were mixed in Eppendorf micro test tubes with 950 µl of 8 M-urea after which 180 µl of 0.2 M-Tris/0.02 M-ethylenediamine tetraacetic acid-disodium (EDTA Na_2) buffer, pH 8.2 and 10 µl of 0.012 M-DTNB were added to give a final volume of 1.2 ml. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The samples were allowed to stand, with occasional mixing, at room temperature for 30 minutes to develop the colour. The samples were then centrifuged for 6 minutes at 13 000*g* and the absorbance of the clear supernatants read at 412 nm.

Determination of non-protein sulfhydryl groups. Aliquots of 600 µl of the platelet suspension or enzyme preparation were mixed in Eppendorf micro test tubes with 480 µl of water and 120 µl of 50% (w/v) trichloroacetic acid to give a final volume of 1.2 ml. The tubes were mixed intermitently for 10 to 15 minutes at room temperature and then centrifuged for 6 minutes at 13 000*g*. A 240 µl sample of the supernatant was mixed with 470 µl of 0.4 M-Tris/0.02 M-EDTA Na_2 buffer,

pH 8.9 and 10 µl of 0.012 M-DTNB was added to give a final volume of 720 µl. The samples were mixed and the absorbance read at 412 nm within 5 minutes of the addition of DTNB.

Determination of protein-bound sulfhydryl groups. The amount of protein-bound thiol is calculated by subtracting the non-protein bound thiol from the total thiol value.

2.2.7.5. Measurement of radioactivity. Aqueous solutions of 1.0 ml containing either ^{3}H or ^{14}C, or both ^{3}H and ^{14}C were counted for radioactivity for 20 minutes in a Beckman LS 230 scintillation counter after mixing the sample with 7.5 ml of Quantafluor, a toluene-based phosphor. With samples containing only ^{3}H or ^{14}C, the counting efficiencies were approximately 33% and 50%, respectively. For samples containing both ^{3}H and ^{14}C, counting efficiencies of approximately 16% and 50%, respectively, were obtained. Results were corrected for channel cross-over and quench corrections were applied for all samples by the external standard method (Howard, 1976) after subtraction of the background (approximately 26 c.p.m.). The average counting error in assays ranged from about 2 to 5%.

2.2.7.6. Analysis of data. Individual guanylate cyclase assays were almost always performed in triplicate in each experiment. Mean values ± the standard error of the mean (S.E.M.) for each experiment are reported when guanylate cyclase activity is expressed as specific activity. However, when activity is expressed as a percentage of a control sample, only mean values are indicated, but in this case, the mean specific activity ± S.E.M. of the control sample is given in the legend of each table or figure. Standard errors of triplicate assays within each experiment were almost always less than ±5% of the mean. In a few experiments in which the guanylate cyclase activity

was near the limit of the sensitivity of the assay (*i.e.* less than about 0.5 nmol of cyclic GMP formed/20 min per mg of protein), the standard errors occasionally ranged from ±5% to ±10% of the mean.

In many of the tables and figures presented, the values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations from one representative experiment. Although the effects of various treatments reported were usually observed with lysates from at least three separate platelet preparations, the guanylate cyclase activities from different experiments were not pooled because of the variability of the basal activities of different platelet lysates (see for example Figs. 3.2.1., 4.1.1. and 4.1.4.). However, when the changes in guanylate cyclase activity were expressed as percentages of basal activity, data from different experiments could be pooled and the mean values ± S.E.M. from these different experiments are reported in the text to indicate the extent of biological variation. The significance of the effects of various treatments or agents on guanylate cyclase activity observed in different experiments were evaluated by either paired or unpaired Student's *t* tests, depending on the data (Daniel, 1974). When results expressed in percentage terms were pooled for statistical purposes, it is assumed in the calculation of *t*, that the values for percentage changes in guanylate cyclase activity are distributed normally. $2P$ values are indicated in the text.

Table 2.2.4.1. *Elution of cyclic [8-^{14}C]GMP from alumina columns*

Assay mixtures containing 2240 d.p.m. of cyclic [8-^{14}C]GMP were prepared and applied to alumina columns as described in Section 2.2.4. Cyclic [8-^{14}C]GMP was eluted with 50 mM-Tris/HCl, pH 7.6 in individual 1.0 ml fractions which were collected into scintillation vials and freeze-dried prior to counting for ^{14}C radioactivity. Fraction 1 contained the sample. The values given are the means ± S.E.M. of 12 determinations. Of the total amount of cyclic 8-^{14}C GMP applied to each column, 84% was collected in all 10 fractions and 60% in fractions 4,5 and 6.

Fraction (1 ml)	^{14}C d.p.m.	% of total cyclic [8-^{14}C] GMP
1	1.1 ± 0.3	0.04
2	7.0 ± 1	0.26
3	361.0 ± 46	13.4
4	833.0 ± 23	31.0
5	512.0 ± 15	19.0
6	264.0 ± 12	9.8
7	134.0 ± 8	5.0
8	71.0 ± 4	2.6
9	42.0 ± 3	1.6
10	25.0 ± 1	1.0

Fig. 2.2.4.1. *Elution of ^{3}H and of cyclic [8-^{14}C]GMP from alumina columns*

Complete assay mixtures containing 931,545 d.p.m. of 1.0 mM-[8-^{3}H]GTP were prepared as described in Section 2.2.3. Zero-incubation-time blanks were prepared by the simultaneous addition of 25 µl of platelet lysate (6.0 mg of protein/ml) and 100 µl of a solution containing 2691 d.p.m. of cyclic [8-^{14}C]GMP and 50 mM-EDTA, pH 7.4 to each assay tube. The samples were quickly mixed and then boiled for 3 min. After boiling, the samples were transferred to an ice bath and 50 mM-Tris/HCl, pH 7.6 was added to give a final volume of 1.0 ml. The samples were applied to alumina columns as described in Section 2.2.4. Cyclic [8-^{14}C]GMP and blank-^{3}H were eluted with 50 mM-Tris/HCl, pH 7.6 in individual 1.0 ml fractions which were collected into scintillation vials and freeze-dried prior to counting for ^{3}H and ^{14}C radioactivity. Fraction 1 contained the sample. The values given are the means ± S.E.M. of triplicate determinations. Of the total amount of [8-^{3}H]GTP applied to each column, 0.149% (1388 d.p.m.) was collected in all 10 fractions and 0.023% (214 d.p.m.) in fractions 4,5 and 6 (●). Of the total amount of cyclic [8-^{14}C]GMP applied to each column, 86% was collected in all 10 fractions and 58% in fractions 4,5 and 6 (O).

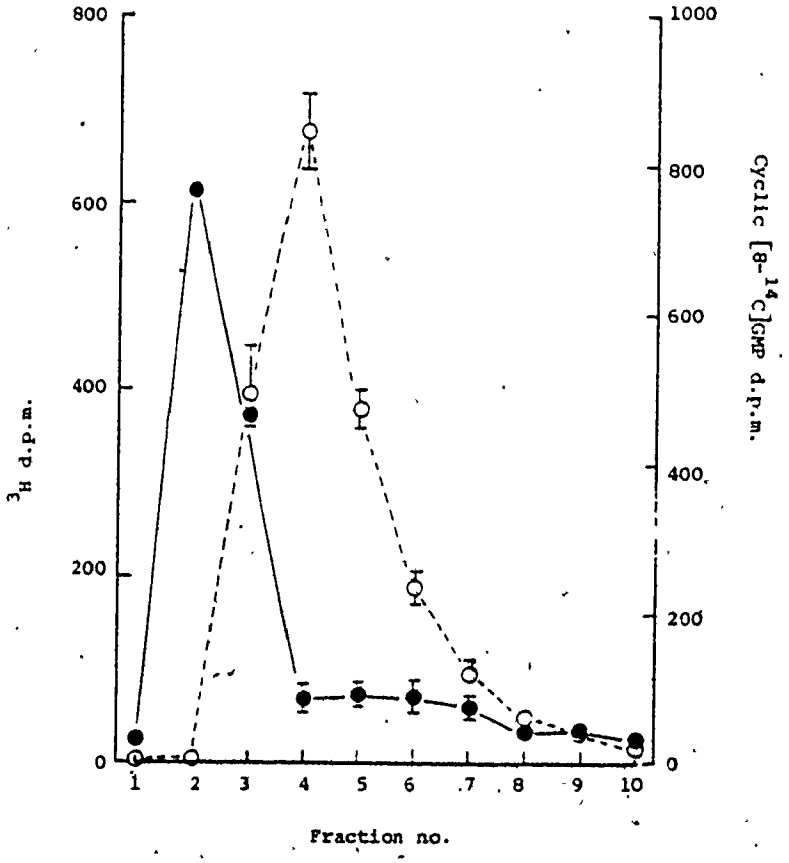


Table 2.2.4.2. *Effect of boiling assay mixtures and of freeze-drying eluted fractions on ^{3}H-blank values*

Complete assay mixtures containing 931,545 d.p.m. of 1.0 mM-[8-^{3}H]GTP were prepared as described in Section 2.2.3. Incubation blanks (A) were prepared by the addition of 25 µl of 150 mM-Tris/HCl, pH 7.4 to assay mixtures in place of platelet lysate. After a 20 min incubation period at 30° C, 100 µl of a solution containing 2691 d.p.m. of cyclic [8-^{14}C]GMP and 50 mM-EDTA, pH 7.4 was added to each assay tube. The samples were transferred to an ice bath and 50 mM-Tris/HCl, pH 7.6 was added to give a final volume of 1.0 ml. The samples were applied to alumina columns as described in Section 2.2.4. Cyclic [8-^{14}C]GMP and blank-^{3}H were eluted with 50 mM-Tris/HCl, pH 7.6 in individual 1.0 ml fractions which were collected into scintillation vials and counted for ^{3}H and ^{14}C radioactivity. Zero-incubation-time blanks (B & C) were prepared by the simultaneous addition of 25 µl of platelet lysate (6.0 mg of protein/ml) and 100 µl of a solution containing 2691 d.p.m. of cyclic [8-^{14}C]GMP and 50 mM-EDTA, pH 7.4 to each assay tube. The samples were quickly mixed and then boiled for 3 min. After boiling the samples were transferred to an ice bath and processed as described above with the exception that fractions from zero-incubation-blank "B" were freeze-dried prior to counting for ^{3}H and ^{14}C radioactivity. For all blanks, fraction 1 contained the sample. The values given are the means ± S.E.M. of triplicate determinations. For blanks A,B and C, 59%, 58% and 64%, respectively, of the total amount of cyclic [8-^{14}C]GMP applied to each column was collected in fractions 4, 5 and 6. For blank A, of the total amount of [8-^{3}H]GTP applied to each column, 0.152% (1416 d.p.m.) was collected in all 10 fractions and 0.024% (224 d.p.m.) in fractions 4,5 and 6; for blank B, 0.149% (1388 d.p.m.) was collected in all 10 fractions and 0.023% (214 d.p.m.) in fractions 4,5 and 6; and for blank C, 0.377% (3410 d.p.m.) was collected in all 10 fractions and 0.085%(792 d.p.m.) in fractions 4,5 and 6.

Treatment	Fraction (1 ml)	^{3}H d.p.m.	% of total [8-^{3}H] GTP
Blank A (unboiled) Incubation blank (no enzyme) (Fractions were not freeze-dried)	1	26 ± 6	0.003
	2	617 ± 3	0.066
	3	373 ± 9	0.040
	4	68 ± 13	0.007
	5	72 ± 12	0.008
	6	72 ± 18	0.008
	7	60 ± 12	0.006
	8	34 ± 4	0.006
	9	37 ± 5	0.004
	10	24 ± 2	0.003
Blank B (boiled) Zero-incubation-time blank (Fractions were freeze-dried)	1	23 ± 5	0.002
	2	651 ± 13	0.070
	3	343 ± 8	0.037
	4	89 ± 10	0.010
	5	79 ± 9	0.008
	6	54 ± 6	0.006
	7	48 ± 4	0.005
	8	45 ± 10	0.005
	9	52 ± 5	0.006
	10	28 ± 4	0.003
Blank C (boiled) Zero-incubation-time blank (Fractions were not freeze-dried)	1	14 ± 1	0.002
	2	1202 ± 28	0.129
	3	491 ± 15	0.053
	4	283 ± 12	0.030
	5	270 ± 11	0.029
	6	246 ± 14	0.026
	7	239 ± 2	0.026
	8	217 ± 13	0.023
	9	231 ± 14	0.025
	10	211 ± 9	0.023

Table 2.2.4.3. *Effect of purification of [8-^{3}H]GTP on the presence of ^{3}H-labeled impurities in eluates from alumina columns*

Alumina columns were prepared and eluted as described in Section 2.2.4. Approximately 1.18 x 10^5 d.p.m. of unpurified [8-^{3}H]GTP was applied to a column in a total volume of 1.0 ml. [8-^{3}H]GTP was purified by t.l.c. in two dimensions on cellulose, by using the solvents described by Haslam & McClenaghan (1974). Approximately 1.07 x 10^6 d.p.m. of this purified material was applied to a column in a total volume of 1.0 ml. The elution of [^{3}H]-labelled material is expressed in terms of ^{3}H-d.p.m./1.0 ml fraction and as a percentage of total [8-^{3}H]GTP applied to each column. All samples were freeze-dried prior to counting for ^{3}H radioactivity.

Substrate preparation	Fraction (1 ml)	^{3}H d.p.m.	% of total [8-^{3}H]GTP
Unpurified [8-^{3}H]GTP	1	86	0.07
	2	8214	6.96
	3	1705	1.45
	4	612	0.52
	5	123	0.10
	6	61	0.05
	7	45	0.04
	8	31	0.02
Purified [8-^{3}H]GTP	1	3	0
	2	490	0.05
	3	361	0.03
	4	52	0.005
	5	39	0.004
	6	35	0.003
	7	26	0.002
	8	30	0.003

Table 2.2.5.1. *Determination of the radiochemical purity of cyclic [^{3}H]GMP isolated from guanylate cyclase assays*

The following is a detailed description of the procedure outlined in Section 2.2.5. for the determination of the radiochemical purity of cyclic [^{3}H]GMP isolated from guanylate cyclase assays. After each step, a sample was counted for ^{3}H and ^{14}C radioactivity. The numbers in parenthesis identify each of these steps which also correspond to the table below. In this experiment, 859,950 d.p.m. of [8-^{3}H]GTP and 5387 d.p.m. of cyclic [8-^{14}C]GMP were added to each assay tube.

Six replicates of a typical guanylate cyclase assay (i.e. 4.62 ± 0.02 nmol of cyclic GMP formed/20 min per mg of protein per assay) were each applied to alumina columns and eluted as described in Section 2.2.4. The 3.0 ml collected from each of six columns were pooled and a 1.5 ml sample was counted for radioactivity prior to freeze-drying (1). The ratio of ^{3}H to ^{14}C of this sample was 1.20. One 3.0 ml sample of the pooled material was freeze-dried, reconstituted to 1.0 ml and counted for radioactivity (2). The ratio of ^{3}H to ^{14}C of this sample was 0.77. As shown in the table, freeze-drying the eluate from alumina columns decreased the ^{3}H d.p.m. in a 3.0 ml sample by about 1185 d.p.m. The recovery of cyclic [8-^{14}C]GMP from the alumina columns was 51%.

Two aliquots of 5.0 ml of the pooled eluate from the alumina columns (not freeze-dried) were chromatographed on anion exchange resin (Dowex AG 1-X2) and cyclic GMP was eluted as described by Haslam & McClenaghan (1974). The 18.0 ml collected from each of 2 columns were pooled and a 0.5 ml sample was counted for radioactivity prior to freeze-drying (3). The ratio of ^{3}H to ^{14}C in this sample was 0.83. (The radioactivity in the total 36 ml of this material corresponds to 10.0 ml of the pooled eluate from the alumina columns. The recovery of added cyclic [8-^{14}C]GMP from the anion exchange resin was 82%). The remaining 35.5 ml sample was freeze-dried and reconstituted with 100 µl of 2.5 mM-cyclic GMP. A 10 µl sample of this material was counted for radioactivity (4), the ratio of ^{3}H to ^{14}C was 0.74. A 60 µl sample was incubated at 30°C with 60 µl of a reaction mixture containing (final concentrations): 0.05 unit cyclic nucleotide phosphodiesterase, 4 mg of bovine serum albumin/ml, 4 mM-$MgSO_4$, 0.25 mM-EDTA and 10 mM-*N*-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid (adjusted to pH 7.5 with NaOH). Aliquots (40 µl) of this reaction mixture (equivalent to 20 µl of the reconstituted anion exchange eluate) were taken at 0, 5 and 60 min which were boiled (to remove denatured protein); and the supernatant chromatographed on cellulose as described by Haslam & McClenaghan (1974). Cyclic GMP and product 5'-GMP were eluted and counted for ^{3}H and ^{14}C radioactivity (5,6,7). After 60 min, all cyclic GMP was converted to 5'-GMP by the cyclic nucleotide phosphodiesterase. The ratio of ^{3}H to ^{14}C in this 5'-GMP was the same as that of the freeze-dried eluates from the columns containing either alumina or anion-exchange resin (i.e. 0.77), indicated that the standard assay procedure isolated radiochemically pure cyclic[^{3}H]GMP.

Step number	Sample	^{3}H d.p.m.	^{14}C d.p.m.	Ratio of ^{3}H to ^{14}C d.p.m.
1	*1.5 ml of pooled eluate from alumina columns*	1633	1363	1.20
	Radioactivity in 3.0 ml	3266	2726	1.20
	Radioactivity in 10.0 ml	10887	9089	1.20
2	*Radioactivity in freeze-dried 3 ml sample from alumina columns*	2081	2701	0.77
3	*0.5 ml of pooled eluate from anion exchange resin*	86	103	0.84
	Radioactivity in 36 ml	6192	7416	0.84
4	*Radioactivity in a 10µl sample of 100µl of reconstituted eluate from anion exchange resin*	553	749	0.74
	Radioactivity in 20µl	1106	1498	0.74
	Radioactivity in 100µl	5530	7490	0.74
	Samples equivalent to 20µl of reconstituted eluate from anion exchange resin from cyclic nucleotide phosphodiesterase incubations:			
5	*Zero-time incubation*			
	Radioactivity in cyclic GMP	755	961	0.79
	Radioactivity in 5'-GMP	12	0	-
6	*5-minute incubation*			
	Radioactivity in cyclic GMP	407	533	0.76
	Radioactivity in 5'-GMP	246	314	0.78
7	*60-minute incubation*			
	Radioactivity in cyclic GMP	7	0	-
	Radioactivity in 5'-GMP	725	946	0.77

Table 2.2.6.1. *Determination of cyclic GMP phosphodiesterase activity present in guanylate cyclase assays*

Cyclic GMP phosphodiesterase activity was assayed as described in Section 2.2.6. and is expressed as the percentage of cyclic [8-^{3}H]GMP broken down after 20 min of incubation at 30°C. The values given for cyclic GMP phosphodiesterase activity are the means ± S.E.M. of three separate experiments in which the lysates contained 6.0 mg of protein/ml.

Concn. of protein in assay (mg)	Phosphodiesterase activity (% of cyclic [8-^{3}H GMP] broken down after 20 min incubation)
0.06	2.1 ± 1.1
0.12	2.2 ± 0.4
0.30	4.3 ± 1.4
0.60	6.7 ± 0.5

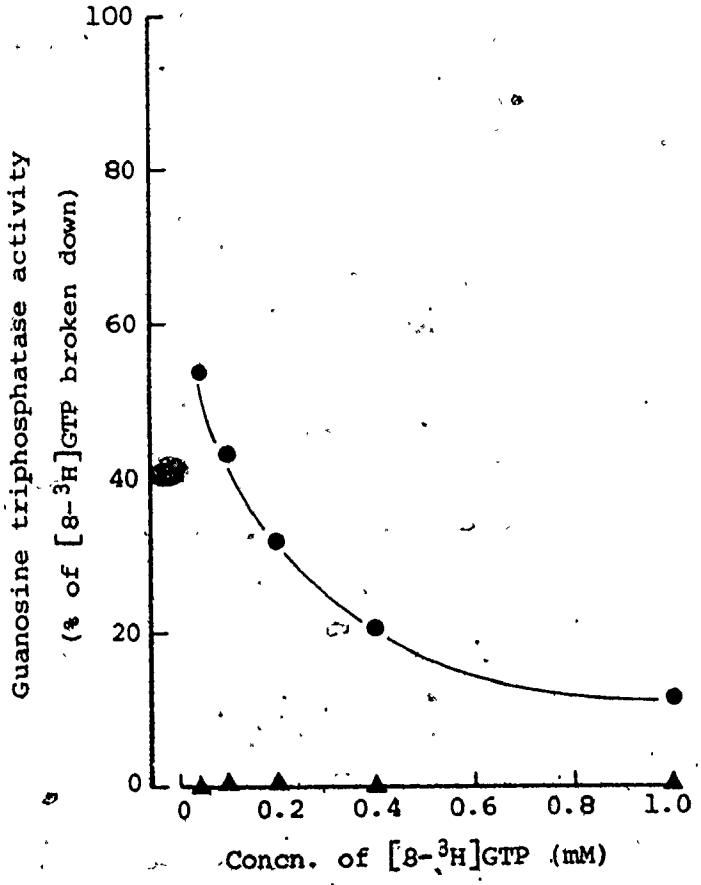
Table 2.2.6.2. *Determination of guanosine triphosphatase activity present in guanylate cyclase assays*

Guanosine triphosphatase activity was assayed as described in Section 2.2.6. In both experiments, the concentration of protein in the platelet lysate was 6.0 mg/ml. The concentration of protein in the assay was varied by the addition of 10, 20, 50 and 100 µl of platelet lysate to the assay tubes. The values given are single determinations. The guanylate cyclase activity assayed with 0.30 mg of protein was 6.60 ± 0.20 nmol of cyclic GMP formed/20 min per mg of protein in Experiment 1 and 4.90 ± 0.40 nmol of cyclic GMP formed/20 min per mg of protein in Experiment 2 (mean ± S.E.M. of triplicate determinations).

Experiment no.	Concn. of protein in assay (mg)	Guanosine triphosphatase activity (% of added [8- H]GTP broken down during 20 min incubation)
1	0.06	9.8
	0.12	13.1
	0.30	24.1
	0.60	45.6
2	0.06	6.6
	0.12	10.6
	0.30	22.1
	0.60	42.3

Fig. 2.2.6.1. *Effect of a phosphocreatine/creatine phosphokinase GTP-regenerating system on the breakdown of [8-^{3}H]GTP during a 10 min incubation at 30°C*

Guanosine triphosphatase activity was assayed as described in Section 2.2.6. In this experiment, the concentration of [8-^{3}H]GTP in the assay mixture ranged from 0.05 to 1.0 mM. The breakdown of added [8-^{3}H]GTP was assayed in the presence (▲) or absence (●) of a phosphocreatine/creatine phosphokinase GTP-regenerating system (see Section 2.2.3.). The values given are single determinations. The platelet lysate contained 4.4 mg of protein/ml and 50 µl was added to each assay tube.



Chapter 3

Properties and Subcellular Distribution of Platelet Guanylate Cyclase

3.1. Results: Subcellular distribution

The apparent subcellular distribution of platelet guanylate cyclase activity varied with the tonicity of the lysis medium. As indicated in Table 3.1.1., between 60 and 75% of the total activity was found in the 100 000*g* supernatant fraction when the cells were lysed by freezing and thawing in an isotonic medium (150 mM-Tris/HCl, pH 7.4). Under these conditions, the specific activity of guanylate cyclase in the supernatant fraction was somewhat greater than in the particulate fraction. However, when the platelets were frozen and thawed in an hypotonic medium (60 mM-Tris/HCl, pH 7.4), about 90% of the enzyme was soluble, and the specific activity of the supernatant was about eight-fold greater than that of the particulate fraction. The tonicity of the assay mixture had no effect on the apparent subcellular distribution of guanylate cyclase activity (Table 3.1.2.); however, the activity in the supernatant and particulate fractions prepared from both hypo- and isotonically freeze-thawed platelet lysates was inhibited on average by about 35% when assays were carried out in an isotonic medium. The distribution of guanylate cyclase activity closely paralleled that of the soluble enzyme marker, lactate dehydrogenase, in both isotonic and hypotonic preparations (Table 3.1.1.). Treatment of the supernatant and the particulate fractions with Lubrol PX (1.0%, w/v) did not change the relative distribution of guanylate cyclase activity (Table 3.1.1. and Fig. 4.4.2.), indicating the absence of latent particle-bound enzyme. These results show that the only particle-bound guanylate cyclase found was due to contamination of particulate fractions with soluble enzyme.

In spite of the fact that platelet guanylate cyclase appears to be a predominantly soluble enzyme, whole platelet lysate was studied in the present investigation primarily because of the possibility that particulate components as well as soluble intermediary factors may be involved in an indirect mechanism of activation. However, the present results do not completely eliminate the possibility that the soluble form of the enzyme may originate from cell membranes but is artifactually rendered soluble by physiological processes or mechanical forces of cell disruption (Goldberg *et al.*, 1973*b*; Limbird & Lefkowitz, 1975; Neer & Sukiennik, 1975). To establish beyond doubt that the guanylate cyclase activity present in the particulate fraction of isotonically lysed platelets is attributable to contamination with soluble enzyme, several properties of these two enzyme preparations were compared.

Table 3.1.1. *Effect of freezing and thawing of platelet suspended in isotonic and hypotonic media on the subcellular distribution of guanylate cyclase analyzed in the presence and absence of Lubrol PX*

A suspension of washed platelets was prepared as described in Section 2.2.1. This suspension was divided into two portions which were centrifuged as described in Section 2.2.1. and the platelet pellets were then resuspended in either isotonic medium (150 mM-Tris/HCl, pH 7.4) or hypotonic medium (60 mM-Tris/HCl, pH 7.4). The platelet suspensions were then lysed by freezing and thawing as described in Section 2.2.2. The lysates were centrifuged at 100 000*g* for two hours at 4°C and each particulate fraction was resuspended to the volume of the corresponding supernatant with the original buffer. A portion of the supernatant and particulate fractions from both lysate preparations was treated with Lubrol PX. The protein concentrations of the enzyme preparations were as follows: isotonic supernatant fraction, 2.2 mg/ml; isotonic particulate fraction, 2.2 mg/ml; hypotonic supernatant fraction, 2.6 mg/ml; and hypotonic particulate fraction 2.0 mg/ml. In each case, 50 µl aliquots of the enzyme preparation were assayed. The final buffer concentration in the assay mixtures was adjusted to be 60 mM-Tris/HCl. pH 7.4 in all cases. Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Lactate dehydrogenase activity was assayed as described in Section 2.2.7.2. 'Total activity' refers to the sum of the activities in the supernatant and particulate fractions.

Lysis medium	Enzyme preparation	Lubrol PX concn. (%, w/v)	Guanylate cyclase activity		Distribution of lactate dehydrogenase (% of total activity)
			nmol cyclic GMP/ 20 min per mg protein	% of total activity	
Isotonic	Supernatant	0	5.22 ± 0.17	61	67
		1.0	16.15 ± 0.45	67	-
	Particulate	0	3.25 ± 0.19	39	33
		1.0	7.82 ± 0.08	33	-
Hypotonic	Supernatant	0	5.16 ± 0.18	92	89
		1.0	18.34 ± 0.49	91	-
	Particulate	0	0.60 ± 0.03	8	11
		1.0	2.56 ± 0.12	9	-

Table 3.1.2. *Effect of tonicity of the assay mixture on the guanylate cyclase activities in supernatant and particulate fractions prepared from platelets lysed in an isotonic or hypotonic medium*

A suspension of washed platelets was prepared as described in Section 2.2.1. This suspension was divided into two portions which were centrifuged as described in Section 2.2.1. and the platelet pellets were then resuspended in either isotonic medium (150 mM-Tris/HCl, pH 7.4) or hypotonic medium (60 mM-Tris/HCl, pH 7.4). The platelet suspensions were then lysed by freezing and thawing as described in Section 2.2.2. The lysates were centrifuged at 48 000*g* for 30 min at 4°C and each particulate fraction was resuspended to the volume of the corresponding supernatant with the original buffer. The protein concentrations of the enzyme preparations were as follows: isotonic supernatant fraction, 3.5 mg/ml; isotonic particulate fraction, 2.5 mg/ml; hypotonic supernatant fraction, 4.3 mg/ml; and, hypotonic particulate fraction, 2.4 mg/ml. In each case, 50 µl aliquots of the enzyme preparation were assayed. The final buffer concentration in the assay mixtures was adjusted to be either 60 mM-Tris/HCl, pH 7.4 (hypotonic) or 150 mM-Tris/HCl, pH 7.4 (isotonic). Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. 'Total activity' refers to the sum of the activities in the supernatant and particulate fractions.

Assay medium	Lysis medium	Enzyme preparation	Guanylate cyclase activity	
			nmol of cyclic GMP/ 20 min per mg of protein	% of total activity
Hypotonic	Isotonic	Supernatant	7.77 ± 0.06	67
		Particulate	3.83 ± 0.26	33
	Hypotonic	Supernatant	5.14 ± 0.08	88
		Particulate	0.07 ± 0.13	12
Isotonic	Isotonic	Supernatant	4.78 ± 0.16	66
		Particulate	2.46 ± 0.15	34
	Hypotonic	Supernatant	3.86 ± 0.14	90
		Particulate	0.43 ± 0.03	10

3.2. Results: Bivalent cation requirements

The maximum guanylate cyclase activity of the platelet lysate was observed in the presence of 4.0 mM-$MnCl_2$ when the GTP concentration was 1.0 mM (Fig. 3.2.1.). Both $MgCl_2$ and $CaCl_2$ were found to be poor substitutes for $MnCl_2$. Only 20 ± 4% (mean ± S.E.M. of six experiments) of maximum activity was expressed with $MgCl_2$ at an optimal concentration of 10.0 mM, whereas almost no activity was measurable with 4.0 mM-$CaCl_2$ (0.7 ± 0.5% of maximum activity, mean ± S.E.M. of three experiments)(Table 3.2.1.). In the presence of lower concentrations of $MnCl_2$ (0.1 or 1.0 mM) together with either $MgCl_2$ (10.0 mM) or $CaCl_2$ (4.0 mM), a synergistic stimulation of guanylate cyclase activity was observed (Table 3.2.1. and 3.2.2.). As shown in Fig. 3.2.2., the guanylate cyclase activity of platelet lysate assayed in the presence of different concentrations of $MgCl_2$ was markedly enhanced by the addition of 1.0 mM-$MnCl_2$. Maximum synergistic stimulation of activity was observed with a concentration of 10.0 mM-$MgCl_2$. In four experiments, 68 ± 5% (mean ± S.E.M.) of maximum activity was expressed with 1.0 mM-$MnCl_2$ and 10.0 mM-$MgCl_2$ (Table 3.2.1.). As shown in Fig. 3.2.3., 1.0 mM-$MnCl_2$ markedly stimulated enzyme activity when assayed in the presence of several concentrations of $CaCl_2$. The greatest stimulation was expressed with $CaCl_2$ at an optimal concentration of 4.0 mM. In three experiments, 65 ± 2% (mean ± S.E.M.) of maximum activity was observed with 1.0 mM-$MnCl_2$ and 4.0 mM-$CaCl_2$ (Table 3.2.1.).

In the absence of added $MnCl_2$, 4.0 mM-$CaCl_2$ had no effect on the guanylate cyclase activity of platelet lysate assayed in the presence of

10.0 mM-$MgCl_2$ (Table 3.2.2.). However, as shown in Table 3.2.3., low concentrations of Ca^{2+} (0.1 to 100 μM) significantly stimulated guanylate cyclase activity when assayed with 10.0 mM-$MgCl_2$ whereas higher concentrations of Ca^{2+} (0.5 to 4.0 mM) had no significant effect.

As observed with platelet lysate, at least 4.0 mM-$MnCl_2$ was required for the expression of maximum guanylate cyclase activity in both the supernatant and particulate fractions obtained after isotonic lysis of platelets (Fig. 3.2.4.). Table 3.2.4. shows that for both enzyme preparations, less than 20% of maximum activity was expressed with 10.0 mM-$MgCl_2$; whereas almost no activity was measurable with 4.0 mM-$CaCl_2$. A synergistic stimulation of guanylate cyclase activity by low concentrations of $MnCl_2$ together with 10.0 mM-$MgCl_2$ or 4.0 mM-$CaCl_2$ was also observed with both platelet fractions (Table 3.2.4.). Thus no significant difference was found in the bivalent cation requirements of these two enzyme preparations.

Table 3.2.1. *Summary of the effects of bivalent cations on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. The concentration of protein in the platelet lysates of the 10 experiments summarized below was 6.2 ± 0.5 mg/ml. The guanylate cyclase activity assayed with 4 mM-$MnCl_2$ was 5.26 ± 0.62 nmol of cyclic GMP formed/20 min per mg of protein (mean ± S.E.M. of 10 experiments). The values given below are the means ± S.E.M. of the percent activity observed with 4 mM-$MnCl_2$ in the number of separate experiments indicated in parentheses.

Bivalent cation concentration (mM)			Guanylate cyclase activity
$MnCl_2$	$MgCl_2$	$CaCl_2$	(% of activity with 4 mM-$MnCl_2$)
1.0	-	-	31.0 ± 4.0 (6)
-	10.0	-	20.0 ± 4.0 (6)
1.0	10.0	-	68.0 ± 5.0 (4)
-	-	4.0	0.7 ± 0.5 (3)
1.0	-	4.0	65.0 ± 2.0 (3)

Fig. 3.2.1. *Effect of increasing concentrations of $MnCl_2$ on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. The protein concentration of the platelet lysate was 8.3 mg/ml and 50 µl aliquots were assayed. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated the S.E.M. are within the limits of the symbols. The guanylate cyclase activity observed with 4.0 mM-$MnCl_2$ was 2.74 ± 0.05 nmol of cyclic GMP formed/20 min per mg of protein.

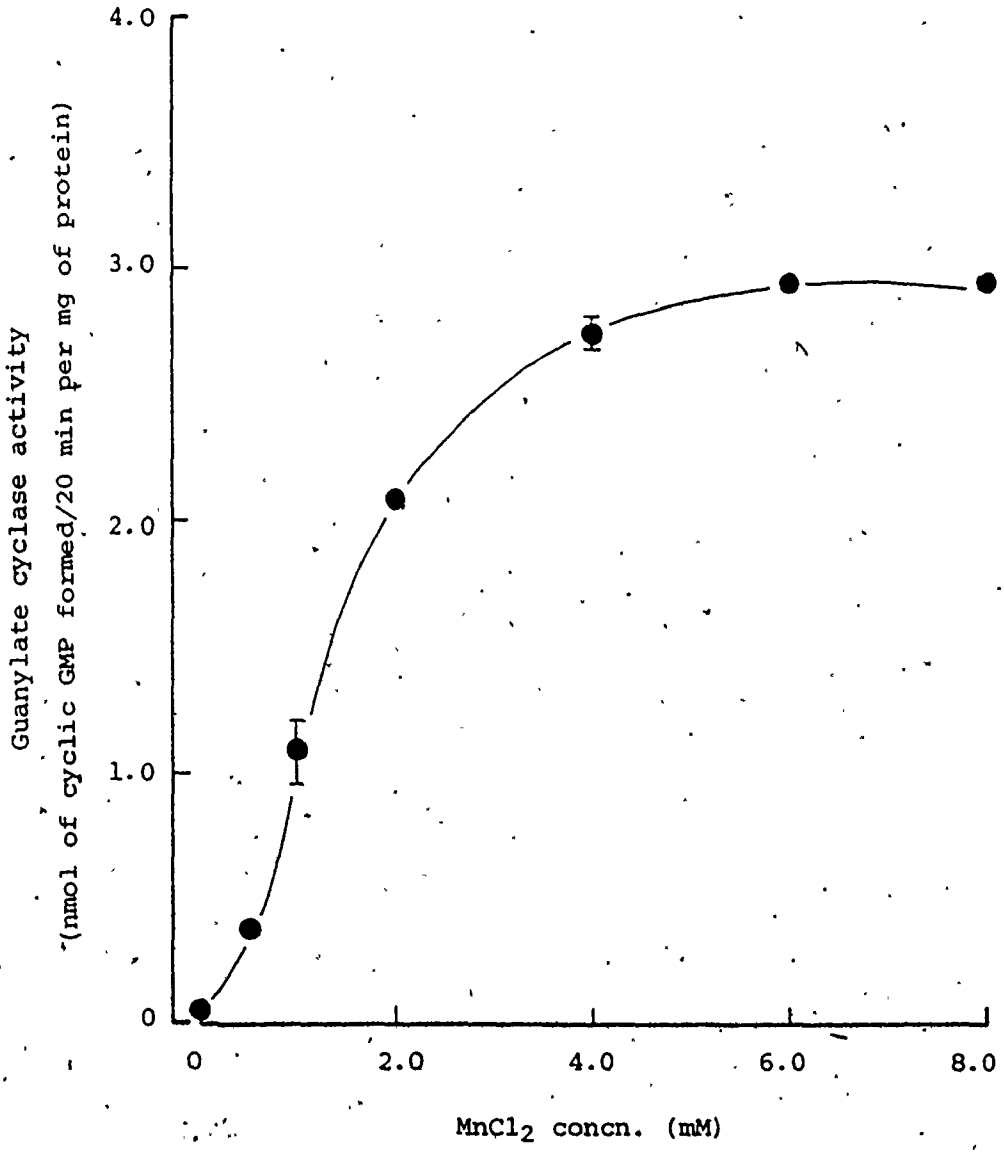


Fig. 3.2.2. *Effect of 1.0 mM-$MnCl_2$ on the guanylate cyclase activity of platelet lysate assayed in the presence of increasing concentrations of $MgCl_2$*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. The protein concentration of the platelet lysate was 8.0 mg/ml and 50 µl aliquots were assayed. The values given are the means of duplicate determinations. Guanylate cyclase activity is expressed in terms of the percentage of activity observed with 4.0 mM-$MnCl_2$ and 0, 1.0, 4.0 and 10.0 mM-$MgCl_2$ (1.82 ± 0.02 nmol of cyclic GMP formed/20 min per mg of protein) (mean ± S.E.M. of 8 values). Guanylate cyclase activity assayed in the absence of $MnCl_2$ (○); activity assayed in the presence of 1.0 mM-$MnCl_2$ (●).

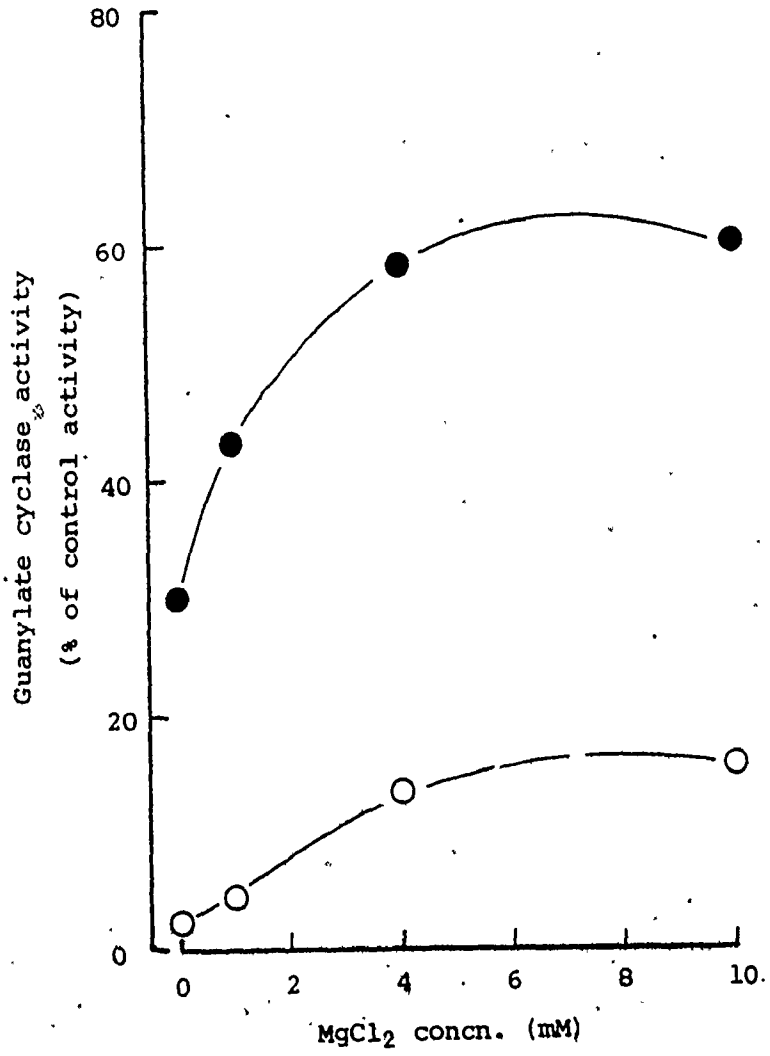


Fig. 3.2.3. *Effect of 1.0 mM-$MnCl_2$ on the guanylate cyclase activity of platelet lysate assayed in the presence of increasing concentrations of $CaCl_2$*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. The protein concentration of the platelet lysate was 5.2 mg/ml and 50 µl aliquots were assayed. The values given are the means of duplicate determinations. Guanylate cyclase activity is expressed in terms of the percentage of activity observed with 4.0 mM-$MnCl_2$ and 0, 1.0, 2.0, and 4.0 mM-$CaCl_2$ (4.04 ± 0.01 nmol of cyclic GMP formed/20 min per mg of protein)(mean ± S.E.M. of 8 values). Guanylate cyclase activity assayed in the absence of $MnCl_2$ (○); activity assayed in the presence of 1.0 mM-$MnCl_2$ (●).

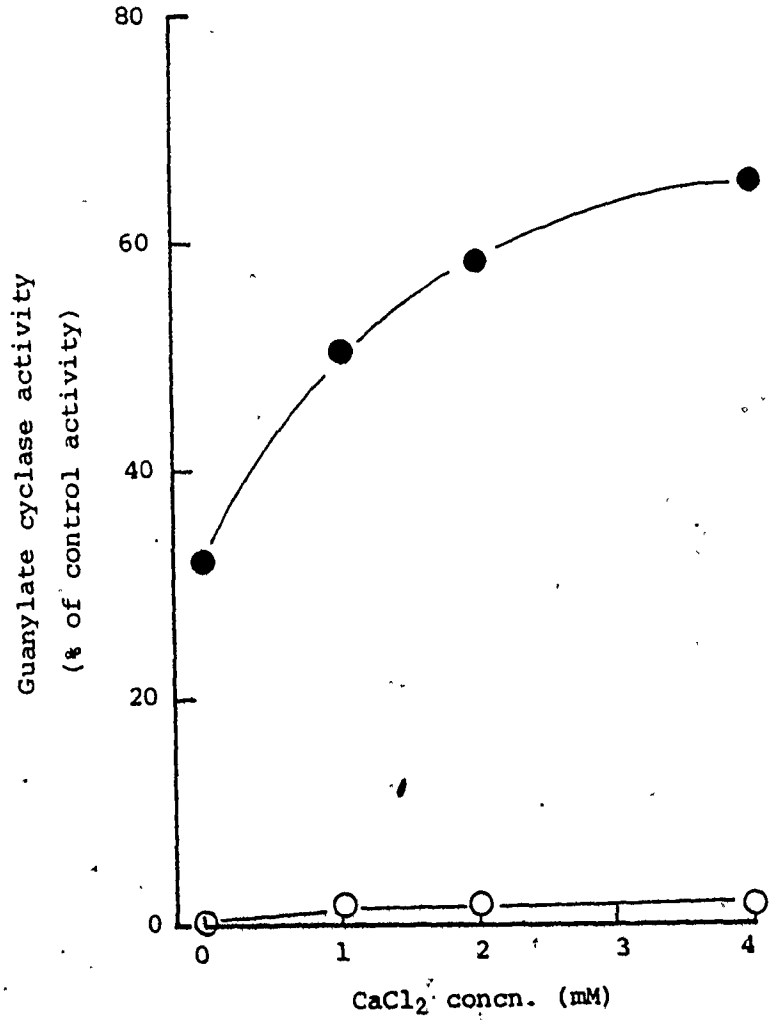


Table 3.2.2. *Effects of bivalent cations on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. The protein concentration of the lysate was 3.5 mg/ml; and 50 µl aliquots were assayed. The values given are the means ± S.E.M. of triplicate determinations.

Bivalent cation concentration (mM)			Guanylate cyclase activity	
$MnCl_2$	$MgCl_2$	$CaCl_2$	nmol of cyclic GMP/ 20 min per mg of protein	% of activity with 4 mM MnCl
4.0	0	0	8.40 ± 0.22	100
4.0	10.0	0	9.53 ± 0.15	113
1.0	0	0	3.03 ± 0.23	36
1.0	10.0	0	6.28 ± 0.13	75
1.0	0	4.0	5.07 ± 0.11	60
1.0	10.0	4.0	7.42 ± 0.25	88
0.1	0	0	0.14 ± 0.06	2
0.1	10.0	0	1.90 ± 0.17	23
0.1	0	4.0	0.73 ± 0.01	9
0.1	10.0	4.0	1.04 ± 0.09	12
0	10.0	0	1.26 ± 0.08	15
0	0	4.0	0.16 ± 0.02	2
0	10.0	4.0	0.83 ± 0.09	10

Table 3.2.3. *Effect of $CaCl_2$ on the guanylate cyclase activity of platelet lysate assayed in the presence of 10.0 mM-$MgCl_2$ and 1.0 mM-EGTA*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP regenerating system was included in the assay mixtures. In these experiments, guanylate cyclase activity was assayed with 10.0 mM-$MgCl_2$ in place of 4.0 mM-$MnCl_2$ in order to simulate physiological conditions. $CaCl_2$ (0.2 to 5.0 mM) was added to assay mixtures containing 1.0 mM-EGTA in order to regulate the concentration of free calcium. The concentration of free calcium in the assay mixtures was calculated by the method described by Portzehl *et al.* (1964). Guanylate cyclase activity is expressed as the percent stimulation of enzyme activity by free calcium. The values given are the means ± S.E.M. of the percent stimulations observed in three different platelet lysate preparations. In each experiment, assays were carried out in triplicate. The protein concentration of the platelet lysates and the maximum guanylate cyclase activities (*i.e.* with 4.0 mM-$MnCl_2$) in each of the experiments summarized below were as follows: (a)Exp. 1: 4.4 mg of protein/ml; 6.0 ± 0.15 nmol of cyclic GMP formed/20 min per mg of protein; (b)Exp. 2: 6.2 mg of protein/ml; 4.30 ± 0.08 nmol of cyclic GMP formed/20 min per mg of protein; (c)Exp.3: 5.0 mg of protein/ml; 5.69 ± 0.02 nmol of cyclic GMP formed/20 min per mg of protein. In each case, 50 µl aliquots of enzyme preparation were assayed. The significance of the effect of $CaCl_2$ on guanylate cyclase activity was determined by paired *t* tests. Statistics were calculated on the basis of the percent stimulation of guanylate cyclase activity. NS indicates values that are not statistically significant.

Concn. of $CaCl_2$ (mM)	Approx. concn. of Ca^{2+} (µM)	Guanylate cyclase activity (% stimulation by Ca^{2+})	Level of significance ($2P<$)
None	None	0	–
0.2	0.033	0 ± 0.9	NS
0.5	0.132	21.3 ± 2.0	0.01
0.9	1.190	44.0 ± 2.1	0.002
1.1	100.0	54.0 ± 6.2	0.02
1.5	500.0	47.0 ± 15.7	NS
5.0	4000.0	11.3 ± 5.3	NS

Fig. 3.2.4. *Effect of increasing concentrations of $MnCl_2$ on the guanylate cyclase activity in supernatant and particulate fractions of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction (●) was 4.6 mg/ml and that of the particulate fraction (▲) was 4.8 mg/ml. In each case, 50 µl aliquots were assayed. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

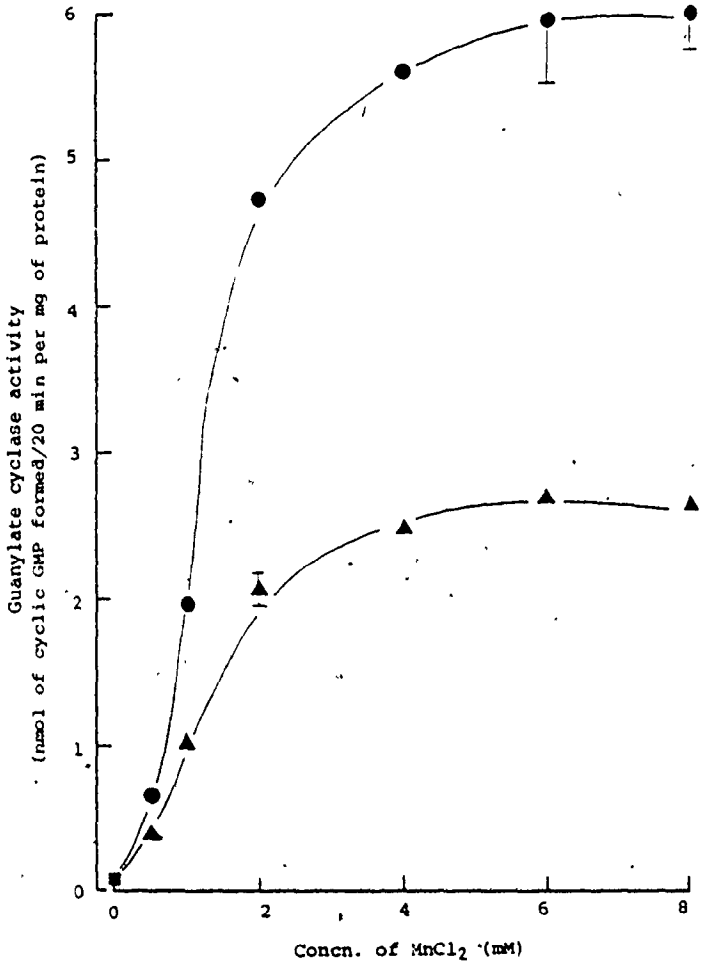


Table 3.2.4. *Effects of bivalent cations on the guanylate cyclase activities in supernatant and particulate fractions of platelet lysate.*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP regenerating system was included in the assay mixtures. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction was 2.6 mg/ml and that of the particulate fraction was 2.0 mg/ml. In each case, 50 µl aliquots of the enzyme preparation were assayed. The values given are the means ± S.E.M. of triplicate determinations.

Bivalent cation concentration (mM)			Guanylate cyclase activity	
$MnCl_2$	$MgCl_2$	$CaCl_2$	nmol of cyclic GMP/ 20 min per mg of protein	% of activity with 4.0 mM $MnCl_2$
A. Supernatant fraction				
4.0	0	0	14.53 ± 0.82	100
4.0	10.0	0	16.12 ± 0.59	111
1.0	0	0	5.15 ± 0.24	35
1.0	10.0	0	11.83 ± 0.39	81
1.0	0	4.0	8.08 ± 0.38	56
1.0	10.0	4.0	11.56 ± 0.64	80
0.1	0	0	0.19 ± 0.05	1
0.1	10.0	0	3.35 ± 0.23	23
0.1	0	4.0	0.99 ± 0.02	7
0.1	10.0	4.0	1.43 ± 0.09	10
0	10.0	0	1.75 ± 0.08	12
0	0	4.0	0.47 ± 0.29	3
0	10.0	4.0	1.00 ± 0.07	7
B. Particulate fraction				
4.0	0	0	2.38 ± 0.09	100
4.0	10.0	0	3.00 ± 0.29	126
1.0	0	0	1.01 ± 0.09	42
1.0	10.0	0	1.89 ± 0.09	79
1.0	0	4.0	1.86 ± 0.12	78
1.0	10.0	4.0	1.84 ± 0.17	77
0.1	0	0	0.13 ± 0.01	5
0.1	10.0	0	0.70 ± 0.01	29
0.1	0	4.0	0.91 ± 0.18	38
0.1	10.0	4.0	1.38 ± 0.38	58
0	10.0	0	0.40 ± 0.16	17
0	0	4.0	0.13 ± 0.10	5
0	10.0	4.0	0.25 ± 0.09	11

3.3. Results: Inhibition of guanylate cyclase activity by factors present in the lysate

The average specific activity of guanylate cyclase measured with 10 or 20 µl of lysate prepared from platelet suspensions containing 100 mg wet weight of platelets/ml was about 10 nmol of cyclic GMP formed/20 min per mg of protein. However, when 50 or 100 µl of lysate was assayed, the specific activity was decreased on average by 30% and 60%, respectively (Fig. 3.3.1.). This effect was not due to either guanosine triphosphatase or cyclic GMP phosphodiesterase activity (see Section 2.2.6.).

Ultrafiltration of the lysate caused highly significant increases in the specific activity of guanylate cyclase in assays of 50 and 100 µl of enzyme, whereas no effect was observed with smaller volumes (Fig. 3.3.1.). Resuspension of ultrafiltered platelet lysate in ultrafiltrate substantially restored the decrease in specific activity observed with 50 or 100 µl of platelet lysate (Fig. 3.3.2.) which indicated that the stimulatory effect of ultrafiltration was not the result of activation of guanylate cyclase. Furthermore, addition of the ultrafiltrate, which contained no measurable protein, to the ultrafiltered lysate (Table 3.3.1.) or to the untreated lysate (Table 3.3.2.) clearly demonstrated the presence of low-molecular-weight inhibitory factors. As shown in Table 3.3.1., treatment of the ultrafiltrate with charcoal completely removed its inhibitory activity which suggested that the observed inhibitory effect might be attributable to platelet adenine nucleotides. Although the comparatively small decrease observed in the inhibitory activity of ultrafiltrate incubated with apyrase

(an average decrease of about 40% in two experiments)(Table 3.3.1.) could be explained by the fact that ATP and AMP inhibit the guanylate cyclase activity of platelet lysate by about the same extent, other data indicated that platelet adenine nucleotides could not completely account for the observed inhibitory effect. Measurement of the concentrations of adenine nucleotides in the lysate ultrafiltrates and of their inhibitory activity against platelet guanylate cyclase showed that only 51 ± 6% (mean ± S.E.M. of five experiments) of the inhibition of the guanylate cyclase activity of platelet lysate by ultrafiltrate could be attributed to adenine nucleotides (Table 3.3.2. and Fig. 3.3.3.). It was similarly found that the concentration of adenine nucleotides present in either the ultrafiltered lysate or platelet lysate was far too low to account for the inhibition of guanylate cyclase activity observed with large volumes of enzyme preparation in the assay mixture (Table 3.3.3.). These results indicate that in addition to adenine nucleotides, other low-molecular-weight factors present in the platelet lysate inhibit guanylate cyclase activity.

The inhibitory effect of ATP and of large volumes of platelet lysate in the assay was also studied in the presence of 10.0 mM-$MgCl_2$, instead of 4.0 mM-$MnCl_2$, to determine whether these effects occurred under more physiological ionic conditions. It was found that in the presence of $MgCl_2$, the inhibition of guanylate cyclase activity by ATP was less than half that observed with $MnCl_2$ (Fig. 3.3.4.); however, the activity of 50 or 100 µl of platelet lysate was decreased by nearly the same extent when assayed with either bivalent cation (Fig. 3.3.5.). Thus, platelet adenine nucleotides could account for no more than one quarter of the inhibition of guanylate cyclase activity observed with 50 or 100 µl of lysate in the

assay with $MgCl_2$ as the sole bivalent cation. These results suggest that under more physiological conditions, other low-molecular-weight factors present in the platelet lysate inhibit guanylate cyclase activity more potently than adenine nucleotides.

The observation that 5.0 mM-dithiothreitol decreased the guanylate cyclase activity of platelet lysate by 73 ± 2% (mean ± S.E.M. of four experiments)($2P<0.001$) suggested that platelet non-protein thiol together with endogenous adenine nucleotides, might fully account for the inhibition of enzyme activity by low-molecular-weight factors present in the lysate. The concentration of non-protein sulfhydryl groups in platelet lysates prepared from freshly drawn blood (see Section 2.2.1.) was found to be 12.6 ± 1.1 nmol/mg of protein (or 2.67 ± 0.14 nmol/10^8 platelets)(mean ± S.E.M. of four experiments). Thus, with 50 or 100 µl of platelet lysate containing 5.5 ± 0.8 mg of protein/ml (mean ± S.E.M. of nine experiments)(see Fig. 3.3.1.), the final concentration of non-protein thiol in the assay mixtures would be about 0.014 and 0.028 mM, respectively. However, these concentrations of glutathione were found to have no significant effect on guanylate cyclase activity. Moreover, with as much as 1.0 mM of added glutathione, the guanylate cyclase activity of platelet lysate was inhibited by only 9 and 21% respectively, in two experiments. These results indicate that platelet non-protein thiol (*i.e.* glutathione) does not inhibit guanylate cyclase activity under the conditions of the assay. Furthermore, although the concentration of non-protein sulfhydryl groups in lysates prepared from four to five-day old platelet concentrates (see Section 2.2.1.) was found to be about 65% lower than in lysates prepared from freshly drawn blood (*i.e.* 4.4 ± 0.4 nmol/mg of protein or 0.91 ± 0.04 nmol/10^8 platelets)

(mean ± S.E.M. of three experiments)(*e.g.* Table 4.1.3.), the guanylate cyclase activity of 50 or 100 µl of this lysate preparation was decreased on average by about 30 and 50%, respectively. These results lend further support to the conclusion that platelet non-protein thiol is not responsible for the observed inhibition of guanylate cyclase activity by ultrafilterable (*i.e.* low-molecular-weight) factors present in the lysate.

The specific activity of guanylate cyclase in the supernatant fraction was decreased to a somewhat greater extent than that in the particulate fraction when 50 or 100 µl of either enzyme preparation was assayed (Fig. 3.3.6.). This difference can more likely be attributed to the presence of a lower concentration of ultrafilterable inhibitory factors in the particulate fraction than to a decrease in the sensitivity of this enzyme preparation to inhibition by these factors, particularly in view of the finding that ATP and ADP inhibited the guanylate cyclase activity in both fractions by approximately the same extent (Fig. 3.3.7. and Table 5.3.1.).

Fig. 3.3.1. *Effect of ultrafiltration of platelet lysate on the specific activity of guanylate cyclase measured with different volumes of enzyme preparation in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysates were ultrafiltered as described in Section 2.2.2. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. In some of these experiments, a phosphocreatine/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures. Although protein concentrations in the lysates and in the ultrafiltered lysates varied in different experiments as indicated below, for statistical purposes paired data were grouped according to the volume of lysate added to the assays, and the significance of the effect of ultrafiltration was determined by paired *t* tests; $*2P<0.01$, $**2P<0.001$. Untreated lysate (●), ultrafiltered lysate (▲); results are from 9 experiments in which the untreated lysates contained 5.5 ± 0.8 mg of protein/ml (mean ± S.E.M.) and the ultrafiltered lysates contained 4.3 ± 0.6 mg of protein/ml (mean ± S.E.M.).

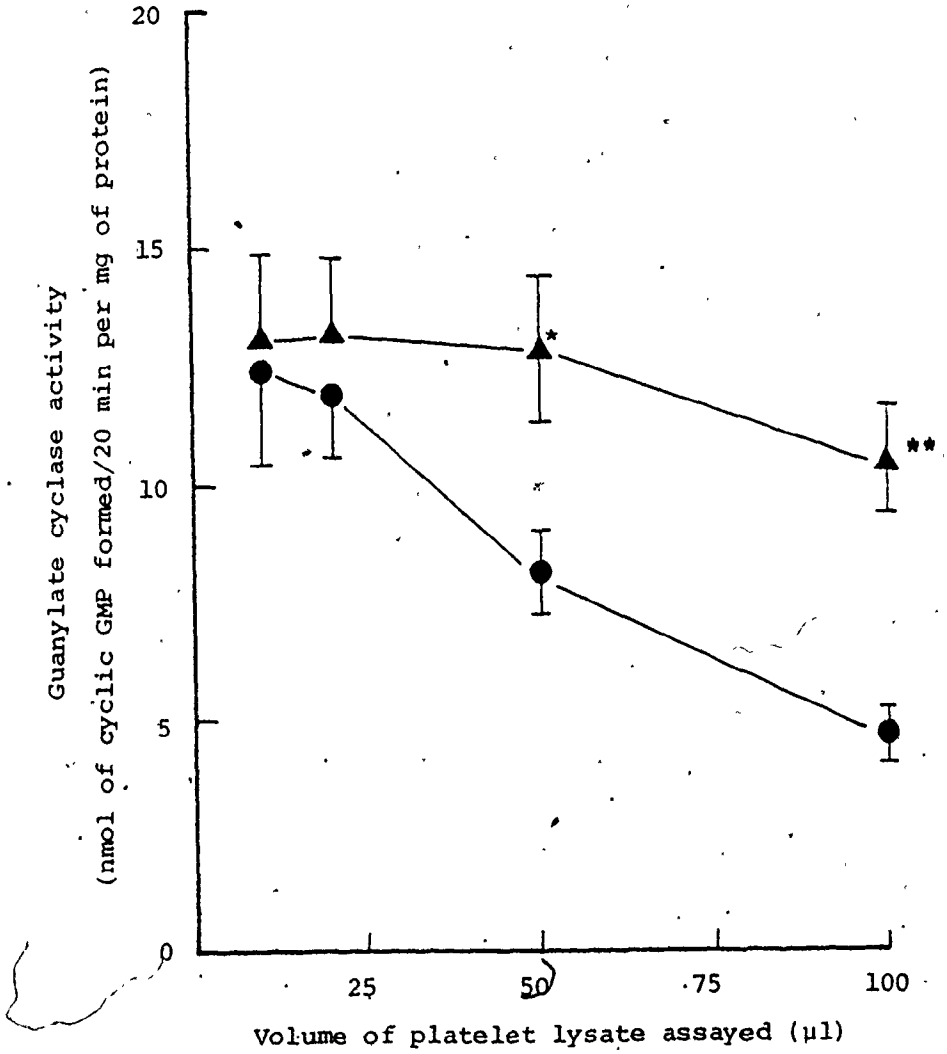


Fig. 3.3.2. *Effect of ultrafiltration and of resuspension of ultrafiltered lysate in ultrafiltrate on the specific activity of guanylate cyclase measured with different volumes of enzyme preparation in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. Platelet lysate was ultrafiltered as described in Section 2.2.2. The volume of ultrafiltrate obtained from each sample of ultrafiltered platelet lysate was measured and then one sample of the ultrafiltered lysate was resuspended in an equivalent volume of 150 mM-Tris/HCl, pH 7.4 while the other was resuspended in the ultrafiltrate. The concentration of protein was 6.0 mg/ml in the platelet lysate (●), 4.8 mg/ml in the ultrafiltered lysate (○) and 4.5 mg/ml in the ultrafiltered lysate resuspended in ultrafiltrate (▲). The data were grouped according to the volume of enzyme preparation added to the assays. The significance of the effect of ultrafiltration (a) or of resuspension of ultrafiltered lysate in ultrafiltrate (b) were determined by unpaired Student's *t* tests; *$2P<0.01$, **$2P<0.001$. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

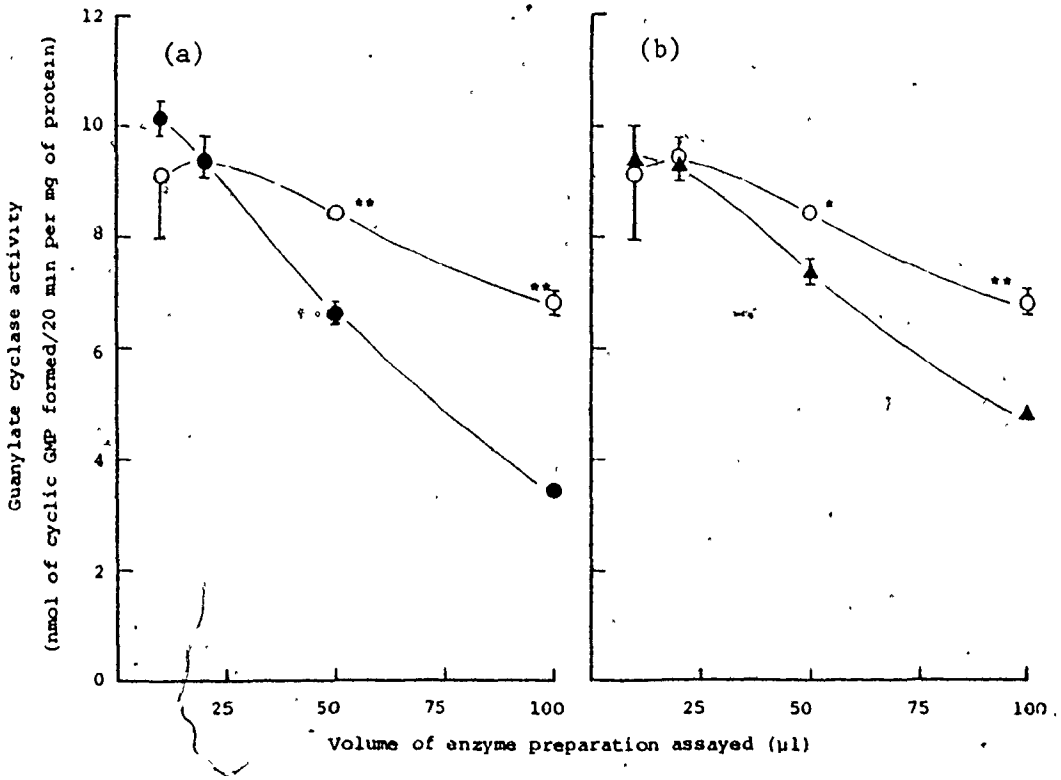


Table 3.3.1. *Effect of various treatments of ultrafiltrate from platelet lysate on its inhibitory activity*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. Platelet lysates were ultrafiltered as described in Section 2.2.2. In these experiments, 20 µl aliquots of ultrafiltered platelet lysate were incubated for 20 min in the presence of 80 µl of 150 mM-Tris/HCl, pH 7.4 or ultrafiltrate that had been subjected to the following treatments: (a) boiled for 3 min; (b) incubation with apyrase; and (c) charcoal absorption. The apyrase treatment was carried out by incubating 500 µl of ultrafiltrate with 50 µl of an apyrase suspension (see Section 2.1.2.) for 15 min at 37°C after which the sample was boiled for 3 min and centrifuged for 4 min at 12 000*g*. The supernatant was transferred to an ice bath until used. Charcoal absorption was carried out by thoroughly mixing 500 µl of ultrafiltrate with 10 mg of activated charcoal. The sample was then centrifuged for 4 min at 12 000*g* and the supernatant stored in an ice bath until used. The guanylate cyclase activities of the ultrafiltered platelet lysates assayed with 80 µl of 150 mM-Tris/HCl, pH 7.4 were 15.94 ± 0.38 nmol of cyclic GMP formed/20 min per mg of protein in Experiment 1; and, 9.08 ± 0.50 nmol of cyclic GMP formed/20 min per mg of protein in Experiment 2. The concentration of protein in the ultrafiltered platelet lysates was 4.0 mg/ml in Exp. 1 and 4.4 mg/ml in Exp. 2. The values given are the means ± S.E.M. of triplicate determinations. The significance of the effect of the treatment of the ultrafiltrate on its inhibitory activity was determined by unpaired *t* tests; *$2P<0.01$, **$2P<0.001$.

Exp. no.	Treatment of ultrafiltrate	Guanylate cyclase activity: nmol of cyclic GMP/20 min per mg of protein	Guanylate cyclase activity: % inhibition by ultrafiltrate
1	None	7.28 ± 0.25	54
	Boiled for 3 min	10.78 ± 0.25 *	32
	Incubation with apyrase	13.31 ± 0.25 **	16
	Charcoal absorption	17.28 ± 1.54 **	0
2	None	3.88 ± 0.04	57
	Boiled for 3 min	4.61 ± 0.06 *	49
	Incubation with apyrase	7.20 ± 0.50 **	21
	Charcoal absorption	9.11 ± 0.50 **	0

Table 3.3.2. *Inhibition of the guanylate cyclase activity of platelet lysate by lysate ultrafiltrates*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Ultrafiltrates were prepared from a portion of platelet lysate as described in Section 2.2.2. The extinction of the ultrafiltrate at 260 nm was measured and the final concentration of adenine nucleotides added to the assay mixtures was calculated. The expected inhibition of guanylate cyclase activity by an equivalent concentration of ATP was calculated from Fig. 3.2.3. In each experiment, 50 µl aliquots were assayed; the concentration of protein in the platelet lysates in each of the experiments shown were as follows: (1)9.2 mg/ml; (2)7.0 mg/ml; (3)5.2 mg/ml; (4)4.2 mg/ml; and, (5) 7.4 mg/ml. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations in Experiments 1, 2 and 3. The values given in Experiments 4 and 5 are the means of duplicate determinations.

Exp. no.	Volume of ultrafiltrate added (µl)	Final concn. of added nucleotides (mM)	Guanylate cyclase activity: nmol of cyclic GMP/ 20 min per mg of protein	Guanylate cyclase activity: % inhibition by ultrafiltrate	Inhibition of activity predicted from Fig. 3.2.3. (%)
1	0	-	3.67 ± 0.04	0	-
	50	0.06	2.30 ± 0.02	36	26
2	0	-	3.60 ± 0.07	0	-
	50	0.05	2.03 ± 0.07	44	16
3	0	-	4.90 ± 0.07	0	-
	50	0.05	3.48 ± 0.12	29	16
4	0	-	6.14	0	-
	50	0.06	3.67	40	20
5	0	-	2.73	0	-
	50	0.06	1.32	52	20

Fig. 3.3.3. *Inhibition of platelet guanylate cyclase activity by ATP*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The concentration of protein in the platelet lysate was 7.1 mg/ml and 50 µl aliquots were assayed. The guanylate cyclase activity of platelet lysate assayed in the absence of ATP was 4.45 ± 0.06 nmol of cyclic GMP formed/20 min per mg of protein (mean ± S.E.M. of triplicate determinations). Guanylate cyclase activity is expressed as the percent inhibition by ATP. The values given are the means of triplicate determinations.

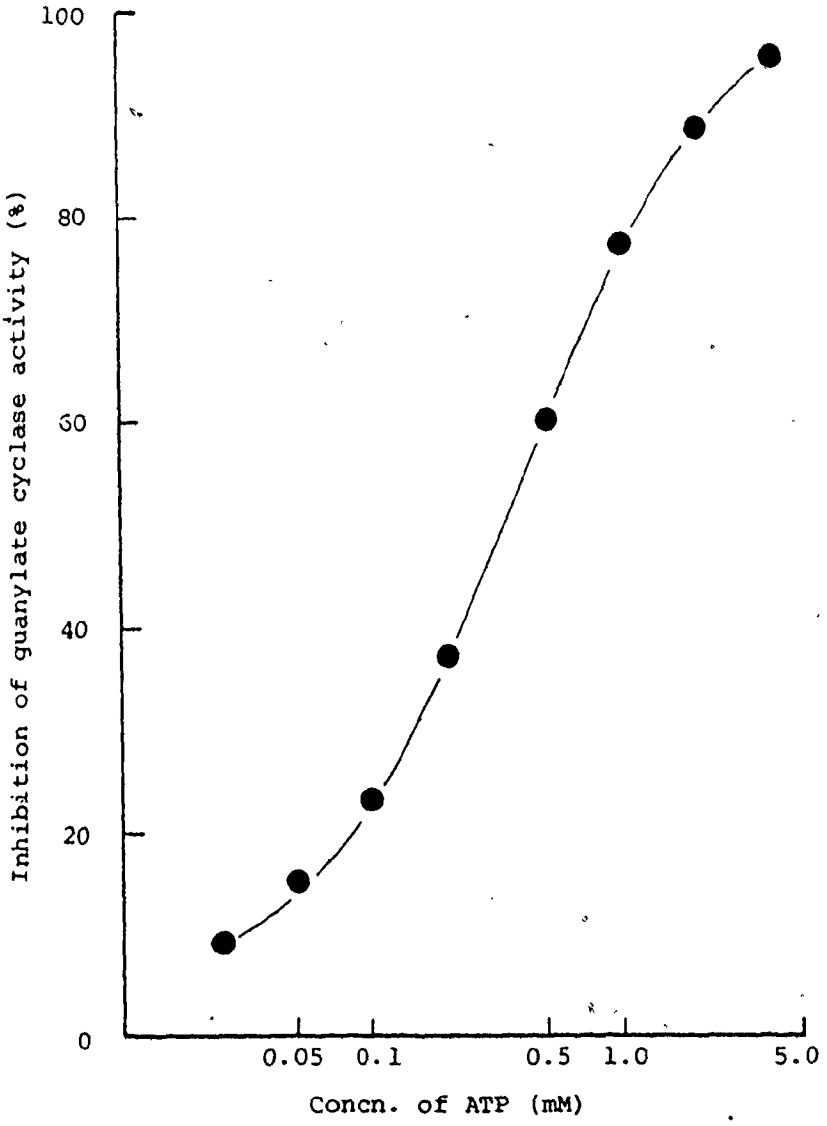


Table 3.3.3. *Concentration of total adenine nucleotides in platelet lysate and in ultrafiltered platelet lysate: Relationship to the inhibition of guanylate cyclase activity observed with increasing concentrations of protein*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was ultrafiltered as described in Section 2.2.2. and the concentration of adenine nucleotides in the platelet lysate and ultrafiltered lysate was assayed as described in Section 2.2.7.3. The concentration of protein in the platelet lysate was 2.8 mg/ml and 2.2 mg/ml in the ultrafiltered lysate. The total concentration of adenine nucleotides assayed (ATP, ADP & AMP) was 108.4 nmol/mg of protein in the platelet lysate and 52.5 nmol/mg of protein in the ultrafiltered lysate. Maximum guanylate cyclase activity was established as that activity observed with the lowest concentration of adenine nucleotides in the assay mixture (i.e. 0.009 mM). Guanylate cyclase activity is expressed as specific activity as well as in terms of the percent inhibition of activity compared to the theoretical maximum. The values given are the means ± S.E.M. of triplicate determinations.

Enzyme preparation	Concn. of protein assayed (mg)	Final concn. of total adenine nucleotides in each assay (mM)	Guanylate cyclase activity: nmol of cyclic GMP/ 20 min per mg of protein	Guanylate cyclase activity: % inhibition compared to maximum	Inhibition of activity predicted from Fig. 3.2.3. (%)
Ultrafiltered lysate	0.044	0.009	17.14 ± 1.23	0	0
	0.110	0.023	16.42 ± 0.23	4	6
	0.220	0.046	13.76 ± 0.28	20	14
Lysate	0.056	0.024	15.24 ± 0.27	12	6
	0.140	0.061	9.82 ± 0.29	49	16
	0.280	0.121	5.42 ± 0.11	68	26

Fig. 3.3.4. *Inhibition of the guanylate cyclase activity of platelet lysate by ATP: Comparison of the inhibitory effect of ATP when assayed in the presence of $MnCl_2$ or $MgCl_2$*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The concentration of protein in the platelet lysate was 7.9 mg/ml and 25 µl aliquots were assayed. In this experiment, 4.0 mM-$MnCl_2$ (●) or 10.0 mM-$MgCl_2$ (▲) was present in the assay mixtures. The guanylate cyclase activity of platelet lysate assayed in the absence of ATP was 6.10 ± 0.10 nmol of cyclic GMP formed/20 min per mg of protein with 4.0 mM-$MnCl_2$, and 2.10 nmol of cyclic GMP formed/20 min per mg of protein with 10.0 mM-$MgCl_2$ (mean ± S.E.M. of triplicate determinations). Guanylate cyclase activity is expressed as the percent inhibition by ATP. The values given are the means of triplicate determinations.

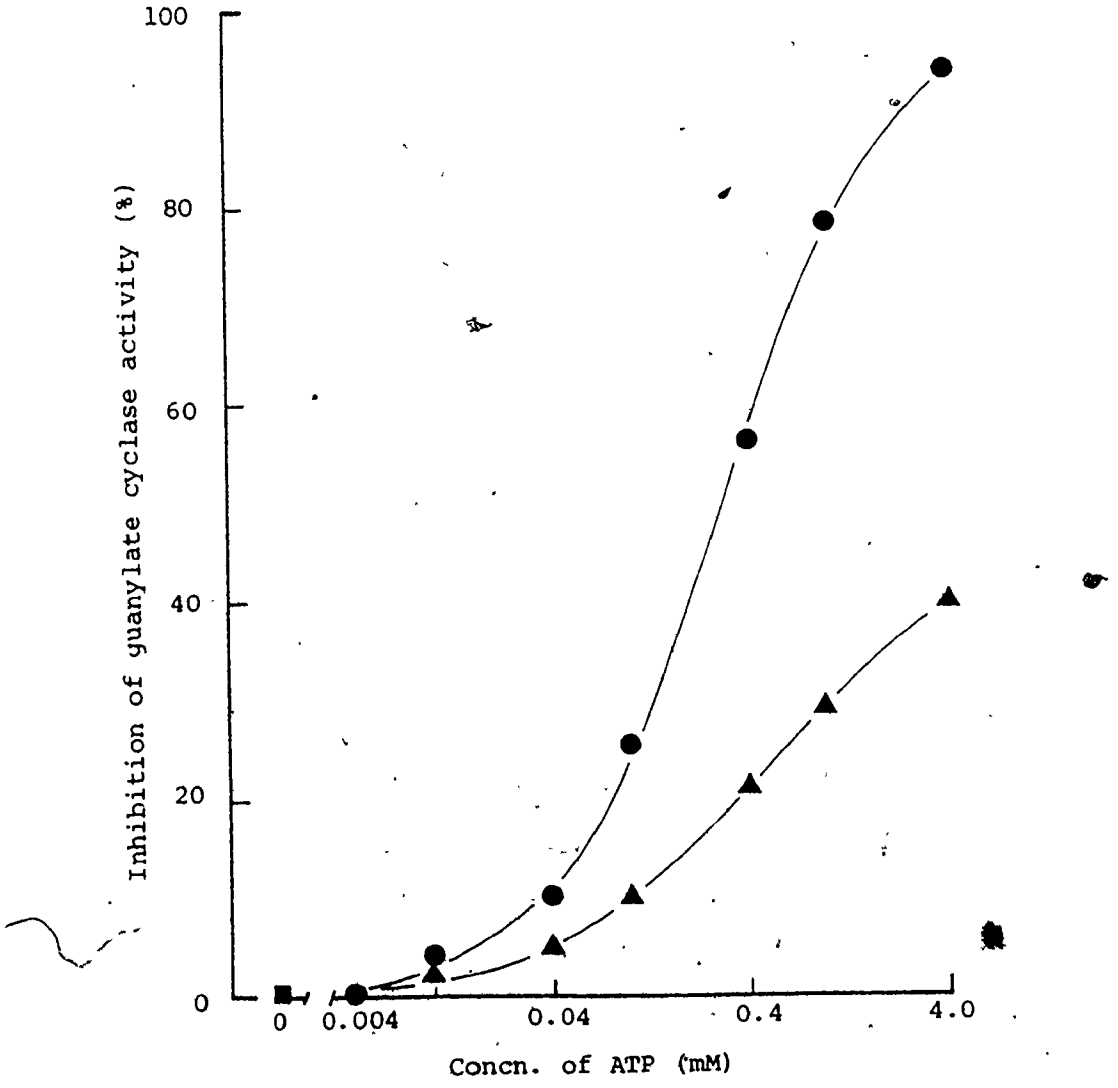


Fig. 3.3.5. *Effect of bivalent cation availability on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The concentration of protein in the platelet lysate was 6.9 mg/ml. Increasing volumes of platelet lysate were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures. In this experiment, 4.0 mM-$MnCl_2$ (●) or 10.0 mM-$MgCl_2$ (▲) was present in the assay mixtures. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

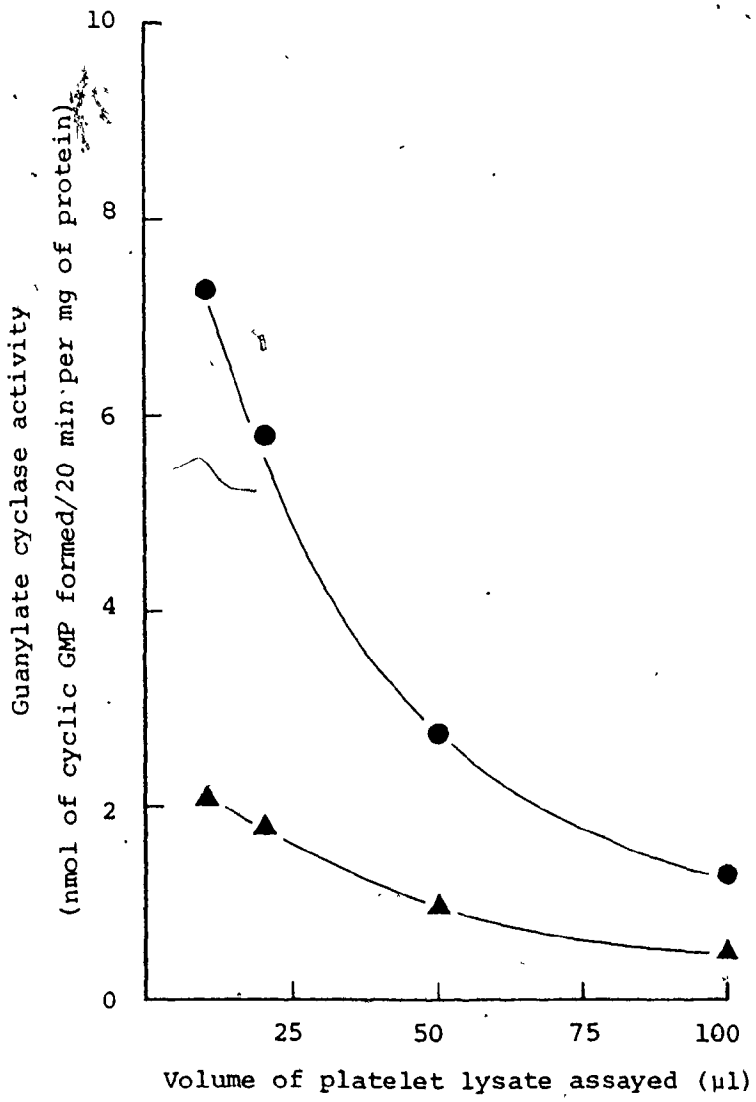


Fig. 3.3.6. *The specific acitivity of guanylate cyclase measured with different volumes of supernatant and particulate fractions in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP regenerating system was included in the assay mixtures. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant (●) was 3.5 mg/ml and that of the particulate fraction (▲) was 2.5 mg/ml. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 or 100 µl to the assay mixtures. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

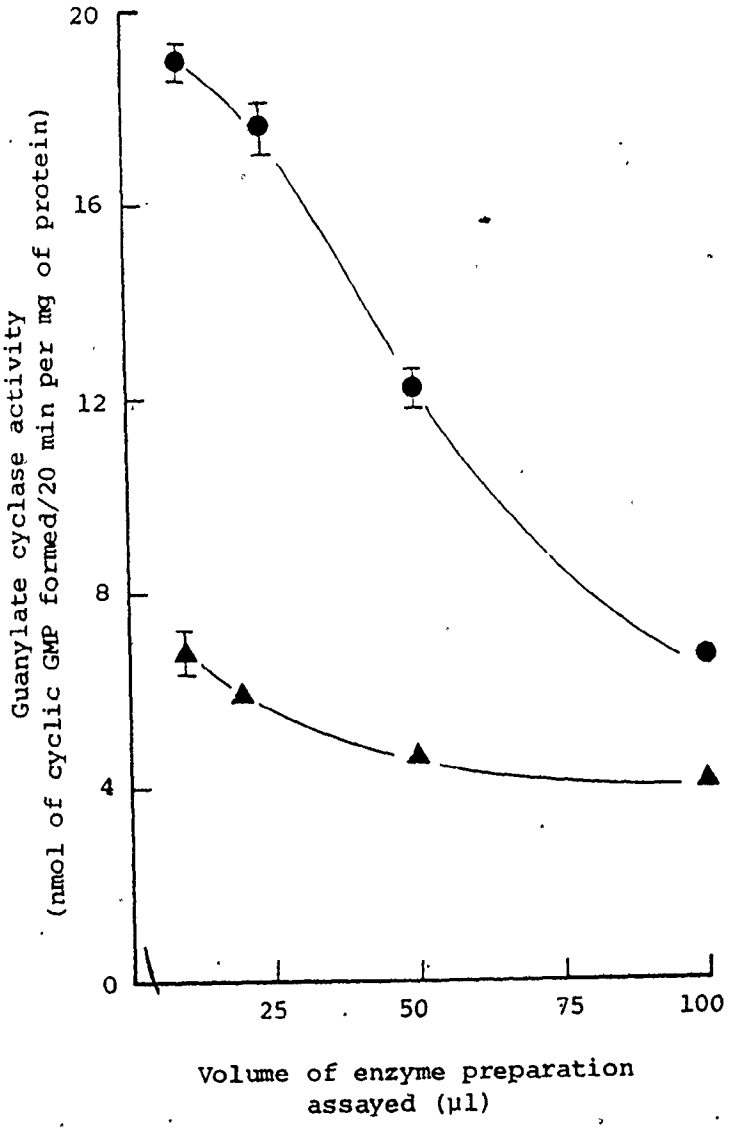
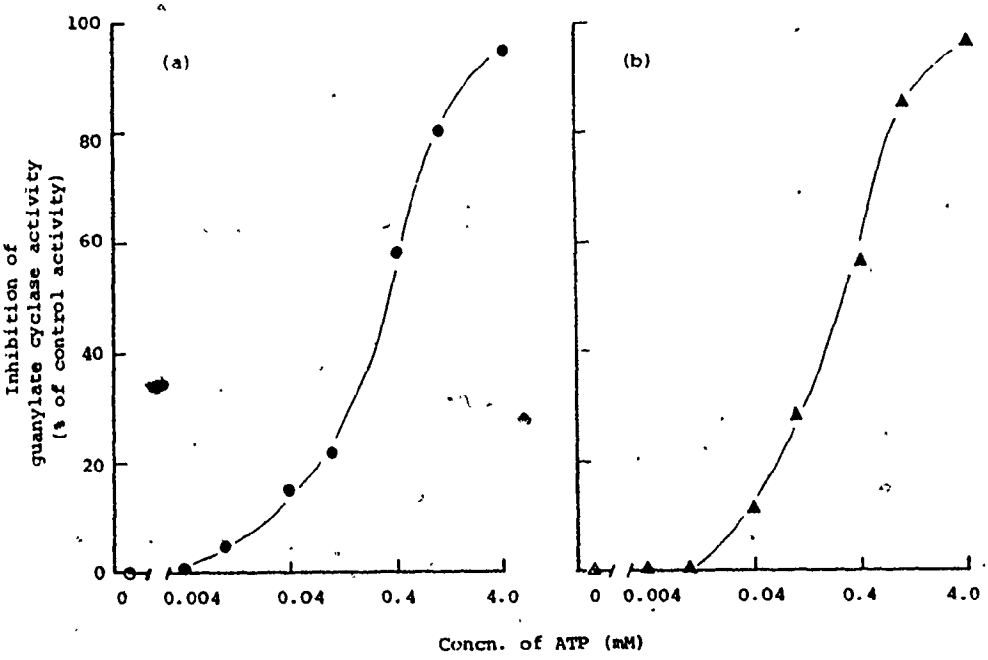


Fig. 3.3.7. *Inhibition of the guanylate cyclase activities in the supernatant and particulate fractions of platelet lysate by ATP*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction (○,●) was 5.6 mg/ml and that of the particulate fraction (△,▲) was 3.7 mg/ml. In each case, 50 µl aliquots of enzyme preparation were assayed. The guanylate cyclase activity in the supernatant fraction assayed in the absence of ATP (○) was 8.25 ± 0.04 nmol of cyclic GMP formed/20 min per mg of protein and 4.26 ± 0.11 nmol of cyclic GMP formed/20 min per mg of protein in the particulate fraction (△) (mean ± S.E.M. of triplicate determinations). Guanylate cyclase activity is expressed as the percent stimulation by ATP.



3.4. *Discussion*

3.4.1. *Subcellular distribution*

Determination of the intracellular distribution of guanylate cyclase is crucial to an understanding of the mechanism of activation of this enzyme. The results of Böhme *et al.* (1974), Rodan & Feinstein (1976), Barber (1976) and Glass *et al.* (1977*a*) indicate that about 90% of the measurable activity of this enzyme is present in the supernatant fraction from disrupted platelets. This distribution pattern was observed whether platelets were disrupted by sonication (Böhme *et al.*, 1974; Rodan & Feinstein, 1976; Glass *et al.*, 1977a), glycerol lysis (Barber, 1976) or freezing and thawing (Böhme *et al.*, 1974). The findings of the present study indicate that guanylate cyclase activity remaining in the particulate fraction was sequestered soluble enzyme that had not been released into the supernatant fraction during lysis. Finally, no latent guanylate cyclase activity was detectable by treatment of particulate fractions with Lubrol PX.

The subcellular distribution of guanylate cyclase in mammalian cells has been found to vary from 90% of the total activity in the high-speed supernatant fraction to 90% in the particulate fraction (Hardman & Sutherland, 1969; Goldberg & Haddox, 1977). Investigation of the properties of the enzyme in these fractions from several tissues has suggested that guanylate cyclase may be present in cells in distinct soluble and particulate forms that could be regulated by independent mechanisms (Kimura & Murad, 1975*a*; Chrisman *et al.*, 1975; Siegal *et al.*, 1976). However, as the platelet enzyme appears to be entirely soluble, the fact that marked

increases in platelet cyclic GMP occur in response to aggregating (Haslam, 1975; Davies *et al.*, 1976; Haslam, 1978) or other stimuli (Haslam *et al.* 1978*a*) suggests that indirect mechanisms may exist by which extracellular stimuli can activate the soluble enzyme in the cytosol.

3.4.2. Factors affecting the basal activity of platelet guanylate cyclase

Basal activity. The average basal guanylate cyclase activity of platelet lysate, assayed under optimal ionic conditions (4.0 mM-$MnCl_2$), was approximately 10 nmol of cyclic GMP formed/20 min per mg of protein at 30°C. This value agrees reasonably well with those reported for platelet guanylate cyclase by other workers (Böhme *et al.*, 1974; Barber, 1976; Glass *et al.*, 1977*a*; Weiss *et al.*, 1978), when allowance is made for the higher temperature (37°C) used by others and the presence of dithiothreitol in the assay medium in one study (Glass *et al.*, 1977*a*). Thus the results confirm that the specific activity of platelet guanylate cyclase is substantially higher than ~~that of any~~ other mammalian cells or tissues studied.

Bivalent cations. The dependence of platelet guanylate cyclase on free Mn^{2+} (*i.e.* $MnCl_2$ in excess of GTP) for the expression of maximum basal activity has also been reported by others (Böhme *et al.*, 1974; Rodan & Feinstein, 1976; Glass *et al.*, 1977*a*). The present results also show that neither $MgCl_2$ nor $CaCl_2$ alone could effectively substitute for $MnCl_2$; in contrast, Böhme *et al.* (1974) reported that the activity with high concentrations of $MgCl_2$ (10.0 mM) was 60% of that assayed in the presence of $MnCl_2$. This value seems high, as only 10 to 20% of maximum activity has been observed with $MgCl_2$ alone in other tissues (Hardman *et al.*, 1973), as in this study.

The synergistic interaction of $MgCl_2$ or $CaCl_2$ with suboptimal concentrations of $MnCl_2$ (*i.e.* $MnCl_2 \leq$ GTP) has not been previously reported for platelet guanylate cyclase, although similar effects have been observed in many other tissues (Hardman *et al.*, 1973; Garbers *et al.*, 1975; Chrisman *et al.*, 1975). It is probable that the stimulation of guanylate cyclase activity by high concentrations of $MgCl_2$ or $CaCl_2$, when $MnCl_2$/GTP molar ratios are one or less, is due in part to the increase in the concentration of free Mn^{2+} resulting from the formation of a Mg·GTP or Ca·GTP complex (Hardman *et al.*, 1973; Garbers *et al.*, 1975). However, in view of the fact that the amount of Mn^{2+} in cells is at least two orders of magnitude less than that of Mg^{2+} (Thiers & Vallee, 1957; Cotzias, 1962) and the concentration of free Ca^{2+} in the cytosol is in the micromolar range (Rasmussen, 1970), it is unlikely that the synergistic interaction of $MgCl_2$ or $CaCl_2$ with millimolar concentrations of $MnCl_2$ is of physiological importance.

Since the concentration of GTP in the platelet cytosol is about 0.8 mM (calculated from Agarwal & Parks, 1975), it follows that the substrate of guanylate cyclase is likely to be Mg·GTP and the activating bivalent cation Mg^{2+}. These considerations suggest that the ionic environment in the platelet may alone limit the basal guanylate cyclase activity to about 15% of the optimum. The effect of calcium on enzyme activity assayed under more physiological ionic conditions (*i.e.* millimolar concentrations of $MgCl_2$ and no added $MnCl_2$) is therefore relevant to the *in vivo* regulation of guanylate cyclase. Micromolar concentrations of free Ca^{2+} have been found to stimulate guanylate cyclase activity in platelets (this study) and in fibroblasts (Wallach & Pastan, 1976a) when Mg^{2+} was

the major bivalent cation. These findings might be attributable to the ability of Ca^{2+} to serve much more effectively as the essential cofactor (*i.e.* activating bivalent cation) for guanylate cyclase, while the more predominant Mg·GTP serves as the actual substrate. Since this activation of guanylate cyclase by Ca^{2+} ions occurs at physiological concentrations of Ca^{2+} ions, Mg^{2+} ions and GTP, it is possible that changes in the cytoplasmic Ca^{2+} distribution brought about by hormones and other substances may thereby indirectly stimulate guanylate cyclase activity and control cyclic GMP levels.

Inhibitory factors present in platelet lysate. The marked decrease in the specific activity of guanylate cyclase observed when 50 µl or more of the lysate was assayed was due to inhibition of the enzyme by relatively low-molecular-weight non-protein factors, which could be removed by ultrafiltration of the lysate. Up to about 50% of this inhibitory activity was accounted for by adenine nucleotides, which are well known to inhibit guanylate cyclase (Hardman & Sutherland, 1969; Kimura & Murad, 1974*a*, 1975a). Because of the release, during freezing and thawing, of adenine nucleotides from platelet dense bodies, which contain about 60% of the total present in platelets (Holmsen & Day, 1971; Agarwal & Parks, 1975), the concentration of adenine nucleotides in platelet lysate is likely to be higher than in similar preparations from other tissues. In intact platelets, the concentration of adenine nucleotides in the metabolic compartment alone is approximately 6.0 mM (Holmsen & Day, 1971). Under optimal ionic conditions (4.0 mM-$MnCl_2$), this concentration of adenine nucleotides would be sufficient to inhibit guanylate cyclase activity by more than 95% (Fig. 3.2.3. and 3.2.4.); whereas under more physiological conditions (10.0 mM-$MgCl_2$), it is unlikely

that activity would be inhibited by much more than about 50% (Fig. 3.2.4.). These results therefore indicate that other inhibitory factors present in the lysate are likely to be substantially more important than adenine nucleotides in limiting enzyme activity in intact platelets. The marked inhibition of guanylate cyclase activity observed with high concentrations of dithiothreitol or glutathione suggested that platelet thiols might contribute to the inhibitory effect; however, the concentration of non-protein sulfhydryl groups in platelet lysate was found to be far too low to have any effect on enzyme activity *in vitro*. In contrast, the concentration of non-protein thiol in intact platelets was estimated to be 4.0 to 5.0 mM, which is enough to inhibit guanylate cyclase activity by about 50%. Although it was not possible to identify the other ultrafilterable non-protein inhibitory factors present in platelet lysate, the results indicate that the combined effects of inhibitory factors and of suboptimal ionic conditions are likely to lower the guanylate cyclase activity in intact platelets to almost negligible values in the absence of activating factors. The similarity of the bivalent cation requirements and sensitivity to inhibition by ATP of the guanylate cyclase activity in supernatant and particulate fractions of platelet lysate indicate that only the soluble form of the enzyme is present in platelets.

Chapter 4

Activation of Platelet Guanylate Cyclase

4.1. Results: Time- and temperature-dependent activation

The guanylate cyclase activity of platelet lysate was not linear with respect to period of incubation. As shown in Fig. 4.1.1.a., the guanylate cyclase activity of platelet lysate increased progressively when assays were incubated at 30°C for up to 40 min. Similar results were obtained when assays were carried out at 37°C. Expression of these data in terms of the specific activity determined for the three intervals, 0 to 5 min, 5 to 20 min and 20 to 40 min, illustrates the acceleration of guanylate cyclase activity during assay (Table 4.1.1.). The average specific activity of the enzyme during the interval between 20 and 40 min after starting the assay was over two-fold greater than the value measured during the first 5 min of incubation. This increase in activity was not dependent on components present in the assay medium, as preincubation of the lysate for 60 min at 30°C also had a similar stimulatory effect (Table 4.1.1. and Fig. 4.1.2.). Furthermore, preincubation of the platelet lysate resulted in a marked reduction of the acceleration of guanylate cyclase activity during assay (Fig. 4.1.1.a. and Table 4.1.1.).

As shown in Fig. 4.1.2., the stimulation of guanylate cyclase activity by preincubation of platelet lysate was significantly greater when the lysate was preincubated at 37°C for up to 120 min. Maximum stimulation of enzyme activity was observed after 120 min of preincubation at 30°C or 37°C. The 'rate of activation' or the increase in the specific activity of guanylate cyclase during the initial 60 min of preincubation at 0°, 30° or 37°C was calculated to be 0.007 ± 0.003 nmol of cyclic GMP/10 min per mg of protein at 0°C (mean ± S.E.M. of four experiments), 0.055 ± 0.004 nmol of

cyclic GMP/10 min per mg of protein at 30°C (mean ± S.E.M. of two experiments) and 0.105 ± 0.007 nmol of cyclic GMP/10 min per mg of protein at 37°C (mean ± S.E.M. of three experiments). Thus, the rate of activation of guanylate cyclase in platelet lysates preincubated at 37°C is approximately twice the rate observed in lysates preincubated at 30°C.

Fig. 4.1.3. shows that while preincubation of the supernatant fraction resulted in a marked stimulation of guanylate cyclase activity, preincubation of the particulate fraction had no significant effect on enzyme activity.

The magnitude of the stimulatory effect of preincubation of platelet lysate at 30°C or at 37°C was a function of the volume of lysate assayed (Fig. 4.1.4.). Average increases of 44 ± 9%, 51 ± 7%, 121 ± 16% and 204 ± 38% were observed with 10, 20, 50 and 100 µl respectively, of lysate preincubated at 30°C for 60 min (mean ± S.E.M. of 12 experiments); whereas, average increases of 98 ± 10%, 131 ± 18%, 223 ± 21% and 348 ± 43% were found with 10, 20, 50 and 100 µl respectively, of lysate preincubated at 37°C for 60 min (mean ± S.E.M. of three experiments). Thus, at all volumes of lysate assayed, the guanylate cyclase activity of lysate preincubated at 37°C was about two-fold greater than that of lysate preincubated at 30°C (Fig. 4.1.4.).

In contrast to untreated platelet lysate, the specific guanylate cyclase activity of lysate preincubated at 30°C or at 37°C did not significantly decrease when more than 20 µl of lysate was assayed (Fig. 4.1.5. and 4.1.6.). This increase in guanylate cyclase activity of 50 and 100 µl of lysate after preincubation was only partly attributable to decreased inhibition by ultrafilterable factors, because ultrafiltrate from

preincubated lysate retained a large proportion of the original inhibitory activity (59 and 89% respectively, in two experiments). Furthermore, preincubated lysate was as sensitive to inhibition by ultrafiltrate as untreated lysate. Although preincubation of platelet lysate at 30°C was found to decrease the concentration of adenine nucleotides in the lysate by 67 ± 2% (mean ± S.E.M. of three determinations), this decrease could not account for the 126 ± 11% (mean ± S.E.M. of three experiments) increase in the guanylate cyclase activity of the preincubated lysate (Table 4.1.2.). ATP, ADP and AMP inhibited the guanylate cyclase activities of platelet lysate and preincubated lysate to the same extent. The concentration of platelet non-protein thiol was also measured in lysate that had been preincubated at 37°C; however, no significant decrease was observed even though guanylate cyclase activity was markedly stimulated (Table 4.1.3.). Moreover, preincubation of lysates prepared from four to five-day old platelet concentrates at 37°C resulted in a marked stimulation of guanylate cyclase activity in spite of the fact that the concentration of non-protein thiol in these lysates was significantly lower than in lysates prepared from freshly drawn blood (Table 4.1.3.). It is therefore doubtful on the basis of these observations that either a change in the concentration of platelet adenine nucleotides or non-protein thiol or a change in the sensitivity of preincubated lysate to inhibition by these factors can account for the marked increase in guanylate cyclase activity observed with large volumes of preincubated lysate in the assay. This conclusion is also supported by the observation that ultrafiltered lysate showed a substantial increase in guanylate cyclase activity during assay (Fig. 4.1.1.b. and Table 4.1.1.).

The effect of dithiothreitol on the stimulation of guanylate cyclase activity by preincubation was studied in order to throw light on this process of enzyme activation. As shown in Fig. 4.1.7., the addition of 5.0 mM-dithiothreitol to platelet lysate not only inhibited the guanylate cyclase activity of the lysate by about 70% but also prevented any increase in activity when present during preincubation of the lysate. However, dithiothreitol inhibited the activity of enzyme that had already been preincubated by the same percentage as control enzyme, so that in this case the preincubation effect could still be detected (Table 4.1.4.). Similar results were obtained with 10.0 mM-glutathione. These observations suggest that activation of platelet guanylate cyclase by preincubation of the lysate may be the result of oxidation of sulfhydryl or other oxidizable groups on the enzyme or on other components involved in the regulation of enzyme activity. To investigate this possibility, the effect of *N*-ethylmaleimide was studied as this agent alkylates sulfhydryl groups (Webb, 1966). As found with dithiothreitol, the addition of concentrations of *N*-ethylmaleimide as low as 0.1 mM to platelet lysate not only inhibited the guanylate cyclase activity of the lysate by about 50% (*e.g.* Fig. 4.1.8.), but also prevented any increase in activity when present during preincubation of the lysate. However, in contrast to the effects of dithiothreitol, low concentrations of *N*-ethylmaleimide (*i.e.* less than 1.0 mM) did not inhibit the guanylate cyclase activity of enzyme that had already been preincubated by the same percentage as the control enzyme (Fig. 4.1.8.). In four experiments, 0.1 mM-*N*-ethylmaleimide was found to inhibit the guanylate cyclase activity of platelet lysate by 48 ± 9% (mean ± S.E.M.), ($2P<0.02$) whereas the activity of preincubated (at 37°C for 60 min) lysate was inhibited by only 25 ± 5% ($2P<0.05$)

On the basis of a paired *t* test, this difference in inhibition of enzyme activity by 0.1 mM-*N*-ethylmaleimide was determined to be statistically significant ($2P<0.05$). However, no significant difference was observed between the inhibition of guanylate cyclase activity of platelet lysate (78 ± 5% inhibition) ($2P<0.001$) and that of preincubated lysate (71 ± 7% inhibition) ($2P<0.001$) by a ten-fold higher concentration of *N*-ethylmaleimide. Thus, activation of guanylate cyclase by preincubation appeared to alter the sensitivity of this enzyme preparation to inhibition by low concentrations of *N*-ethylmaleimide.

The guanylate cyclase activity of preincubated platelet lysate was assayed with Mg^{2+} as the sole cation in order to determine whether activation by preincubation affected the requirement of this enzyme preparation for Mn^{2+} for the expression of maximum activity. Table 4.1.5. shows that in the presence of 4.0 mM-$MnCl_2$, preincubation of the lysate stimulated guanylate cyclase activity by an average of 90%; whereas in the presence of 10.0 mM-$MgCl_2$, activity was increased by an average of only 27%. Thus, full expression of the stimulatory effect of preincubation of the lysate on guanylate cyclase activity was dependent on the presence of an optimal concentration of $MnCl_2$. It follows that the effectiveness of Mg^{2+} as a substitute for Mn^{2+} was markedly decreased with preincubated lysate (Table 4.1.5.).

Fig. 4.1.1. *Changes in the guanylate cyclase activities of platelet lysate, ultrafiltered lysate and preincubated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate (●), ultrafiltered lysate (▲) and preincubated lysate (30°C)(■) were prepared as described in Section 2.2.2. The values given are the means ± S.E.M. of results pooled from the following numbers of experiments: platelet lysate, 12; ultrafiltered lysate, 6; and, preincubated lysate, 10. The mean values ± S.E.M. for the protein assayed were: platelet lysate, 200 ± 15 µg; ultrafiltered lysate, 145 ± 20 µg; and preincubated lysate, 185 ± 10 µg. Incubations were for 5, 20 and 40 min. In some of these experiments, a phosphocreatine/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures. The data shown are the same as in Table 4.1.1.

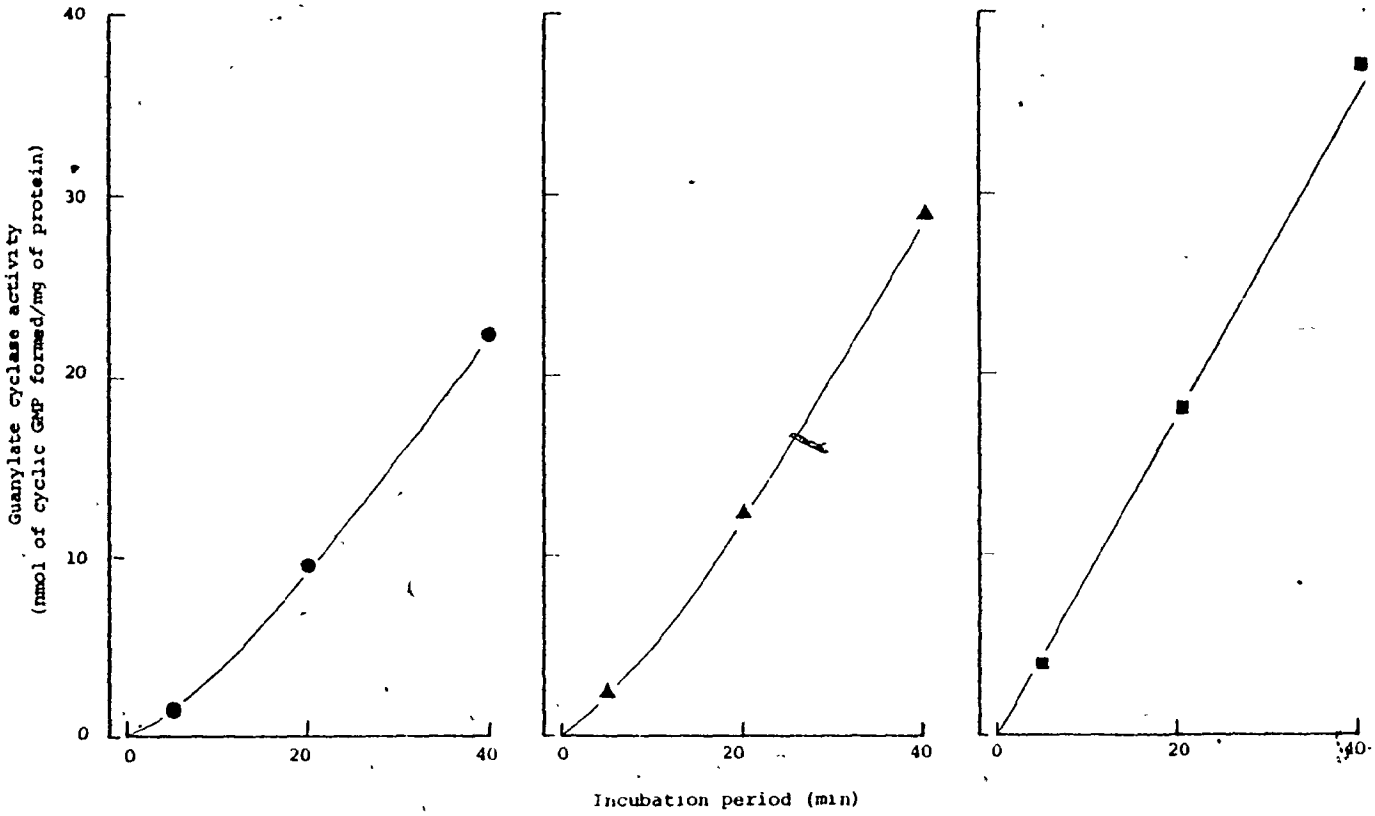


Table 4.1.1. *Changes in the guanylate cyclase activities of platelet lysate, ultrafiltered lysate and preincubated lysate.*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate, ultrafiltered lysate and preincubated lysate were prepared as described in Section 2.2.2. Results were pooled from the number of experiments listed below; the values given are the means ± S.E.M. The mean values ± S.E.M. for the protein assayed were: platelet lysate 200 ± 15 μg; ultrafiltered lysate, 145 ± 20 μg; and preincubated lysate, 185 ± 10 μg. Incubations were for 5, 20 and 40 min. In some of these experiments, a phosphocreating/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures. Guanylate cyclase activity is expressed in terms of specific activity determined for three intervals: 0 to 5 min, 5 to 20 min, and 20 to 40 min. The ratio of the specific activities (20 to 40 min/0 to 5 min) reflects the acceleration of guanylate cyclase activity in each of these preparations. The data shown are the same as in Fig. 4.1.1.

Enayme preparation	No. of experiments	Guanylate cyclase activity (nmol of cyclic GMP/min per mg of protein)			Ratio of guanylate cyclase activities
		0 to 5 min	5 to 20 min	20 to 40 min	(20 to 40 min/0 to 5 min)
Lysate	12	0.28 ± 0.03	0.49 ± 0.02	0.61 ± 0.04	2.18
Ultrafiltered lysate	6	0.48 ± 0.08	0.60 ± 0.06	0.78 ± 0.09	1.63
Preincubated lysate	10	0.64 ± 0.06	0.87 ± 0.09	0.87 ± 0.12	1.36

Fig. 4.1.2. *Stimulation of guanylate cyclase activity by preincubation of platelet lysate: Effect of temperature*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate (0°C)(●) and preincubated lysate, 30°C (▲), 37°C (■) were prepared as described in Section 2.2.2. The protein concentration of the enzyme preparations was 6.6 mg/ml and 25 µl aliquots were assayed. The values given are the means ± S.E.M. of triplicate determinations.

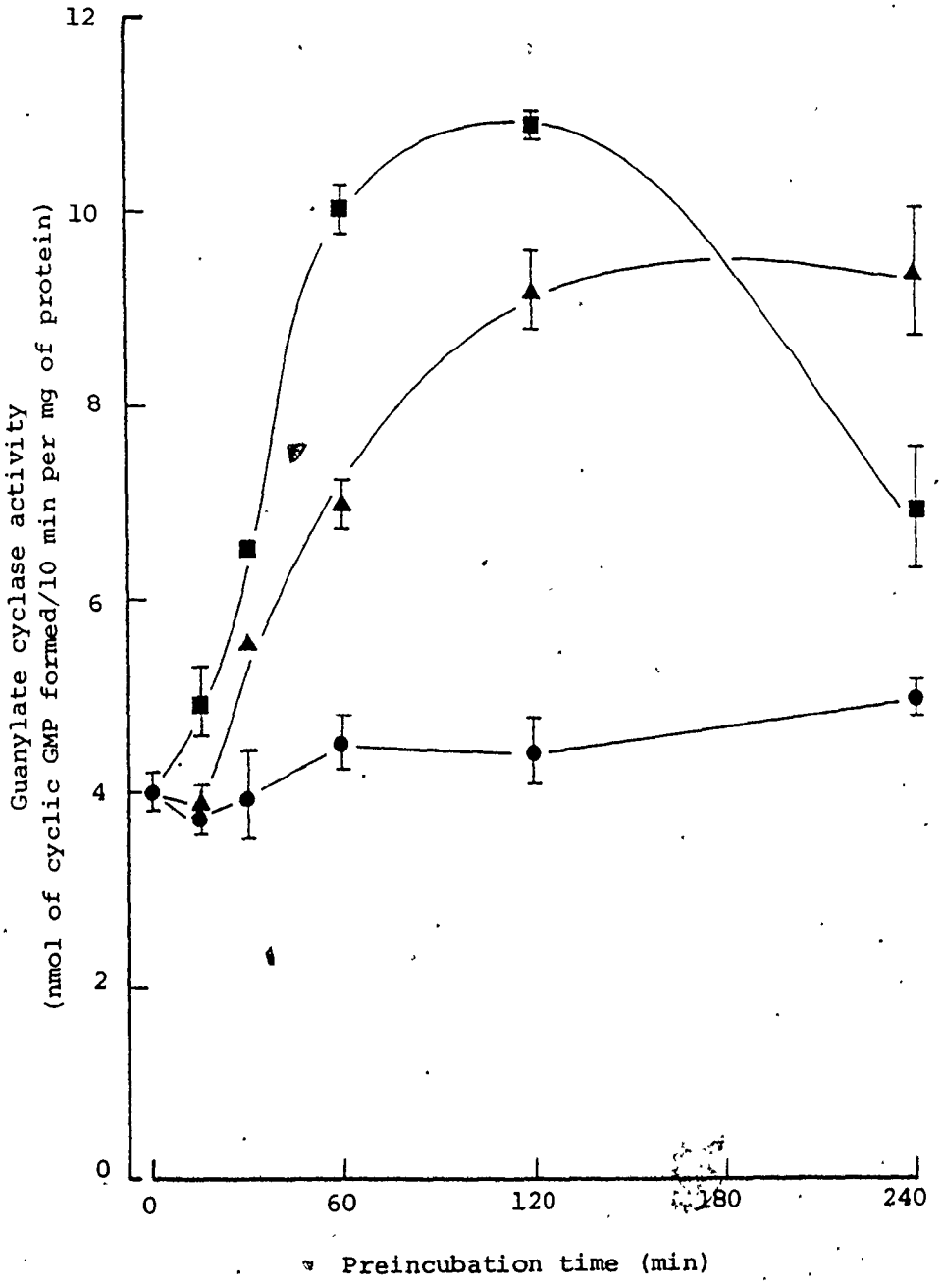


Fig. 4.1.3. *Effect of preincubation of supernatant and particulate fractions of platelet lysate at 0° and at 37°C on guanylate cyclase activity*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction was 4.5 mg/ml and that of the particulate fraction was 3.5 mg/ml. In each case, 50 µl aliquots of enzyme preparation were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols. (a) Supernatant fraction preincubated at 0°C (○) and at 37°C (●); and, (b)particulate fraction preincubated at 0°C (△) and at 37°C (▲).

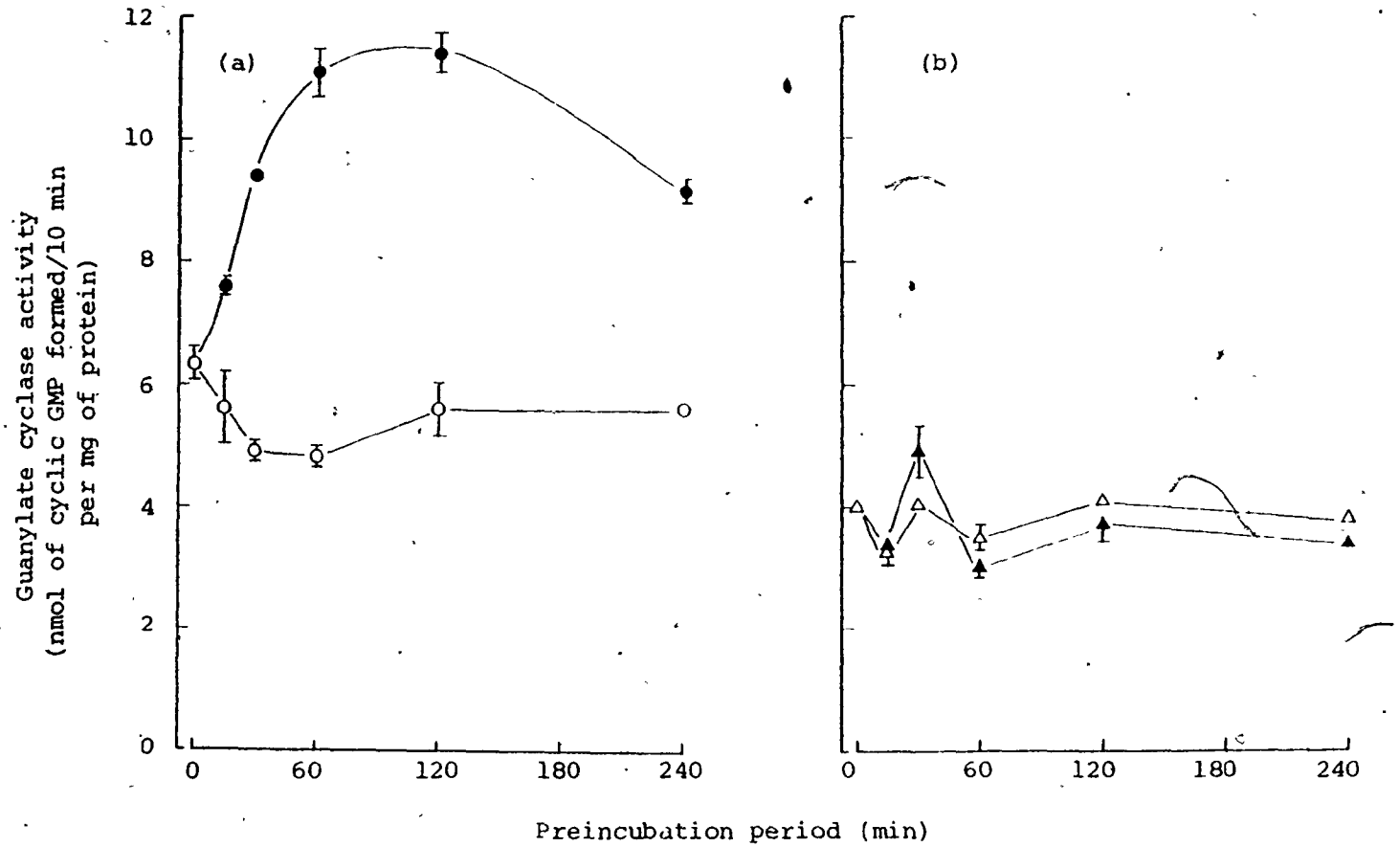


Fig. 4.1.4. *Stimulation of guanylate cyclase activity by preincubation of platelet lysate: Effect of temperature and of volume of preincubated lysate assayed*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures. Platelet lysate was prepared as described in Section 2.2.2. and preincubated for 60 min at 30°C (●) or at 37°C (▲). The values given are the means ± S.E.M. of results pooled from the following numbers of experiments: 30°C preincubation, 12; and, 37°C preincubation, 3. The mean values ± S.E.M. for the protein concentration of platelet lysates preincubated at 30°C was 6.3 ± 0.9 mg/ml and 6.0 ± 0.8 mg/ml for lysate preincubated at 37°C. In some of the experiments with lysate preincubated at 30°C, a phosphocreatine/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures.

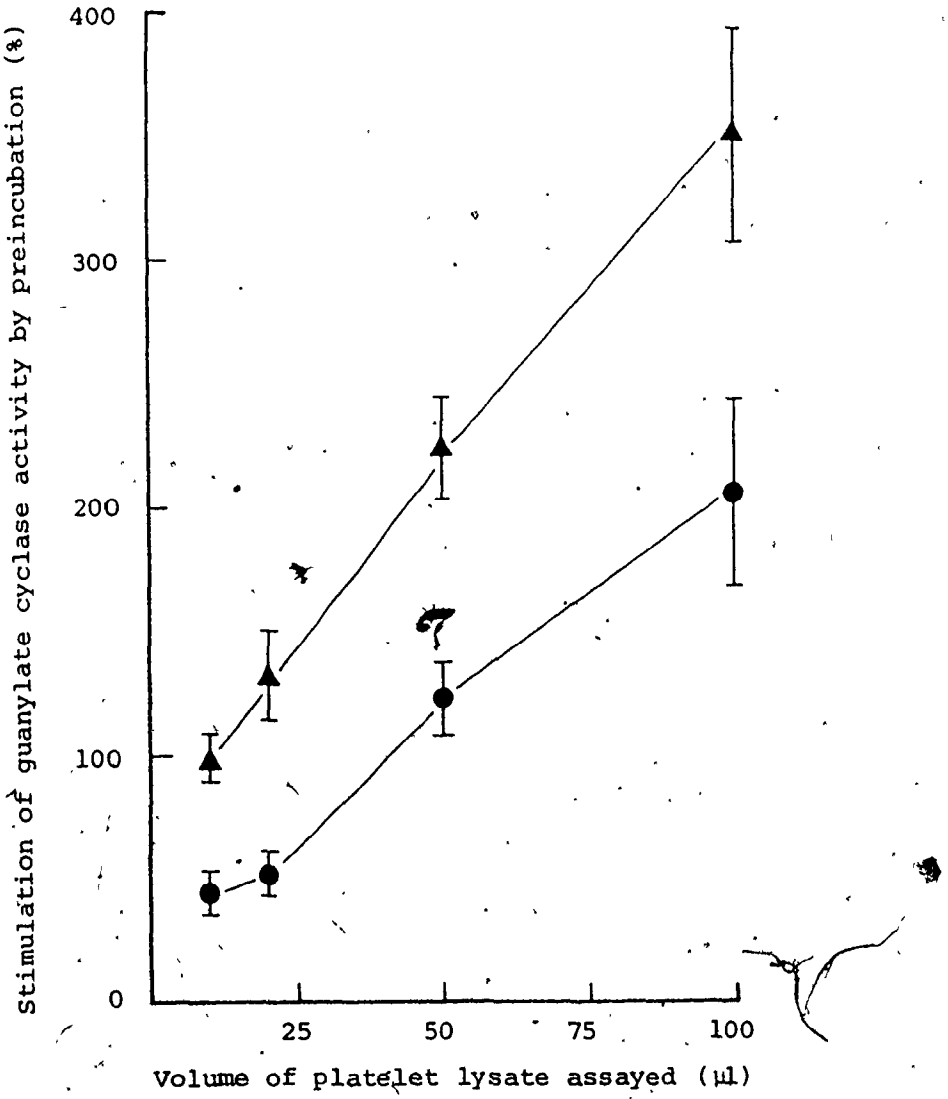


Fig. 4.1.5. *Effect of preincubation of platelet lysate at 30°C on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared and preincubated at 30°C as described in Section 2.2.2. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. In some of these experiments, a phosphocreatine/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures. For statistical purposes, paired data were grouped according to the volume of lysate added to the assays, and the significance of the effect of preincubation was determined by paired *t* tests: *$2P<0.01$, **$2P<0.001$. The values given are the means ± S.E.M. of results pooled from 12 experiments. The protein concentration of the platelet lysate (●) and preincubated lysate (▲) was 6.3 mg/ml (mean ± S.E.M.).

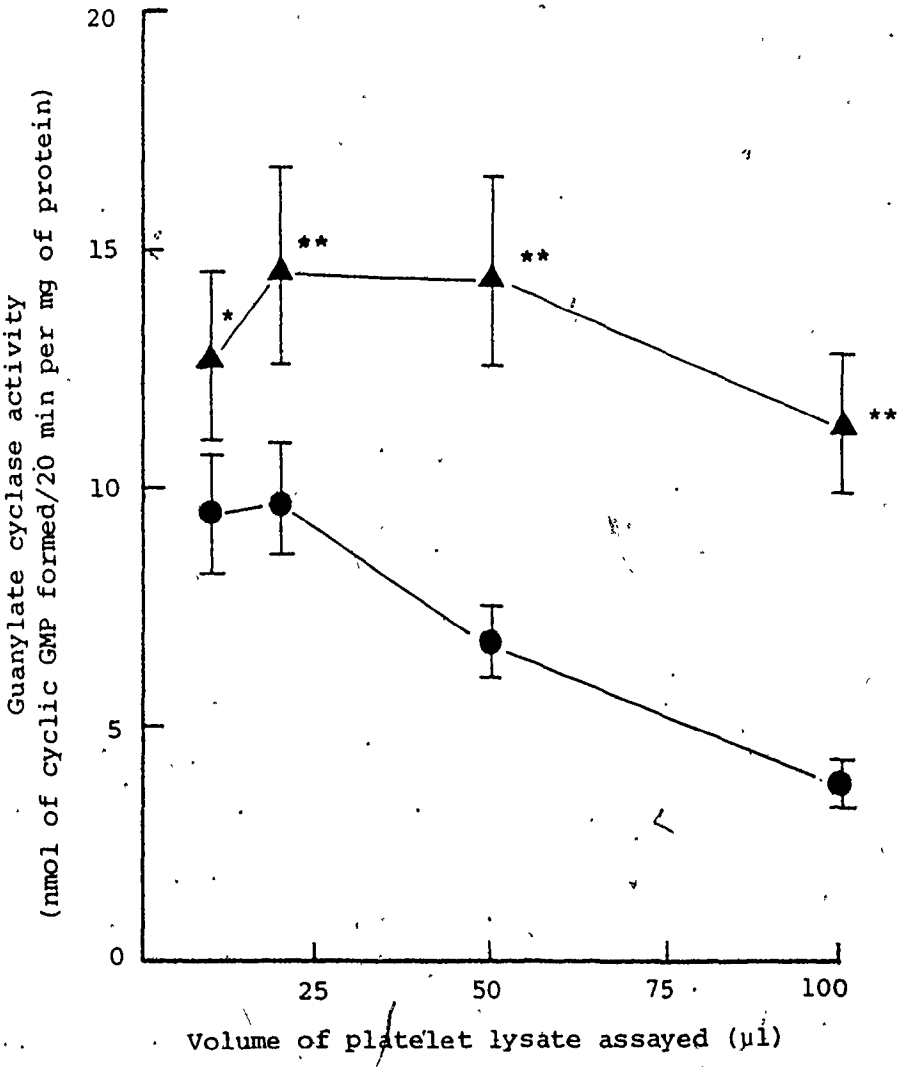


Fig. 4.1.6. *Effect of preincubation of platelet lysate at 37°C on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared from a stored platelet concentrate and preincubated at 37°C as described in Section 2.2.2. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. The protein concentration of the enzyme preparations was 4.8 mg/ml. The values given are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols. Platelet lysate (●), preincubated lysate (▲).

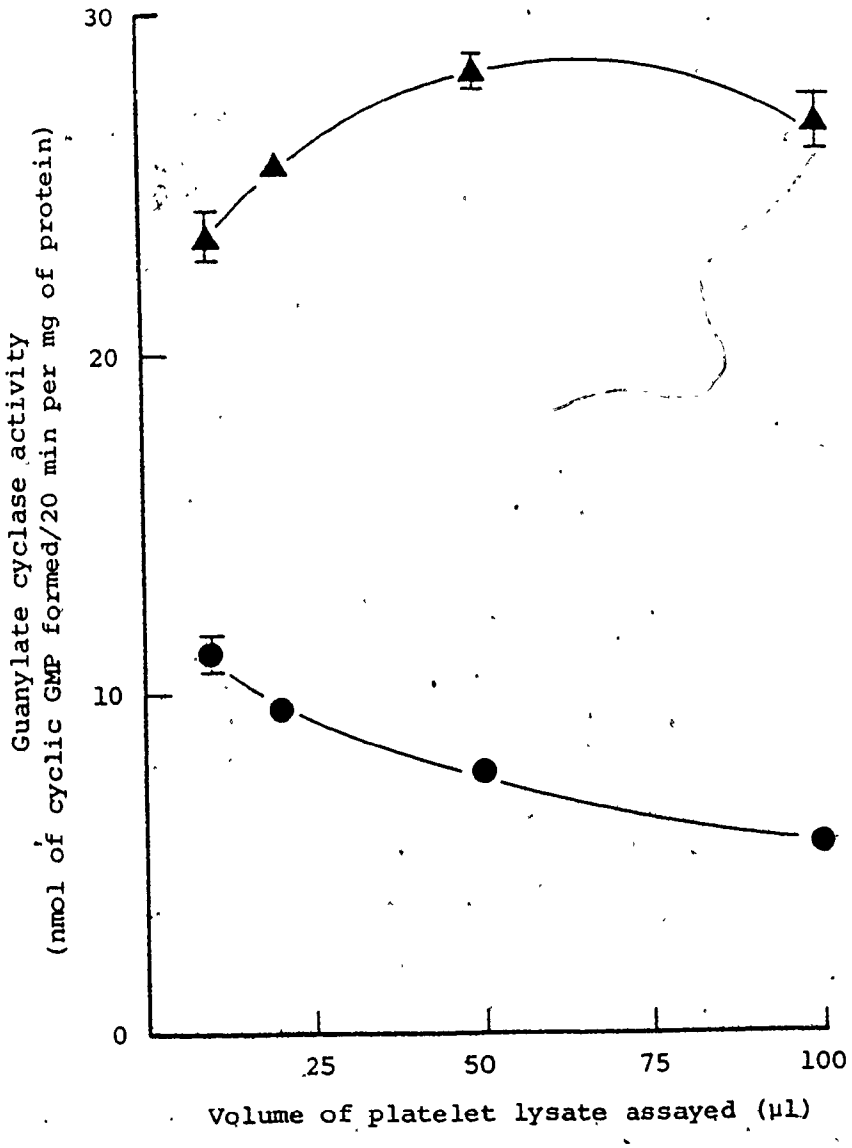


Table 4.1.2. *Effect of preincubation of platelet lysate on the concentration of adenine nucleotides present in the lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysates were prepared and preincubated at 30°C as described in Section 2.2.2. The protein concentration of the lysates and preincubated lysates were as follows: (a)Experiment 1, 3.2 mg/ml; (b)Experiment 2, 3.3 mg/ml; and, (c)Experiment 3, 2.8 mg/ml. In each case, 50 µl aliquots were assayed. The total concentration of ATP, ADP and AMP present in the platelet lysates and preincubated lysates was assayed as described in Section 2.2.7.3.; the values given are the results of single determinations. The concentration of adenine nucleotides present in the platelet lysates (mean ± S.E.M. of the three experiments shown) was 107.8 ± 1.4 nmol/mg of protein before preincubation, and 35.6 ± 2.1 nmol/mg of protein after preincubation (a total reduction of 67 ± 2%). The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Preincubation stimulated guanylate cyclase activity by 126 ± 11% (mean ± S.E.M. of the three experiments shown).

Exp. no.	Enzyme preparation	Concn. of adenine nucleotides present in the:		Guanylate cyclase activity	
		platelet lysate (nmol/mg of protein)	assay mixture (mM)	nmol cyclic GMP/ 20 min per mg protein	% stimulation by preincubation
1	Lysate	104.6	0.067	7.30 ± 0.27	-
	Preincubated lysate	28.4	0.018	15.91 ± 0.27	118
2	Lysate	110.3	0.073	10.61 ± 0.08	-
	Preincubated lysate	35.7	0.024	26.73 ± 0.25	152
3	Lysate	108.4	0.061	9.21 ± 0.29	-
	Preincubated lysate	36.6	0.020	19.19 ± 0.48	108

Table 4.1.3. *Effect of preincubation of platelet lysate on the concentration of non-protein and protein-bound sulfhydryl groups in the lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysates were prepared and preincubated at 37°C as described in Section 2.2.2. In Experiments 1 and 2, lysates were prepared from freshly drawn blood; and in Experiments 3 and 4, lysates were prepared from 4 to 5-day old platelet concentrates. The protein concentrations of the lysates and preincubated lysates were as follows: (a)Exp. 1, 6.2 mg/ml; (b)Exp. 2, 6.9 mg/ml; (c)Exp. 3, 4.8 mg/ml; and, (d)Exp. 4, 5.0 mg/ml. In Exps. 1, 2 and 3, 50 µl aliquots were assayed, and in Exp. 4, 25 µl aliquots were assayed for guanylate cyclase activity. The concentration of non-protein and of protein-bound sulfhydryl groups present in the platelet lysates was assayed as described in Section 2.2.7.4.; the values given are the results of single determinations. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Preincubation stimulated guanylate cyclase activity by 200 ± 24% (mean ± S.E.M. of the four experiments shown).

Exp. no.	Enzyme preparation	Concn. of sulfhydryl groups (nmol/mg of protein)		Guanylate cyclase activity	
		non-protein	protein	nmol cyclic GMP/ 20 min per mg protein	% stimulation by preincubation
1	Lysate	15.0	165.0	4.61 ± 0.13	-
	Preincubated lysate	14.0	164.0	13.16 ± 0.13	185
2	Lysate	12.5	161.5	2.70 ± 0.06	-
	Preincubated lysate	9.5	153.0	8.14 ± 0.41	202
3	Lysate	5.0	140.0	7.55 ± 0.12	-
	Preincubated lysate	4.4	130.0	28.17 ± 0.38	273
4	Lysate	3.5	109.4	13.68 ± 0.32	-
	Preincubated lysate	2.4	107.1	32.64 ± 0.16	139

Fig. 4.1.7. *Effect of dithiothreitol on the stimulation of guanylate cyclase activity by preincubation of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was prepared as described in Section 2.2.2. Dithiothreitol (5 mM-final concentration in the lysate, 1 mM-final concentration in the assay mixture) was added to one portion of the lysate. Both the untreated (●) and the dithiothreitol-treated (▲) lysates were preincubated at 30°C for 0, 10, 20, 40 and 80 min and the guanylate cyclase activity assayed. The protein concentration of both enzyme preparations was 3.4 mg/ml and 50 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols. Preincubation of the untreated lysate stimulated guanylate cyclase activity by 68% after 80 min.

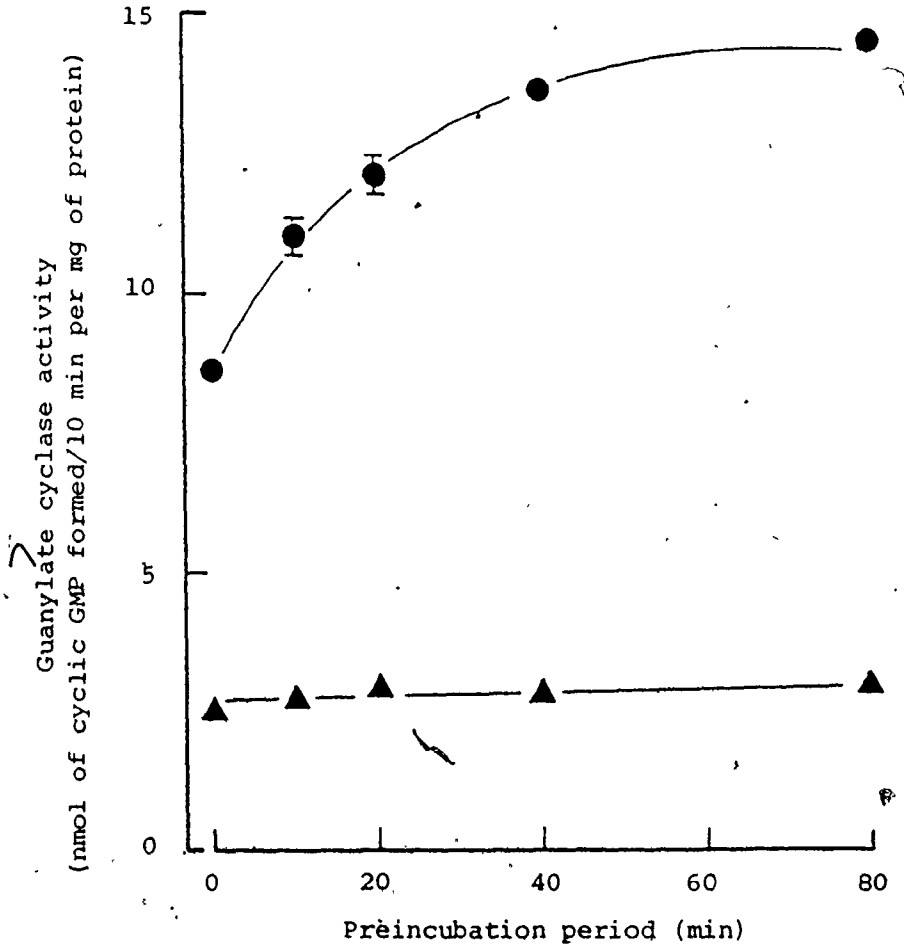


Table 4.1.4. *Effect of dithiothreitol on the guanylate cyclase activities of platelet lysate and of preincubated platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was prepared and preincubated at 30°C as described in Section 2.2.2. Dithiothreitol (2 mM-final concn. in the lysate; 0.4 mM-final concn. in the assay mixture) was added to one portion of the untreated lysate and to one portion of the preincubated lysate. The protein concentration of all enzyme preparations was 3.3 mg/ml, and 50 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations.

Enzyme preparation	Addition to the enzyme preparation	Guanylate cyclase activity	
		nmol cyclic GMP/ 20 min per mg protein	% of control activity
Lysate	None	18.24 ± 0.55	100
	Dithiothreitol	3.75 ± 0.29	21
Preincubated lysate	None	32.71 ± 0.48	207
	Dithiothreitol	9.13 ± 0.27	50

Fig. 4.1.8. *Effect of N-ethylmaleimide on the guanylate cyclase activities of platelet lysate and of preincubated platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that incubations were continued for 10 min. Platelet lysate was prepared and preincubated at 37°C as described in Section 2.2.2 The protein concentration of the platelet lysate (●) and preincubated lysate (▲) was 8.3 mg/ml and 25 µl aliquots were assayed. N-ethylmaleimide was added to assay mixtures at the concentrations indicated on the abcissa. The values given are the means of triplicate determinations. The guanylate cyclase activity of the untreated platelet lysate was 2.83 ± 0.05 nmol of cyclic GMP formed/10 min per mg of protein, and that of the preincubated lysate was 7.2 ± 0.13 nmol of cyclic GMP formed/10 min per mg of protein (mean ± S.E.M. of triplicate determinations)(preincubation stimulated guanylate cyclase activity by 154%).

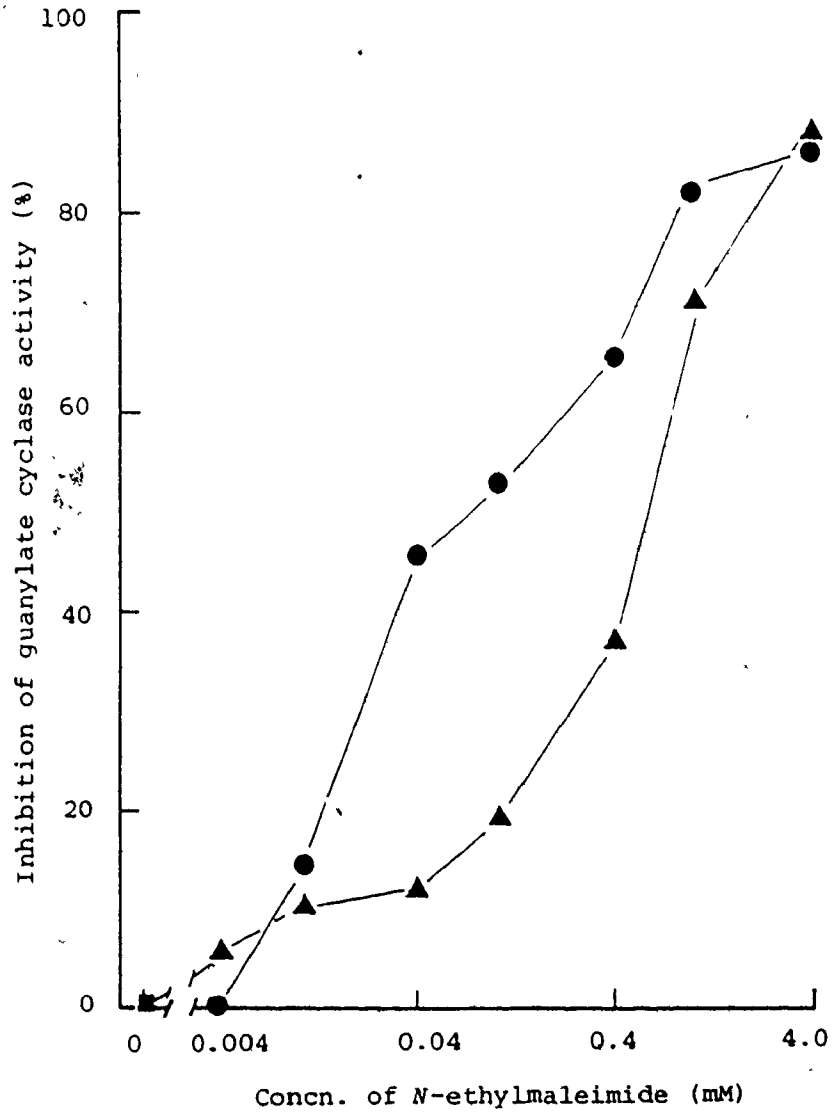


Table 4.1.5. *Effect of preincubation on the bivalent cation requirements of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared and preincubated at 37°C as described in Section 2.2.2. The protein concentrations of the enzyme preparations were as follows: (a)Exp. 1, 6.9 mg/ml, 20 µl aliquots were assayed; and, (b)Exp. 2, 6.2 mg/ml, 25 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. In the presence of 4.0 mM-$MnCl_2$, preincubation stimulated the guanylate cyclase activity of platelet lysate by an average of 90% in the two experiments shown; whereas in the presence of 10.0 mM-$MgCl_2$, preincubation stimulated activity by an average of only 27%.

Exp. no.	Enzyme preparation	Bivalent cation present in assay	Guanylate cyclase activity	
			nmol cyclic GMP/ 20 min per mg protein	% stimulation by preincubation
1	Lysate	$MnCl_2$ (4.0 mM)	5.77 ± 0.07	-
	Preincubated lysate		11.18 ± 0.36	94
	Lysate	$MgCl_2$ (10.0 mM)	1.80 ± 0.36	-
	Preincubated lysate		2.24 ± 0.22	24
2	Lysate	$MnCl_2$ (4.0 mM)	7.83 ± 0.25	-
	Preincubated lysate		14.55 ± 0.38	86
	Lysate	$MgCl_2$ (10.0 mM)	1.08 ± 0.05	-
	Preincubated lysate		1.40 ± 0.07	30

4.2. Results: Effects of oxidants

The suggestion that activation of guanylate cyclase by preincubation may be the result of oxidation of sulfhydryl groups on the enzyme and/or on regulatory components led to the investigation of the effects of several oxidants on the activity of platelet guanylate cyclase.

At concentrations of 1.0 mM or less, oxidized glutathione had no significant effect on guanylate cyclase activity; whereas at higher concentrations, activity was markedly inhibited (*e.g.* 4.0 and 10.0 mM inhibited activity by 65 and 100%, respectively in one experiment). As shown in Fig. 4.2.1., guanylate cyclase was stimulated by about 40% with an optimal concentration of 0.04 mM-4,4'-dithiodipyridine, whereas activity was inhibited by the same percentage with only a slightly higher concentration of this disulfide. Although a biphasic response was also observed with diamide (Fig. 4.2.4.), the maximum stimulatory effect of this agent was about twice that observed with 4,4'-dithiodipyridine. In five experiments, the guanylate cyclase activity of platelet lysate was stimulated by 87 ± 11% (mean ± S.E.M.) ($2P<0.005$) with an optimal concentration of 0.4 mM-diamide.

Preincubation of platelet lysate nearly abolished the stimulatory effect of diamide (Fig. 4.2.2.). In three experiments, 0.4 mM-diamide was found to have no significant effect on the guanylate cyclase activity of preincubated lysate (*i.e.* 2 ± 7% stimulation)(mean ± S.E.M.). As shown in Fig. 4.2.3., the stimulatory effect of diamide progressively decreased with increased periods of preincubation of the lysate at 37°C. As these results suggested that the stimulation of guanylate cyclase activity by diamide

may be dependent on the availability of specific sulfhydryl groups on the enzyme and/or on regulatory components, the effect of diamide on the activity of *N*-ethylmaleimide-treated lysate was studied. Fig. 4.2.4. shows that diamide did not stimulate the activity of lysate treated with 0.1 mM-*N*-ethylmaleimide to nearly the same extent as that of control lysate. Moreover, as observed with preincubated lysate (Fig. 4.2.2.), high concentrations of diamide markedly inhibited the guanylate cyclase activity of *N*-ethylmaleimide-treated lysate (Fig. 4.2.4.). The close similarity in the response of preincubated and of *N*-ethylmaleimide-treated lysates to diamide suggested that inhibition of the stimulatory effect of this agent may be attributable to oxidation by preincubation or alkylation by *N*-ethylmaleimide of specific sulfhydryl groups on or associated with the enzyme.

In addition to 4,4'-dithiodipyridine and diamide, the guanylate cyclase activity of platelet lysate was markedly stimulated by the oxidant, *tert*-butylhydroperoxide (Fig. 4.2.5.). With an optimal concentration of 1.0 mM-*tert*-butylhydroperoxide, activity was stimulated by 165 ± 19% (mean ± S.E.M. of 12 experiments) ($2P<0.001$). Thus, the stimulatory effect of this agent was approximately two-fold greater than the effect of diamide and about four-fold greater than that of 4,4'-dithiodipyridine. Furthermore, in contrast to the effect of high concentrations of 4,4'dithiodipyridine or diamide, no inhibition of activity was observed with as much as 10.0 mM-*tert*-butylhydroperoxide.

As observed with diamide, the stimulation of guanylate cyclase activity by *tert*-butylhydroperoxide progressively decreased with increased periods of preincubation of the lysate at 37°C (Fig. 4.2.6.). Furthermore, after preincubation of the lysate at 37°C for 240 min, both diamide

(Fig. 4.2.3.) and *tert*-butylhydroperoxide (Fig. 4.2.6.) were found to significantly inhibit enzyme activity. On the basis of these findings, the effect of *tert*-butylhydroperoxide on the guanylate cyclase activity of *N*-ethylmaleimide-treated lysate was studied. As shown in Table 4.2.1., *tert*-butylhydroperoxide did not increase the activity of lysate treated with 0.1 mM-*N*-ethylmaleimide. Thus, *N*-ethylmaleimide was effective in blocking the stimulatory effects of both diamide and of *tert*-butylhydroperoxide. It was also found that addition of 0.1 mM-*N*-ethylmaleimide to *tert*-butylhydroperoxide-treated lysate did not result in a marked inhibition of guanylate cyclase activity (Table 4.2.1.). Hence, the guanylate cyclase activity of both preincubated and *tert*-butylhydroperoxide-treated lysates exhibited a significant decrease in the sensitivity to inhibition by low concentrations of *N*-ethylmaleimide. Together, these results indicated that preincubation, diamide and *tert*-butylhydroperoxide activate platelet guanylate cyclase by similar mechanisms. The similarity of the effects of preincubation and of *tert*-butylhydroperoxide was further suggested by the observation that the specific activity of guanylate cyclase measured with 50 or 100 µl of *tert*-butylhydroperoxide-treated lysate in the assay was not inhibited in percentage terms to nearly the same extent as that of control lysate (Fig. 4.2.7.).

The guanylate cyclase activity of *tert*-butylhydroperoxide-treated lysate was also assayed with Mg^{2+} as the sole cation in order to determine whether activation affected the requirement of this enzyme preparation for Mn^{2+} for the expression of maximum activity. Table 4.2.2. shows that in the presence of 4.0 mM-$MnCl_2$, *tert*-butylhydroperoxide stimulated the activity of platelet lysate by an average of 114%; whereas in the presence of

10.0 mM-$MgCl_2$, activity was increased by an average of only 15%. Thus, as observed with preincubated lysate, full expression of the stimulatory effect of *tert*-butylhydroperoxide on guanylate cyclase activity was dependent on the presence of an optimal concentration of $MnCl_2$. It also follows that the effectiveness of Mg^{2+} as a substitute for Mn^{2+} was markedly decreased with *tert*-butylhydroperoxide-treated lysate (Table 4.2.4.).

Since *tert*-butylhydroperoxide had been reported to oxidize glutathione in erythrocytes by an enzymic process (Srivastava *et al.*, 1974), its effect on platelet non-protein sulfhydryl groups was determined. Table 4.2.3. shows that *tert*-butylhydroperoxide decreased the concentration of non-protein thiol by an average of 95% in lysates prepared from freshly drawn blood. However, it is doubtful that the stimulatory effect of *tert*-butylhydroperoxide can be attributed to oxidation of glutathione because the guanylate cyclase activity in lysates substantially depleted of non-protein thiol (*i.e.* lysates prepared from four to five-day old platelet concentrates) was increased on average, by about the same percentage as the activity in lysates that had much higher concentrations of non-protein thiol (Table 4.2.3.). *Tert*-butylhydroperoxide was found to have no marked effect on platelet protein thiol.

Fig. 4.2.1. *Effect of 4,4'-dithiodipyridine on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The effect of 4,4'-dithiodipyridine on guanylate cyclase activity is expressed as a percentage of the control activity (i.e. with no added 4,4'-dithiodipyridine). The data shown were pooled from two identical experiments in which guanylate cyclase assays were carried out in triplicate; thus the values given are the means ± S.E.M. of 6 determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols. The guanylate cyclase activity of the untreated lysate (i.e. control lysate) was 8.84 ± 0.26 nmol of cyclic GMP formed/10 min per mg of protein in Experiment 1, and 2.51 ± 0.06 nmol of cyclic GMP formed/10 min per mg of protein in Experiment 2. The protein concentrations of the platelet lysates were as follows: (a)Experiment 1, 3.8 mg/ml, 25 µl aliquots were assayed; and, (b)Experiment 2, 6.7 mg/ml, 25 µl aliquots were assayed. Platelet lysate assayed in the presence of 4,4'-dithiodipyridine (●), platelet lysate assayed in the absence of 4,4'-dithiodipyridine (○).

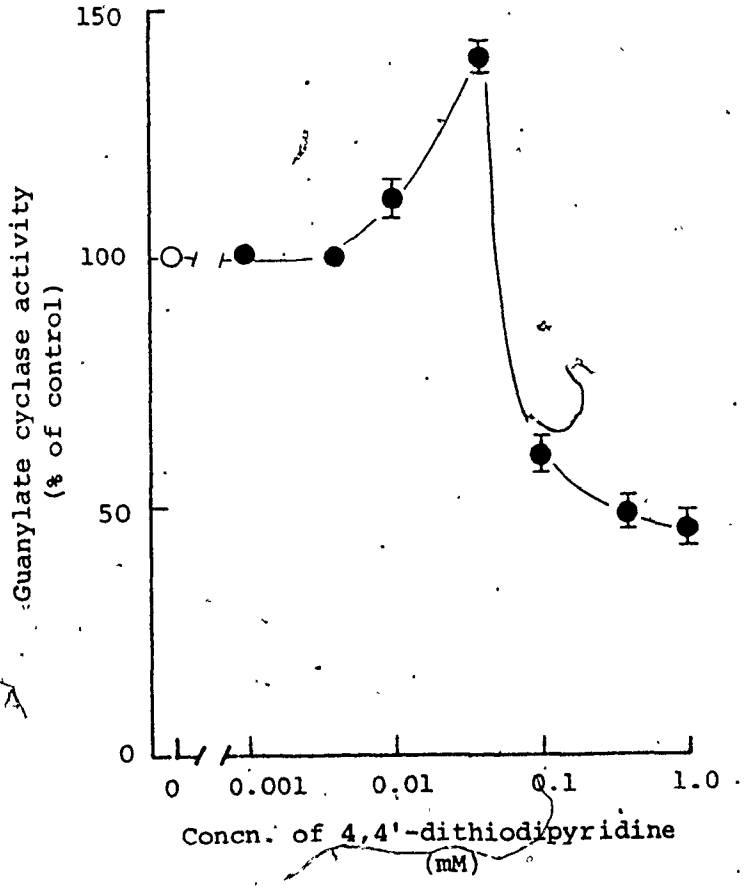


Fig. 4.2.2. *Effect of diamide on the guanylate cyclase activity of platelet lysate and of preincubated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared and preincubated at 37°C as described in Section 2.2.2. The protein concentration of the platelet lysate and preincubated lysate was 8.3 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the platelet lysate (○) was 2.83 ± 0.05 nmol of cyclic GMP formed/10 min per mg of protein and that of the preincubated lysate (△) was 7.20 ± 0.13 nmol of cyclic GMP formed/10 min per mg of protein. Preincubation stimulated the guanylate cyclase activity of the platelet lysate by 154%. Guanylate cyclase activity is expressed as a percentage of the control activity of each enzyme preparation (i.e. 0 mM-diamide). The values given are the means of triplicate determinations. Platelet lysate (●), preincubated lysate (▲) assayed in the presence of diamide.

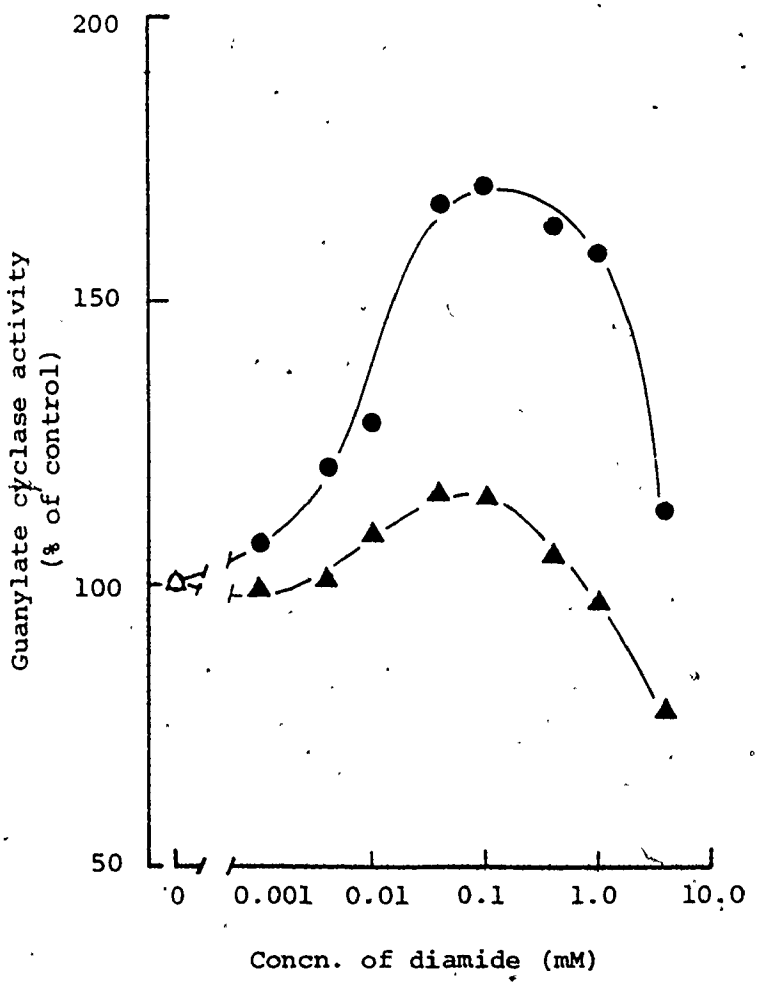


Fig. 4.2.3. *Effect of diamide on the guanylate cyclase activity of platelet lysate preincubated at 0° and at 37°C for increasing periods of time*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The platelet lysate was divided into two portions which were either kept on ice (circles) or preincubated (triangles) for 0, 15, 30, 60, 120 and 240 min at 37°C. The protein concentration of the platelet lysate and preincubated lysate was 7.3 mg/ml and 25 µl aliquots were assayed. Diamide was added to the assay mixtures at a final concentration of 0.4 mM (closed symbols). Platelet lysate (○), platelet lysate assayed in the presence of diamide (●), preincubated lysate (△), preincubated lysate assayed in the presence of diamide (▲). The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

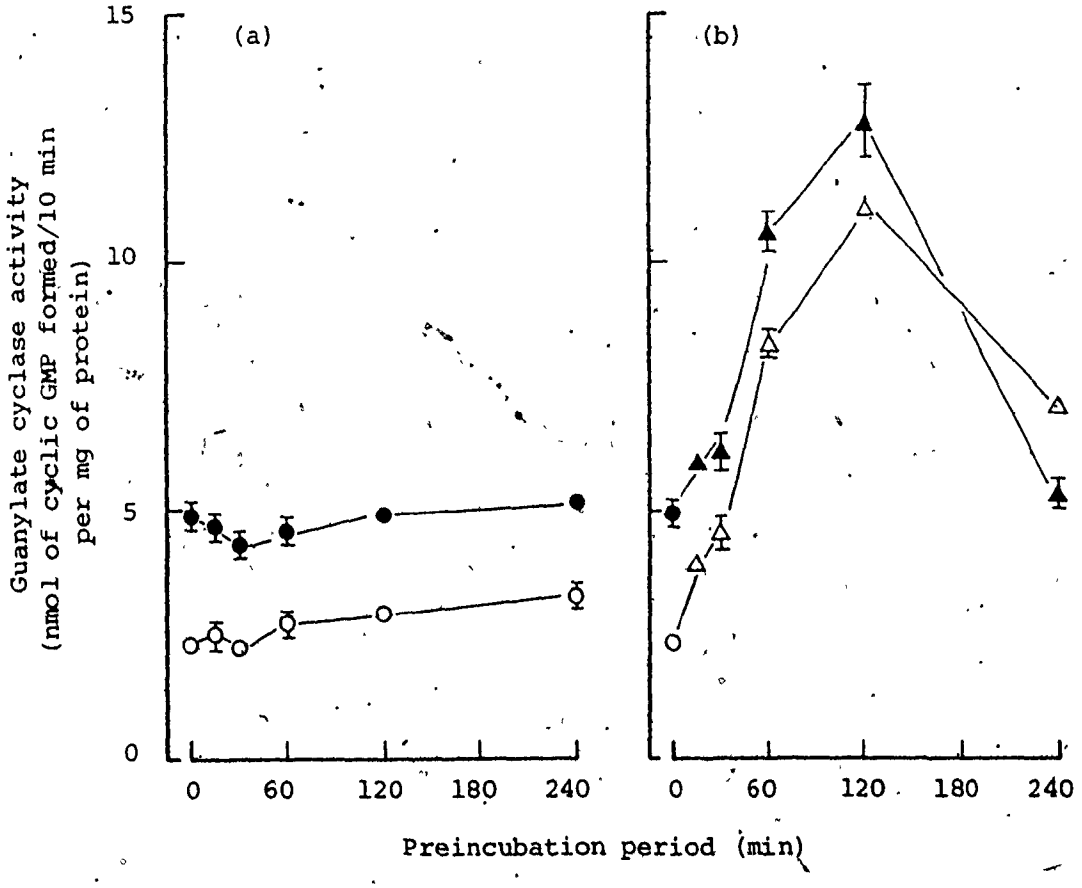


Fig. 4.2.4. *Effect of diamide on the guanylate cyclase activity of platelet lysate pretreated with N-ethylmaleimide*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2.. N-ethylmaleimide (0.1 mM-final concentration in the lysate, 0.01 mM-final concentration in the assay mixture) was added to one portion of the lysate. The protein concentration of the enzyme preparations was 6.0 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the untreated lysate (○) was 2.14 ± 0.05 nmol of cyclic GMP formed/10 min per mg of protein and that of the N-ethylmaleimide-treated lysate (△) was 1.23 ± 0.05 nmol of cyclic GMP formed/10 min per mg of protein. N-ethylmaleimide inhibited the guanylate cyclase activity of the platelet lysate by 43%. Guanylate cyclase activity is expressed as a percentage of the control activity of each enzyme preparation (i.e. 0 mM-diamide). The values given are the means of triplicate determinations. This experiment has been carried out once. Platelet lysate (●), N-ethylmaleimide-treated lysate (▲) assayed in the presence of diamide.

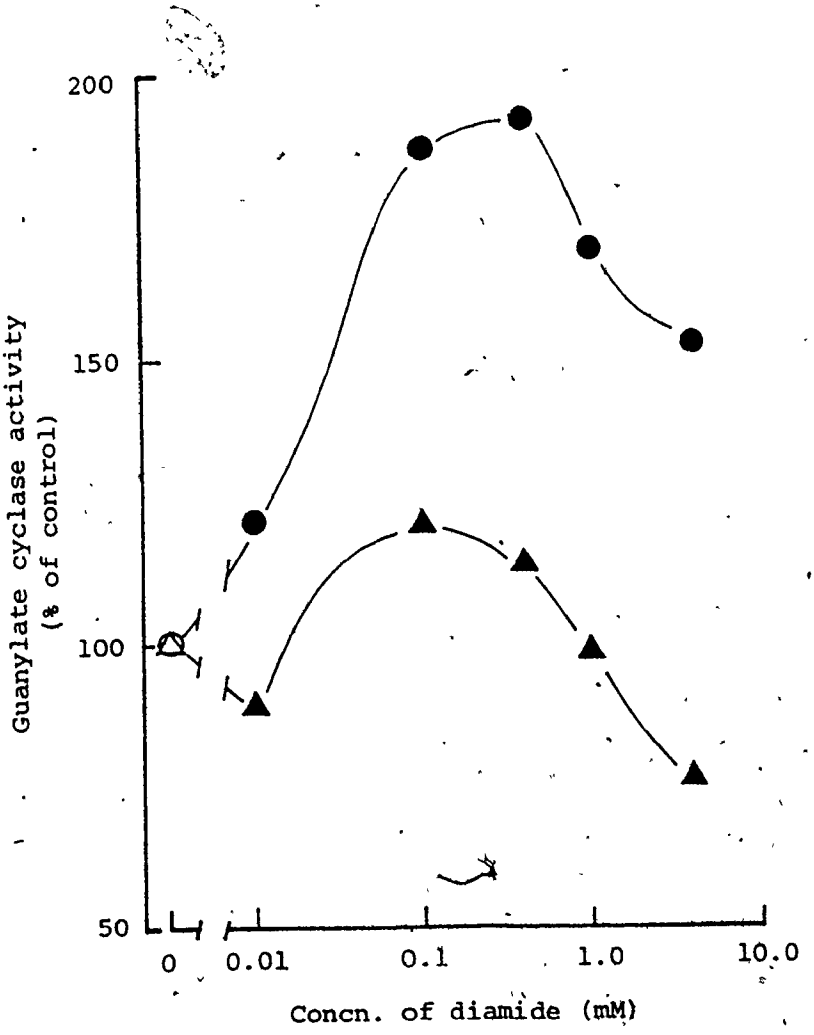


Fig. 4.2.5. *Effect of tert-butylhydroperoxide on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 6.2 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the untreated lysate (O) was 2.51 ± 0.06 nmol of cyclic GMP formed/10 min per mg of protein. Guanylate cyclase activity is expressed as a percentage of the control activity (i.e. 0 mM-tert-butylhydroperoxide). The values given are the means of triplicate determinations. Platelet lysate assayed in the presence of tert-butylhydroperoxide (●).

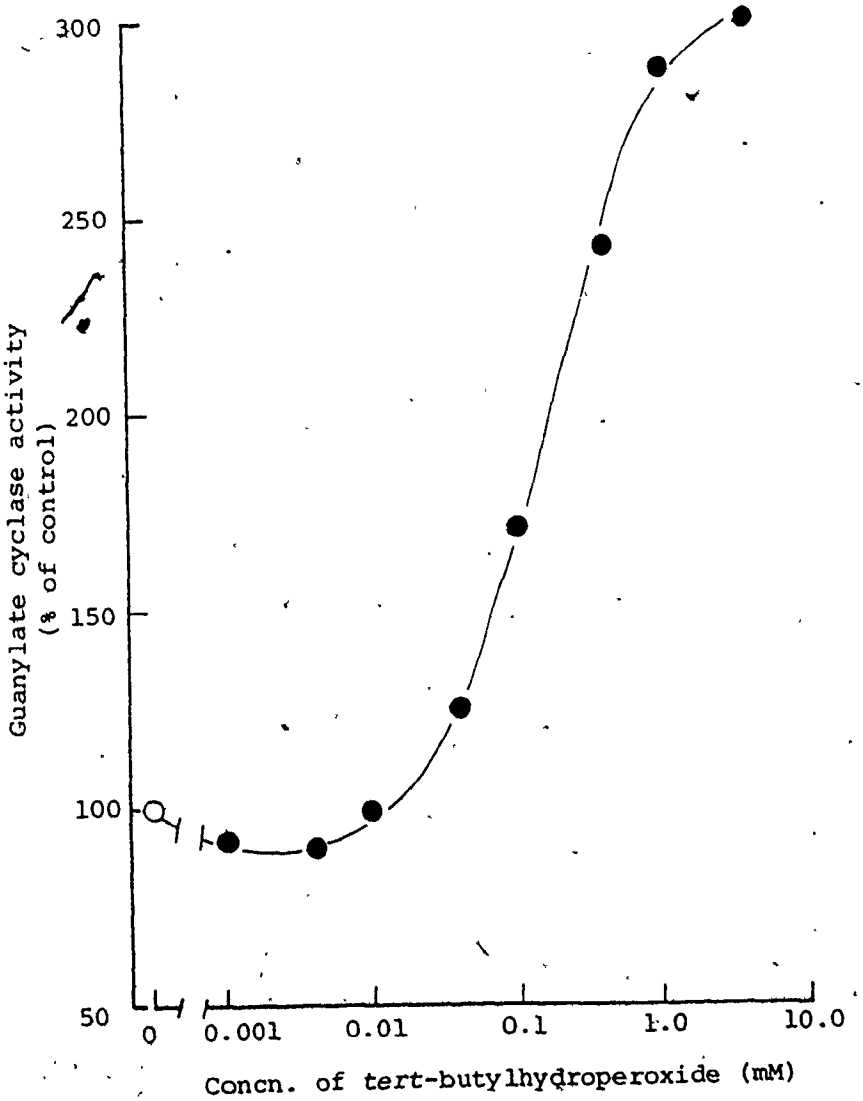


Fig. 4.2.6. *Effect of tert-butylhydroperoxide on the guanylate cyclase activity of platelet lysate preincubated at 0° and at 37°C for increasing periods of time*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The platelet lysate was divided into two portions which were either kept on ice (circles) or preincubated (triangles) for 0, 15, 30, 60, 120 and 240 min at 37°C. The protein concentration of the platelet lysate and preincubated lysate was 7.3 mg/ml and 25 µl aliquots were assayed. *Tert*-butylhydroperoxide was added to the assay mistures at a final concentration of 1 mM (closed symbols). Platelet lysate (○), platelet lysate assayed in the presence of *tert*-butylhydroperoxide (●), preincubated lysate (△), preincubated lysate assayed in the presence of *tert*-butylhydroperoxide (▲). The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

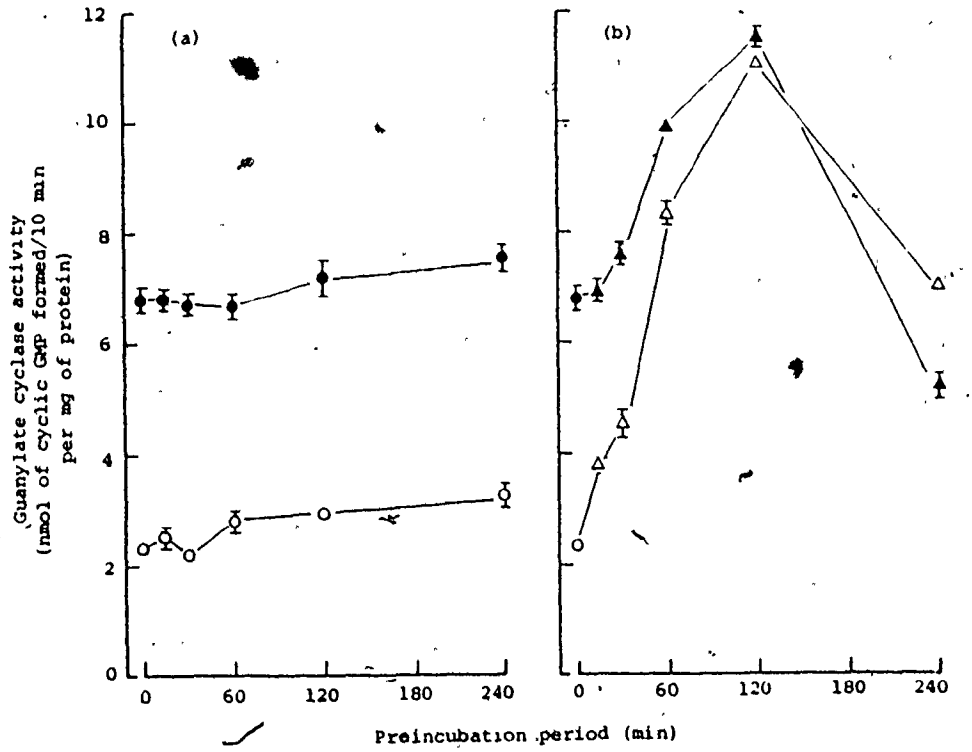


Table 4.2.1. *Effect of tert-butylhydroperoxide and N-ethylmaleimide on the guanylate cyclase activities of platelet lysate, preincubated lysate, tert-butylhydroperoxide- and N-ethylmaleimide-treated lysates*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared and preincubated at 37°C as described in Section 2.2.2. *Tert*-butylhydroperoxide (*t*-BHP)(1.0 mM-final concn. in the lysate) or *N*-ethylmaleimide (*N*-EM)(0.1 mM-final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. Guanylate cyclase activity is expressed as a percentage of control activity. The values given are the means of data from two experiments.

Enzyme preparation	Addition to the assay mixture	Concn. (mM)	Guanylate cyclase activity (% of control activity)
Lysate	None	-	100
	t-BHP	1.0	156
	N-EM	0.1	55
Preincubated lysate	None	-	211
	t-BHP	1.0	150
	N-EM	0.1	164
t-BHP-treated lysate	None	-	155
	N-EM	0.1	129
N-EM-treated lysate	None	-	54
	t-BHP	1.0	57

Fig. 4.2.7. *Effect of tert-butylhydroperoxide on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. Approximately 10 min prior to assay, *tert*-butylhydroperoxide was added to one portion of the platelet lysate to give a final concentration of 1.0 mM. The protein concentration of the platelet lysate (○) and the *tert*-butylhydroperoxide-treated lysate (●) was 4.8 mg/ml. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

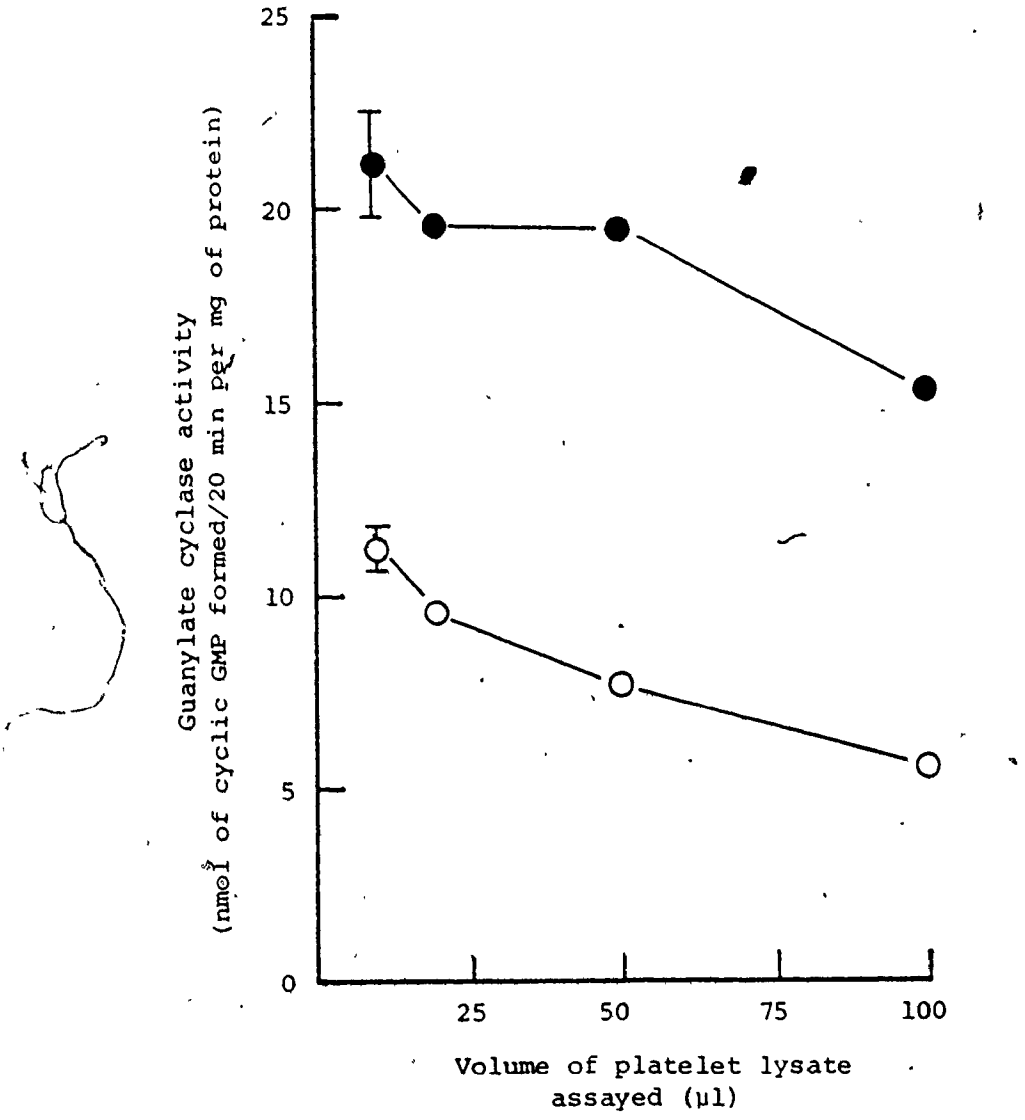


Table 4.2.2. *Effect of tert-butylhydroperoxide on the bivalent cation requirements of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3., and platelet lysate was prepared as described in Section 2.2.2. *Tert*-butylhydroperoxide (*t*-BHP)(1.0 mM-final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. The protein concentrations of the enzyme preparations were as follows: (a)Exp. 1, 6.9 mg/ml, 20 µl aliquots were assayed; and, (b)Exp. 2, 6.2 mg/ml, 25 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. In the presence of 4.0 mM-$MnCl_2$, *tert*-butylhydroperoxide stimulated the guanylate cyclase activity of platelet lysate by an average of 114% in the two experiments shown; whereas in the presence of 10.0 mM-$MgCl_2$, activity was stimulated by an average of only 15%.

Exp. no.	Enzyme preparation	Addition to the assay mixture	Bivalent cation present in assay	Guanylate cyclase activity	
				nmol cyclic GMP/ 20 min per mg protein	% stimulation by *t*-BHP
1	Lysate	None	$MnCl_2$ (4.0 mM)	5.77 ± 0.07	-
	t-BHP-treated lysate	None		13.56 ± 0.43	135
	Lysate	None	$MgCl_2$ (10.0 mM)	1.80 ± 0.30	-
	t-BHP-treated lysate	None		2.09 ± 0.14	16
2	Lysate	None	$MnCl_2$ (4.0 mM)	7.83 ± 0.25	-
		t-BHP (1.0 mM)		15.04 ± 0.19	92
	Lysate	None	$MgCl_2$ (10.0 mM)	1.08 ± 0.05	-
		t-BHP (1.0 mM)		1.22 ± 0.08	13

Table 4.2.3. *Effect of tert-butylhydroperoxide on the concentration of non-protein and protein-bound sulfhydryl groups in the lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3., and platelet lysates were prepared as described in Section 2.2.2. In Experiments 1 and 2, lysates were prepared from freshly drawn blood, and in Experiments 3 and 4, lysates were prepared from 4 to 5 day-old platelet concentrates. Approximately 10 min prior to assay, *tert*-butylhydroperoxide (*t*-BHP)(1.0 mM-final concn. in the lysate) was added to one portion of the lysate. The protein concentrations of the platelet lysates and the *tert*-butylhydroperoxide-treated lysates were as follows: (a)Exp. 1, 6.2 mg/ml; with 50 µl aliquots assayed; (b)Exp. 2, 6.9 mg/ml; with 25 µl aliquots assayed; (c)Exp. 3, 4.8 mg/ml; with 50 µl aliquots assayed; and, (d)Exp. 4, 6.0 mg/ml; with 25 µl aliquots assayed. The concentration of non-protein and protein-bound sulfhydryl groups present in the platelet lysates was assayed as described in Section 2.2.7.4.; the values given are the results of single determinations. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. The asterisk (*) indicates guanylate cyclase assays carried out for 10 instead of 20 min. *Tert*-butylhydroperoxide stimulated guanylate cyclase activity by 185 ± 25% (mean ± S.E.M. of the four experiments shown).

Exp. no.	Enzyme preparation	Concn. of sulfhydryl groups (nmol/mg of protein)		Guanylate cyclase activity	
		non-protein	protein	nmol cyclic GMP/ 20 min per mg protein	% stimulation by *t*-BHP
1	Lysate	15.0	165.0	4.61 ± 0.13	-
	t-BHP-treated lysate	1.0	164.0	16.86 ± 0.18	266
2	Lysate	12.5	162.0	5.77 ± 0.07	-
	t-BHP-treated lysate	0.5	156.0	13.56 ± 0.43	135
3	Lysate	5.0	143.3	7.55 ± 0.12	-
	t-BHP-treated lysate	0.6	134.4	19.43 ± 0.28	157
4	Lysate	4.8	135.2	2.14 ± 0.05*	-
	t-BHP-treated lysate	0.5	132.4	6.05 ± 0.17*	182

4.3. Results: Effects of sodium azide and sodium nitroprusside

Sodium azide (NaN_3), a potent metabolic inhibitor and strong nucleophile (Mittal & Murad, 1977a), greatly increased the guanylate cyclase activity of platelet lysate (Fig. 4.3.1.). With 10.0 mM-sodium azide, enzyme activity was stimulated by 335 ± 29% (mean ± S.E.M. of 14 experiments) ($2P<0.001$). Fig. 4.3.2. shows that sodium azide stimulated the activity in the supernatant fraction to a much greater extent than that in the particulate fraction.

As shown in Fig. 4.3.3., the specific activity of guanylate cyclase in sodium azide-treated lysate was decreased by about the same percentage as in the control lysate when 50 or 100 µl of enzyme was assayed. Thus, in contrast to preincubation or *tert*-butylhydroperoxide, sodium azide had no effect on the inhibition of guanylate cyclase activity by low-molecular-weight factors present in the lysate.

Table 4.3.1. shows that when sodium azide was added to the lysate immediately before assay, the activation of guanylate cyclase activity observed during the first 5 min of assay was much less than observed subsequently. This acceleration of activity during assay was greatly decreased when sodium azide was added to lysate that had already been preincubated and was decreased even more when the lysate was preincubated in the presence of sodium azide. However, preincubation of the lysate did not diminish the maximum activity of guanylate cyclase in the presence of sodium azide (Table 4.3.1.).

Activation of guanylate cyclase by sodium azide resulted in both

a significant increase in the effectiveness of Mg^{2+} as the sole cation and a marked decrease in the dependence of this enzyme preparation on Mn^{2+} for the expression of maximum activity (Table 4.3.4.). In percentage terms, the stimulatory effect of sodium azide was over two-fold greater when assays were carried out in the presence of 10.0 mM-$MgCl_2$ instead of with 4.0 mM-$MnCl_2$ (Table 4.3.4.). No change in the inhibition by ATP was observed with enzyme activated by sodium azide.

The effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate was studied as this agent is a potent oxidant of sulfhydryl groups (Grunert & Phillips, 1951; Leussing *et al.*, 1960). As shown in Fig. 4.3.4., enzyme activity was greatly increased with each concentration of this agent tested. In nine experiments, 1.0 mM-sodium nitroprusside stimulated platelet guanylate cyclase activity by 943 ± 112% (mean ± S.E.M.) ($2P<0.001$). Fig. 4.3.5. shows that the stimulatory effect of sodium nitroprusside, like that of sodium azide, was substantially larger in the supernatant fraction than in the particulate fraction.

As observed with preincubated or *tert*-butylhydroperoxide-treated lysate, the specific activity of sodium nitroprusside-treated lysate was not decreased when 50 or 100 µl of enzyme was assayed (Fig. 4.3.6.). It is of interest to note that this effect of sodium nitroprusside is in contrast to that found with enzyme activated by sodium azide (Fig. 4.3.3.).

As shown in Fig. 4.3.7., the stimulatory effect of sodium nitroprusside progressively decreased with increased periods of preincubation of the lysate at 30°C in the absence of sodium nitroprusside (identical results were obtained when lysate was preincubated at 37°C). After 120 min of preincubation at 30°C or at 37°C, sodium nitroprusside had no significant

effect on guanylate cyclase activity. The decrease in the stimulatory effect of sodium nitroprusside was approximately exponential with respect to preincubation time.

On the basis of the many similarities observed between the properties of guanylate cyclase in preincubated and *tert*-butylhydroperoxide-treated lysates, the effect of sodium nitroprusside on the activity of the latter enzyme preparation was studied. Table 4.3.2. shows that sodium nitroprusside stimulated the guanylate cyclase activity of *tert*-butylhydroperoxide-treated lysate by an average of about 30% (53 and 2% respectively, in two experiments). On the other hand, *tert*-butylhydroperoxide inhibited the activity of sodium nitroprusside-treated lysate by an average of only 16% (Table 4.3.2.). The observation that preincubation and *tert*-butylhydroperoxide-treatment of platelet lysate either blocked or subsequently reduced the stimulatory effect of sodium nitroprusside suggested that activation of guanylate cyclase by the latter agent may involve the oxidation of sulfhydryl groups on the enzyme and/or on a regulatory component, though there must be differences to account for the greater activity of sodium nitroprusside-treated lysate. To investigate this possibility, the effect of sodium nitroprusside on the activity of lysate treated with *N*-ethylmaleimide or dithiothreitol was studied. As shown in Table 4.3.3., sodium nitroprusside stimulated the guanylate cyclase activities of untreated-, *N*-ethylmaleimide (0.1 mM)- and dithiothreitol (1.0 mM)-treated lysates to almost the same level of activity (*i.e.* 585, 483 and 515% respectively, of control activity). Thus, in percentage terms, the magnitude of the stimulatory effect of sodium nitroprusside was substantially greater when added to *N*-ethylmaleimide- or dithiothreitol-treated lysate (Table 4.3.3.).

Fig. 4.3.8. compares the stimulatory effect of different concentrations of sodium nitroprusside on the activity of untreated- and dithiothreitol-treated lysates. In addition to these observations, it was found that 0.1 mM-*N*-ethylmaleimide inhibited the guanylate cyclase activity of sodium nitroprusside-treated lysate by nearly 75% less than it inhibited the activity of untreated lysate, whereas a ten-fold higher concentration of this agent decreased the activities of both enzyme preparations by about the same percentage (Table 4.3.3.). In contrast, 4.0 mM-excess dithiothreitol had no effect on the activity of sodium nitroprusside-treated lysate.

As observed with sodium azide, activation of guanylate cyclase by sodium nitroprusside resulted in both a significant increase in the effectiveness of Mg^{2+} as the sole cation and a marked decrease in the dependence of this enzyme preparation on Mn^{2+} for the expression of maximum activity (Table 4.3.4.). In percentage terms, the stimulatory effect of sodium nitroprusside was over two-fold greater when assays were carried out in the presence of 10.0 mM-$MgCl_2$ instead of with 4.0 mM-$MnCl_2$ (Table 4.3.4.). Thus, the bivalent cation requirements of guanylate cyclase activated by either of these agents are markedly different from those of enzyme activated by preincubation, disulfides or *tert*-butylhydroperoxide.

Fig. 4.3.1. *Effect of NaN_3 on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3, and platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 6.6 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the platelet lysate (○) was 9.21 ± 1.03 nmol of cyclic GMP formed/20 min per mg of protein. Guanylate cyclase activity is expressed as the percent stimulation by NaN_3. The values given are the means of triplicate determinations. Platelet lysate assayed in the presence of NaN_3 (●).

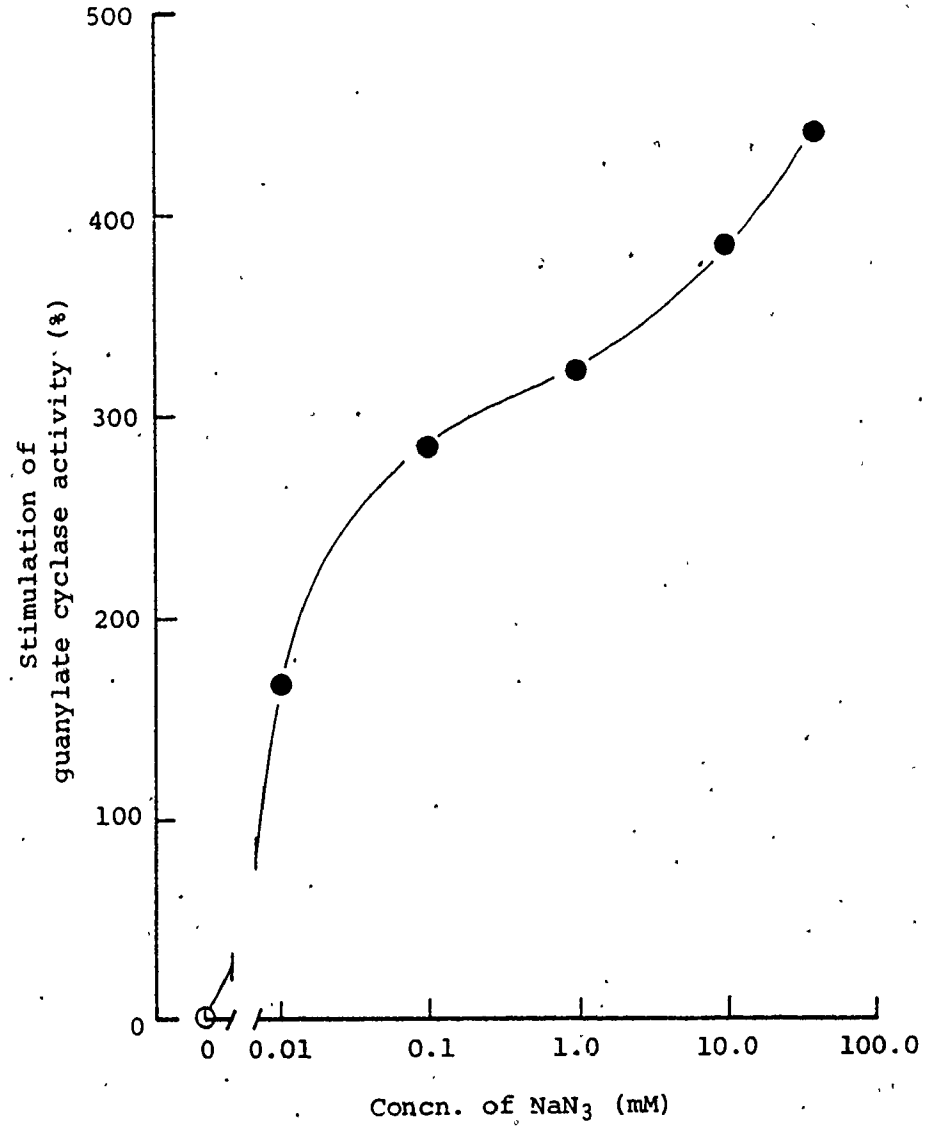


Fig. 4.3.2. *Effect of NaN_3 on the guanylate cyclase activities in the supernatant and particulate fractions of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction (O,●) was 4.5 mg/ml and that of the particulate fraction (△,▲) 3.3 mg/ml. In each case, 50 µl aliquots were assayed. The guanylate cyclase activity of the supernatant fraction assayed in the absence of NaN_3 (O) was 15.07 ± 1.07 nmol of cyclic GMP formed/20 min per mg of protein, and 5.20 ± 0.28 nmol of cyclic GMP formed/20 min per mg of protein in the particulate fraction (△)(mean ± S.E.M. of triplicate determinations). Guanylate cyclase activity is expressed as the percent stimulation by NaN_3. The values given are the means of triplicate determinations.

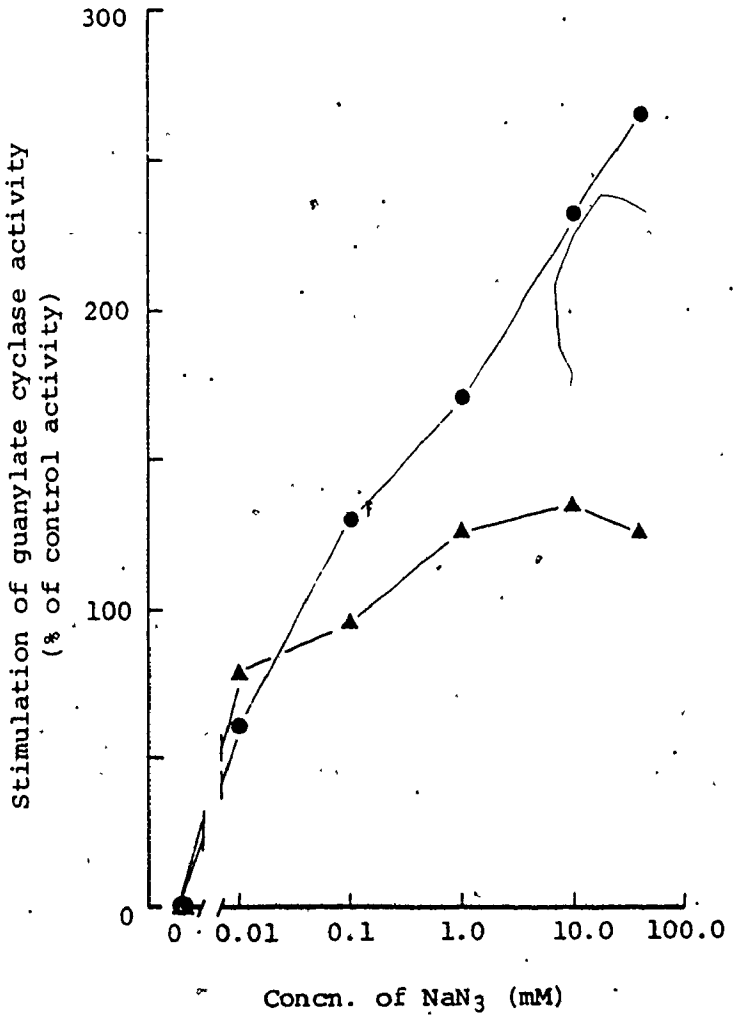


Fig. 4.3.3. *Effect of NaN_3 on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. Approximately 10 min prior to assay, NaN_3 was added to one portion of the platelet lysate to give a final concentration of 10.0 mM. The protein concentration of the platelet lysate (○) and the NaN_3-treated lysate (●) was 3.2 mg/ml. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

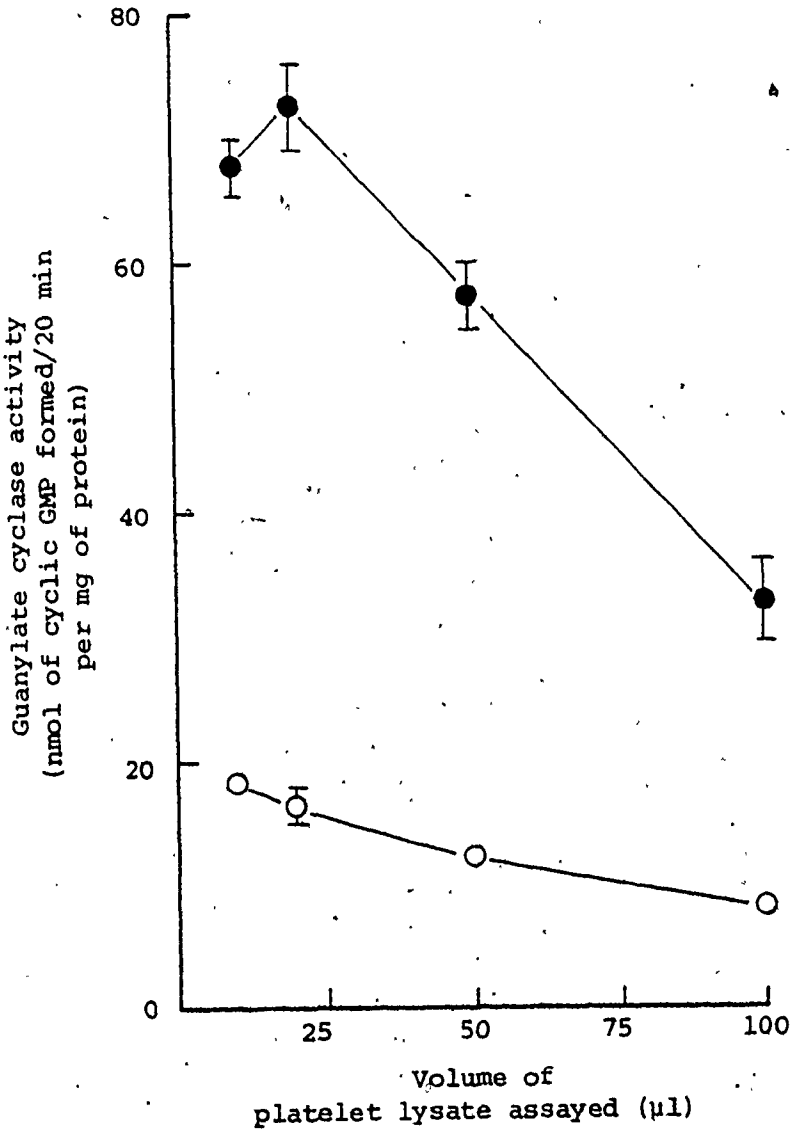


Table 4.3.1. *Effect of NaN_3 on the guanylate cyclase activities of platelet lysate and of preincubated lysate assayed for different periods*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was prepared and preincubated at 30°C as described in Section 2.2.2. NaN_3 (10.0 mM-final concn. in the lysate) was added to the lysate at 0°C approximately 10 min prior to assay, or was preincubated at 30°C as indicated below. The protein concentration of the enzyme preparations was 4.0 mg/ml, and 50 µl aliquots were assayed. Incubations were carried out for 5 or 20 min. Guanylate cyclase activity is expressed in terms of specific activity determined for the two sequential intervals; 0 to 5 min and 5 to 20 min. The ratio of the specific activities (5 to 20 min/0 to 5 min) reflects the acceleration of guanylate cycalse activity. The values given for guanylate cyclase activity are the means of triplicate determinations.

Enzyme preparation	Addition to the enzyme preparation	Preincubation at 30°C with addition (min)	Guanylate cyclase activity				Ratio of activity (5 to 20 min/0 to 5 min)
			0 to 5 min		5 to 20 min		
			nmol cyclic GMP/min per mg of protein	% of control activity	nmol cyclic GMP/min per mg of protein	% of control activity	
Lysate	None	-	0.14	100	0.30	100	2.14
	NaN_3	-	0.43	307	2.03	677	4.72
Preincubated lysate	None	0	0.34	100	0.44	100	1.29
	NaN_3	0	0.88	259	2.06	468	2.34
	NaN_3	60	1.93	258	2.36	536	1.22

Fig. 4.3.4. *Effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 7.7 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the platelet lysate (○) was 4.97 ± 0.10 nmol of cyclic GMP formed/20 min per mg of protein. Guanylate cyclase activity is expressed as the percent stimulation by sodium nitroprusside. The values given are the means of triplicate determinations. Platelet lysate assayed in the presence of sodium nitroprusside (●).

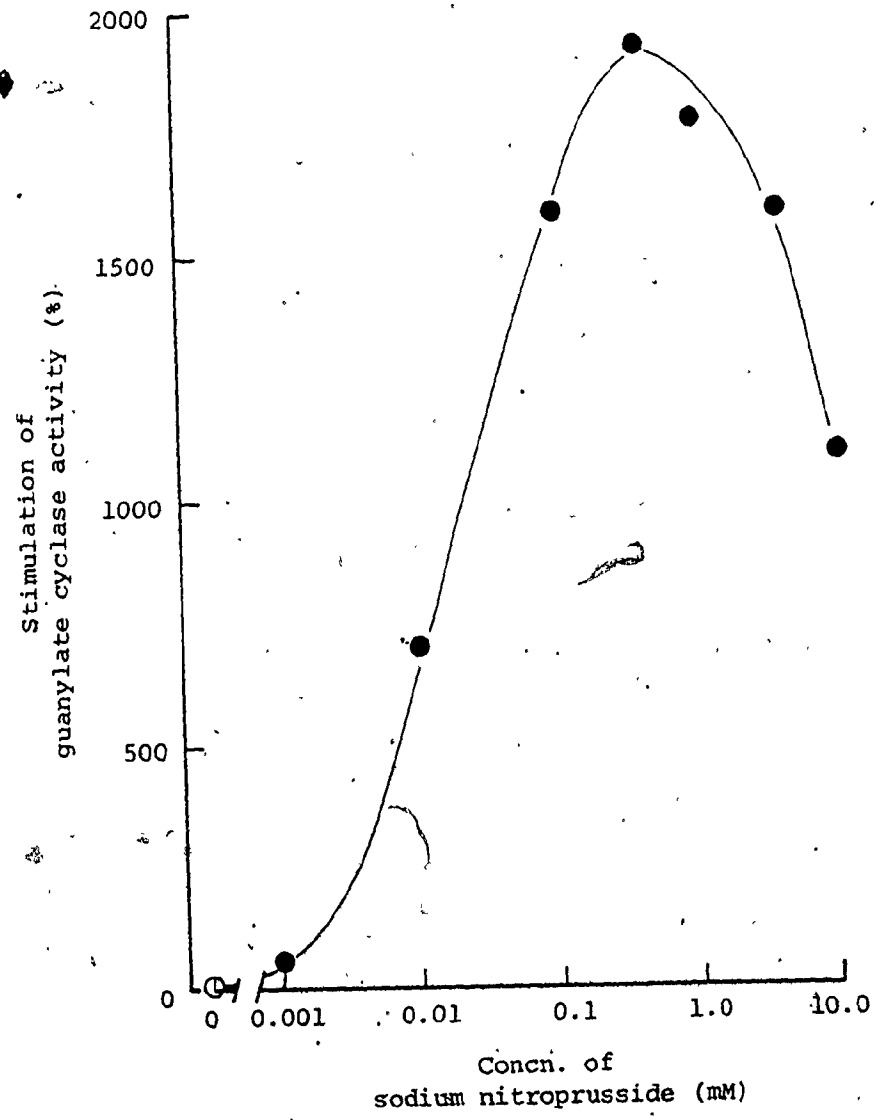


Fig. 4.3.5. *Effect of sodium nitroprusside on the guanylate cyclase activities in the supernatant and particulate fractions of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction (○,●) was 4.8 mg/ml and that of the particulate fraction (△,▲) was 3.5 mg/ml. In each case, 50 µl aliquots were assayed. The guanylate cyclase activity of the supernatant fraction assayed in the absence of sodium nitroprusside (○) was 15.07 ± 1.07 nmol of cyclic GMP formed/20 min per mg of protein, and 5.20 ± 0.28 nmol of cyclic GMP formed/20 min per mg of protein in the particulate fraction (△). Guanylate cyclase activity is expressed as the percent stimulation by sodium nitroprusside. The values given are the means of triplicate determinations. Supernatant and particulate fractions assayed in the presence of sodium nitroprusside, (●), (▲), respectively

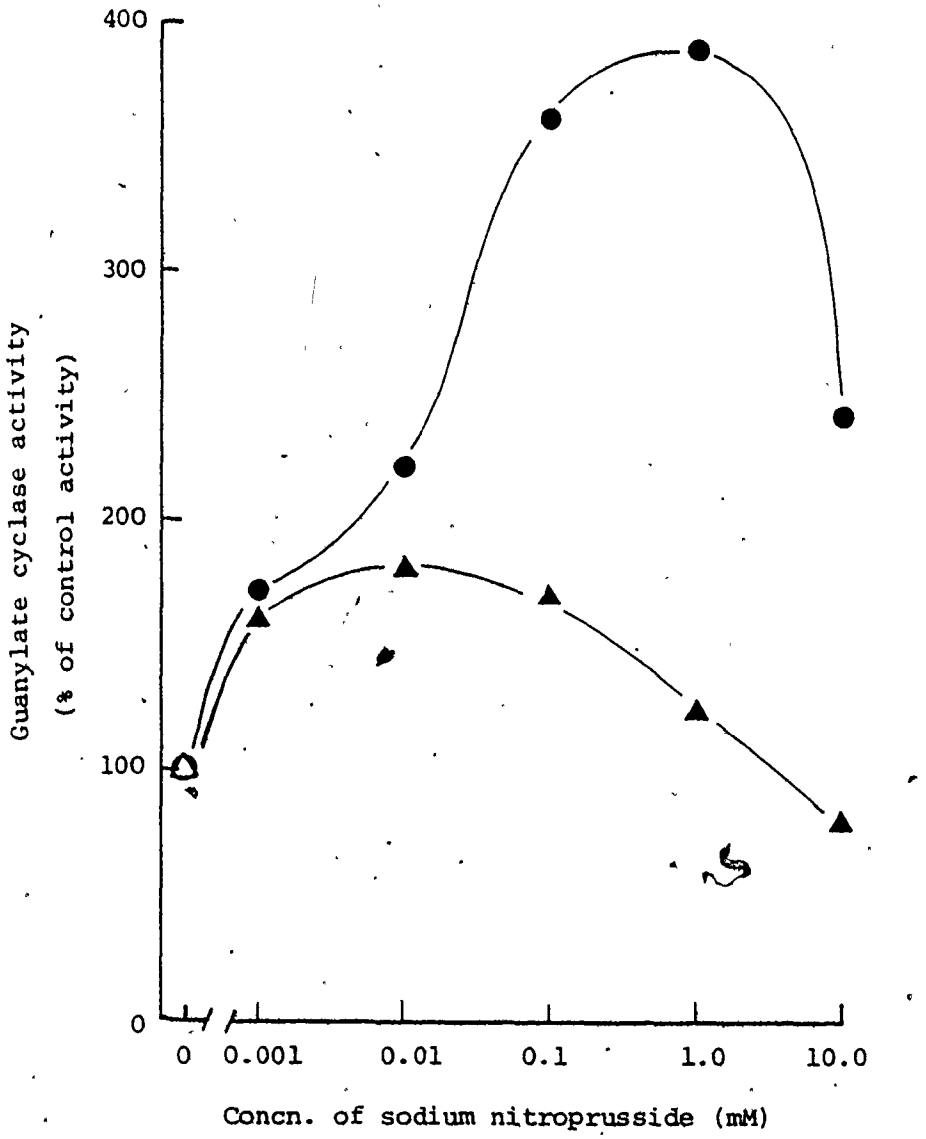


Fig. 4.3.6. *Effect of sodium nitroprusside on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. Approximately 10 min prior to assay, sodium nitroprusside was added to one portion of the platelet lysate to give a final concentration of 10.0 mM. The protein concentration of the platelet lysate (○) and the sodium nitroprusside-treated lysate (●) was 6.6 mg/ml. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols. This experiment has been carried out once.

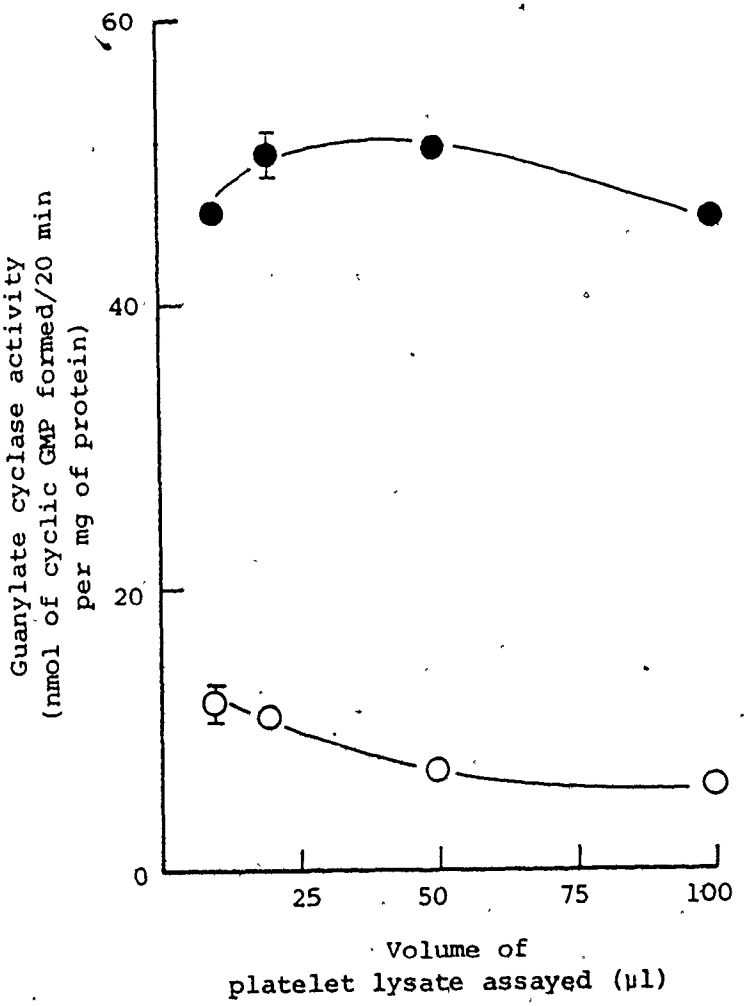


Fig. 4.3.7. *Effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate preincubated at 0° and at 30°C for increasing periods of time*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The platelet lysate was divided into two portions which were either kept on ice (circles) or preincubated (triangles) for 0, 20, 40, 60, 120 and 240 min at 30°C. The protein concentration of the platelet lysate and the preincubated lysate was 6.6 mg/ml and 25 µl aliquots were assayed. Sodium nitroprusside was added to the assay mixtures at a final concentration of 1.0 mM (closed symbols). Platelet lysate (○), platelet lysate assayed in the presence of sodium nitroprusside (●), preincubated lysate (△), preincubated lysate assayed in the presence of sodium nitroprusside (▲). The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

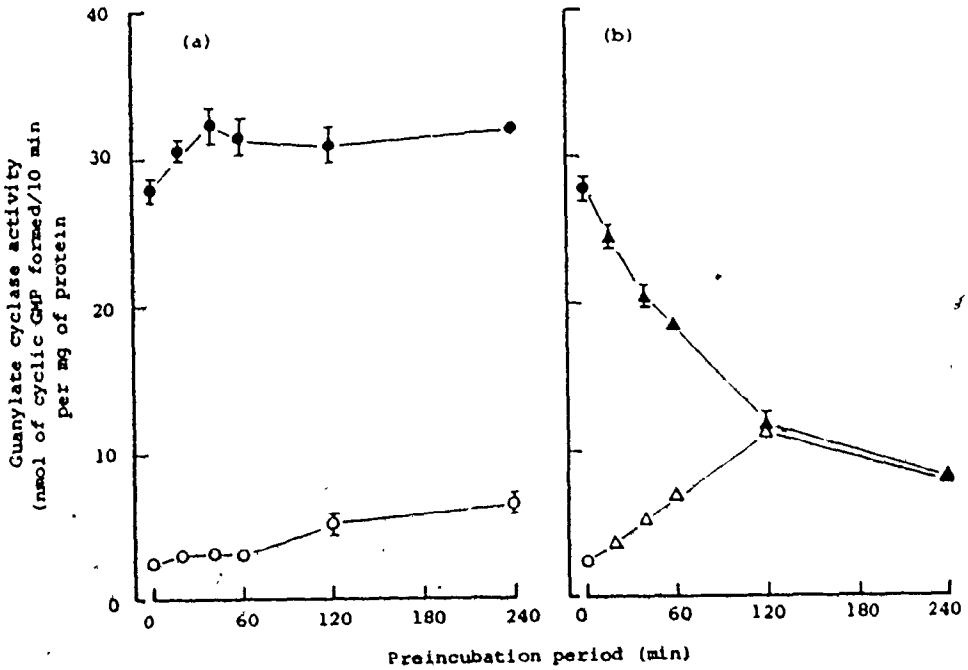


Table 4.3.2. *Effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate, preincubated lysate and tert-butylhydroperoxide-treated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. Platelet lysate was prepared and preincubated at 37°C as described in Section 2.2.2. *Tert*-butylhydroperoxide (*t*-BHP)(1.0 mM-final concn. in the lysate) or sodium nitroprusside (SNP)(1.0 mM-final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. Guanylate cyclase activity is expressed as a percentage of control activity and as percentage of the change in activity of each treatment. The values given are the means of data from two experiments.

Enzyme preparation	Addition to the assay mixture	Concn. (mM)	Guanylate cyclase activity	
			% of control activity	% change in activity
Lysate	None	-	100	-
	SNP	1.0	585	+485
Preincubated lysate	None	-	211	-
	SNP	1.0	183	-13
t-BHP-treated lysate	None	-	155	-
	SNP	1.0	198	+28
SNP-treated lysate	None	-	579	-
	t-BHP	1.0	530	-16

Table 4.3.3. *Effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate, N-ethylmaleimide- and dithiothreitol-treated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. *N*-ethylmaleimide (*N*-EM)(0.1 mM-final concn. in the lysate), dithiothreitol (DTT)(1.0 mM-final concn. in the lysate), or sodium nitroprusside (SNP)(1.0 mM-final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. Guanylate cyclase activity is expressed as a percentage of control activity. The values given are the means of data from two experiments.

Enzyme preparation	Addition to the assay mixture	Concn. (mM)	Guanylate cyclase activity	
			% of control activity	% change in activity
Lysate	None	-	100	-
	SNP	1.0	585	+485
	N-EM	0.1	55	-45
		1.0	18	-88
N-EM-treated lysate	None	-	54	-
	SNP	1.0	483	+808
DTT-treated lysate	None	-	41	-
	SNP	1.0	515	+1153
SNP-treated lysate	None	-	579	-
	N-EM	0.1	508	-12
		1.0	135	-77

Fig. 4.3.8. *Effect of sodium nitroprusside on the guanylate cyclase activity of platelet lysate and dithiothreitol-treated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. Approximately 10 min prior to assay, dithiothreitol (1.0 mM-final concentration in the lysate, 0.1 mM-final concentration in the assay mixture) was added to one portion of the platelet lysate. The protein concentration of the platelet lysate (○,●) and the dithiothreitol-treated lysate (△,▲) was 6.6 mg/ml and 25 µl aliquots were assayed. The guanylate cyclase activity of the lysate (○) was 9.21 ± 1.03 nmol of cyclic GMP formed/20 min per mg of protein, and that of the dithiothreitol-treated lysate (△) was 2.91 ± 0.24 nmol of cyclic GMP formed/20 min per mg of protein. Guanylate cyclase activity is expressed as the percent stimulation by sodium nitroprusside. The values given are the means of triplicate determinations. Platelet lysate assayed in the presence of sodium nitroprusside (●), dithiothreitol-treated lysate assayed in the presence of sodium nitroprusside (▲).

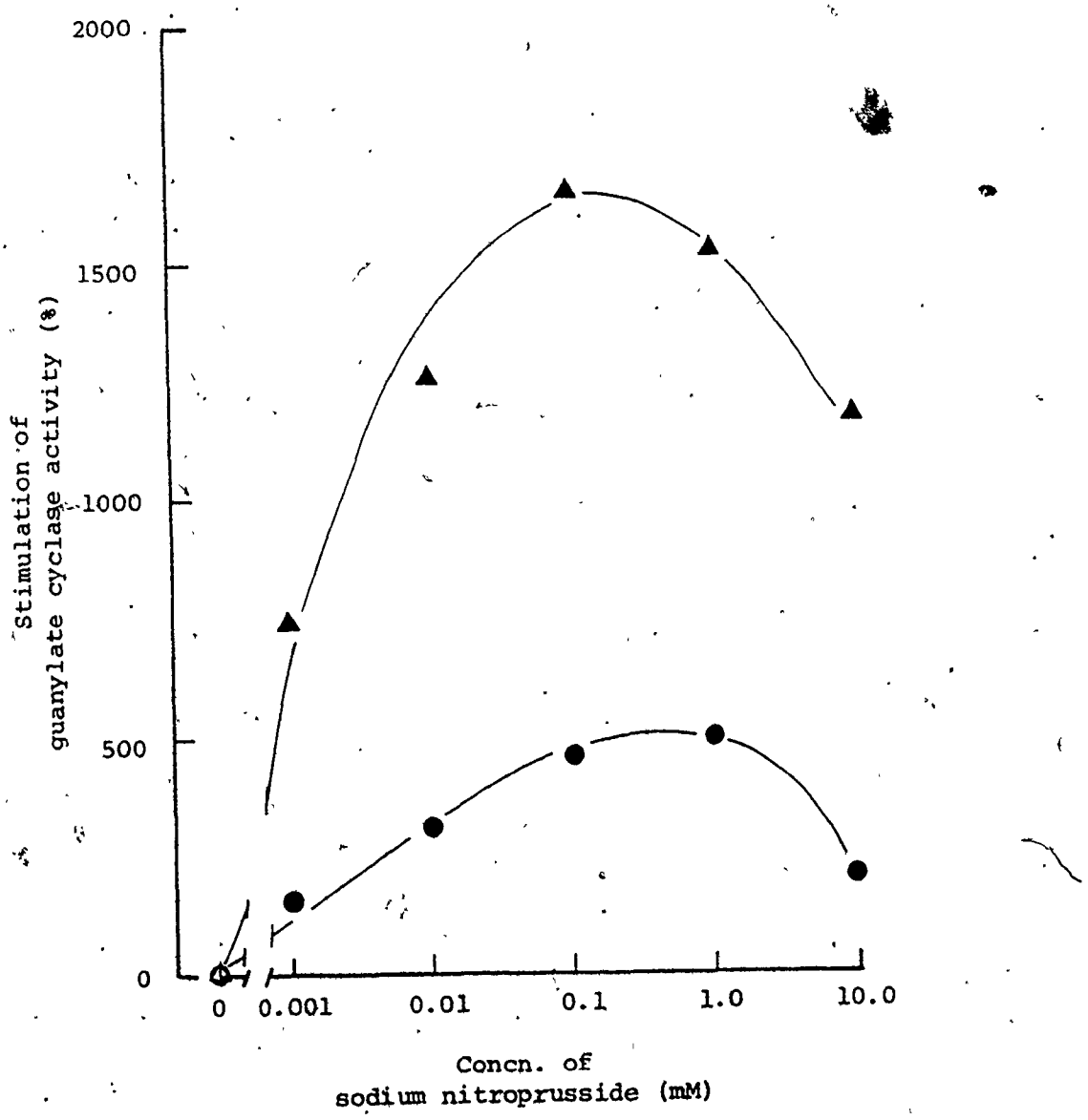


Table 4.3.4. *Effect of NaN_3 and sodium nitroprusside on the bivalent cation requirements of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3., and platelet lysate was prepared as described in Section 2.2.2. NaN_3 (10.0 mM-final concn. in the lysate) or sodium nitroprusside (SNP) (1.0 mM-final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. The protein concentrations of the enzyme preparations were as follows: (a) Experiment 1, 6.9 mg/ml, 20 µl aliquots were assayed; and (b) Experiment 2, 6.2 mg/ml, 25 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. In the presence of 4.0 mM-$MnCl_2$, NaN_3 stimulated the guanylate cyclase activity of platelet lysate by an average of 411% in the two experiments shown; whereas in the presence of $MgCl_2$, NaN_3 stimulated enzyme activity by an average of 1056%. With sodium nitroprusside, guanylate cyclase activity was stimulated by an average of 1160% in the presence of $MnCl_2$, and by an average of 2453% in the presence of $MgCl_2$.

Exp. no.	Enzyme preparation	Addition to the assay mixture	Bivalent cation present in assay	Guanylate cyclase activity	
				nmol cyclic GMP/ 20 min per mg protein	% stimulation
1	Lyate	None	$MnCl_2$ (4.0 mM)	5.77 ± 0.07	-
	NaN_3-treated lysate	None		22.66 ± 0.29	293
	SNP-treated lysate	None		79.00 ± 0.38	1222
	Lysate	None	$MgCl_2$ (10.0 mM)	1.80 ± 0.30	-
	NaN_3-treated lysate	None		10.97 ± 0.14	509
	SNP-treated lyate	None		37.01 ± 0.29	1956
2	Lysate	None	$MnCl_2$ (4.0 mM)	7.83 ± 0.25	-
		NaN_3 (10.0 mM)		49.26 ± 3.76	529
		SNP (1.0 mM)		93.80 ± 1.24	1098
	Lysate	None	$MgCl_2$ (10.0 mM)	1.08 ± 0.05	-
		NaN_3 (10.0 mM)		18.38 ± 0.05	1602
		SNP (1.0 mM)		32.95 ± 0.63	2950

4.4. Results: Effects of non-ionic detergents

The effects of two non-ionic detergents, Lubrol PX and Triton X-100, on the guanylate cyclase activity of platelet lysate were studied (Fig. 4.4.1.). Lubrol PX was chosen for use in all subsequent studies as this non-ionic detergent was about four-fold more effective than Triton X-100 at all concentrations tested (Fig. 4.4.1.). In 16 experiments, the guanylate cyclase activity of platelet lysate was stimulated by 256 ± 16% (mean ± S.E.M.) with an optimal concentration of 1.0% (w/v)-Lubrol PX (44 mM). Optimal activation by 1.0% (w/v)-Lubrol PX was observed whether this concentration of detergent was included in the assay mixture (see for example, Figs. 4.4.1., 4.4.4. and Tables 4.4.3., 4.4.4.) or added directly to the lysate before assay (*e.g.* Fig. 4.4.3. and Tables 4.4.1., 4.4.4.). Thus, the extent of activation by Lubrol PX depended on the highest concentration to which the enzyme was exposed and not on the final concentration in the assay mixture.

Fig. 4.4.2. shows that the guanylate cyclase activity in the supernatant and particulate fractions of both isotonically and hypotonically lysed platelets was stimulated by an optimal concentration of 1.0% (w/v)-Lubrol PX. Although the stimulatory effect of Lubrol PX was slightly different in each of these enzyme preparations, no change in the relative distribution of guanylate cyclase activity was observed with 1.0% (w/v) of detergent (see also Table 3.1.1.).

Fig. 4.4.3. shows that the specific activity of guanylate cyclase of Lubrol PX-treated lysate was decreased by about the same percentage as

that of control lysate when 50 or 100 µl of enzyme was assayed. Hence, in contrast to activation by preincubation, *tert*-butylhydroperoxide and sodium nitroprusside, activation by Lubrol PX had no effect on the inhibition of guanylate cyclase activity by low-molecular-weight factors present in the lysate.

The guanylate cyclase activity of Lubrol PX-treated lysate, unlike that of untreated lysate, did not increase during assay (Table 4.4.1.). Treatment of lysate that had been preincubated at 30°C for 60 min with Lubrol PX did not result in an additive stimulation of enzyme activity; instead, the specific activity of this preparation was either equal to or slightly lower than that of Lubrol PX-treated lysate that had not been preincubated (Tables 4.4.1. and 4.4.2.). As shown in Fig. 4.4.4., the stimulation of guanylate cyclase activity by Lubrol PX diminished with increasing periods of preincubation at 37°C. After 120 min of preincubation at 37°C, Lubrol PX significantly inhibited the activity of platelet lysate. Similar results were obtained with lysate preincubated at 30°C. Preincubation at 30°C in the presence of Lubrol PX resulted in a substantial inhibition of guanylate cyclase activity (Table 4.4.1.).

To investigate the properties of Lubrol PX-activated guanylate cyclase, the effects of dithiothreitol and of *N*-ethylmaleimide were studied. Lubrol PX stimulated the activity of platelet lysate pretreated with dithiothreitol (Table 4.4.2.) or with *N*-ethylmaleimide (Table 4.4.3.) by approximately the same extent, in percentage terms, as untreated lysate. Thus, in contrast to activation of platelet guanylate cyclase by preincubation or *tert*-butylhydroperoxide, activation by Lubrol PX was not blocked by either dithiothreitol or *N*-ethylmaleimide. These observations suggest

that activation by Lubrol PX does not involve oxidation of sulfhydryl groups on the enzyme and/or on regulatory components.

The effect of Lubrol PX on the guanylate cyclase activity of lysate treated with *tert*-butylhydroperoxide, sodium azide and sodium nitroprusside was also studied. Table 4.4.3. shows that when each of these enzyme preparations was assayed in the presence of Lubrol PX, the resulting level of guanylate cyclase activity was about the same as that observed with detergent alone. Thus, in the case of *tert*-butylhydroperoxide-treated lysate, enzyme activity was slightly stimulated by Lubrol PX; whereas in the case of sodium azide- or sodium nitroprusside-treated lysate, activity was markedly inhibited. Addition of these agents to Lubrol PX-treated lysate either had no significant effect (*i.e.* sodium azide and sodium nitroprusside) or only a small inhibitory effect (*i.e. tert*-butylhydroperoxide) (Table 4.4.3.).

Assays were also carried out with Mg^{2+} as the sole cation in order to determine whether activation of guanylate cyclase by Lubrol PX affected the requirement of this enzyme preparation for Mn^{2+} for the expression of maximum activity. Table 4.4.4. shows that in the presence of 4.0 mM-$MnCl_2$, Lubrol PX stimulated activity by an average of 279%; whereas in the presence of 10.0 mM-$MgCl_2$, activity was increased by an average of only 74%. Thus, total expression of the stimulatory effect of Lubrol PX was dependent on the presence of an optimal concentration of $MnCl_2$, and the effectiveness of Mg^{2+} as a substitute for Mn^{2+} was markedly decreased with Lubrol PX-treated lysate (Table 4.4.4.). Therefore, with respect to bivalent cation requirements, preincubation, *tert*-butylhydroperoxide and Lubrol PX have similar effects on platelet guanylate cyclase.

Fig. 4.4.1. *Effect of Lubrol PX and Triton X-100 on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. Platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the lysate was 6.6 mg/ml and 50 µl aliquots were assayed. Platelet lysate was added to assay mixtures containing Lubrol PX or Triton X-100 at the final concentrations indicated on the abscissa. Guanylate cyclase activity is expressed as the percentage stimulation by Lubrol PX or Triton X-100. The values given are the means of triplicate determinations. In this experiment, the guanylate cyclase activity of platelet lysate assayed in the absence of non-ionic detergent was 5.20 ± 0.22 nmol of cyclic GMP formed/20 min per mg of protein. No addition (△), Lubrol PX (●), Triton X-100 (▲).

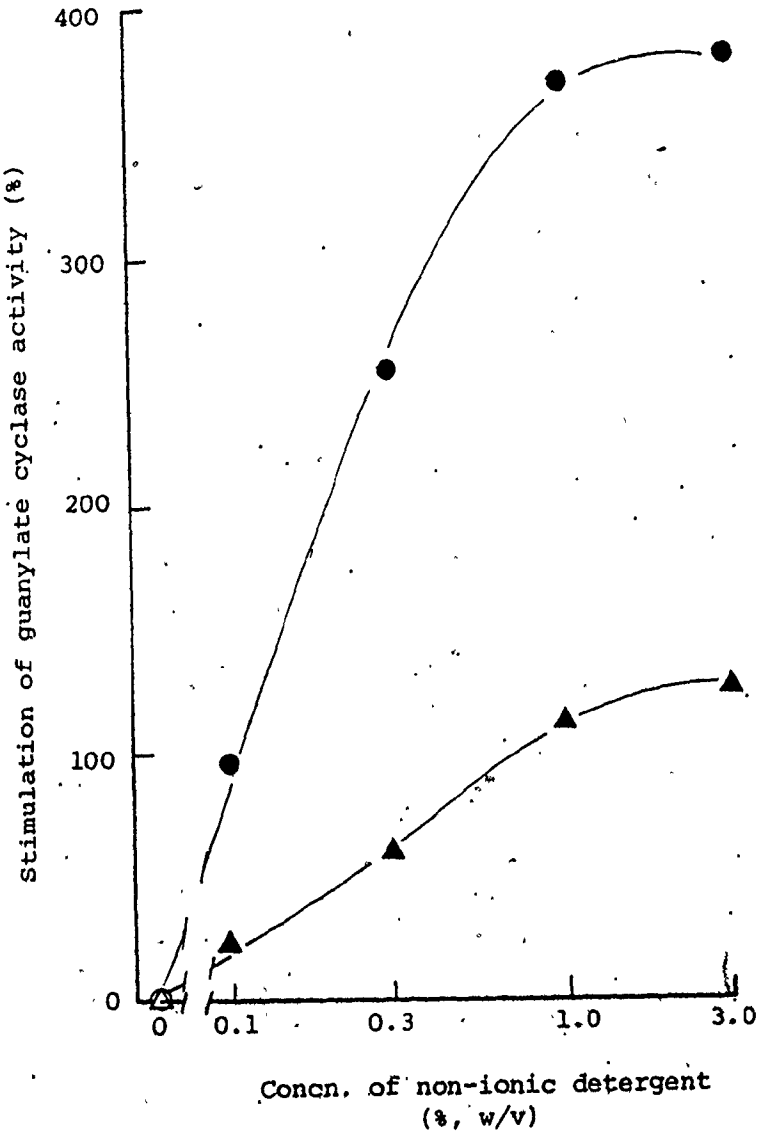


Fig. 4.4.2. *Effect of Lubrol PX on the guanylate cyclase activities in supernatant and particulate fractions prepared from platelets lysed in isotonic or hypotonic medium*

A suspension of washed platelets was prepared as described in Section 2.2.1. This suspension was divided into two portions which were centrifuged as described in Section 2.2.1. and the pellets were then resuspended in either isotonic (150 mM-Tris/HCl, pH 7.4)(a) or hypotonic (60 mM-Tris/HCl, pH 7.4) medium (b). The platelet suspensions were then lysed by freezing and thawing as described in Section 2.2.2. The lysates were centrifuged at 48 000*g* for 30 min at 4 C and each particulate fraction was resuspended to the volume of the corresponding supernatant with the original buffer. The protein concentrations of the enzyme preparations were as follows: (a) isotonic supernatant fraction (●), 3.0 mg/ml; isotonic particulate fraction (▲), 2.5 mg/ml; (b)hypotonic supernatant fraction (○), 2.5 mg/ml, and hypotonic particulate fraction (△), 1.2 mg/ml. In each case, 50 µl aliquots of the enzyme preparation were assayed. Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. The guanylate cyclase activities of the enzyme preparations assayed in the absence of Lubrol PX (a,■)(b,□) were as follows: isotonic supernatant, 2.62 ± 0.16; isotonic particulate, 1.08 ± 0.06; hypotonic supernatant, 8.08 ± 0.37; hypotonic particulate, 1.11 ± 0.11 nmol of cyclic GMP formed/20 min per mg of protein (mean ± S.E.M. of triplicate determinations). Guanylate cyclase activity is expressed as a percentage of control activity. The values given are the means of triplicate determinations. The data shown in (a) and (b) are from separate experiments.

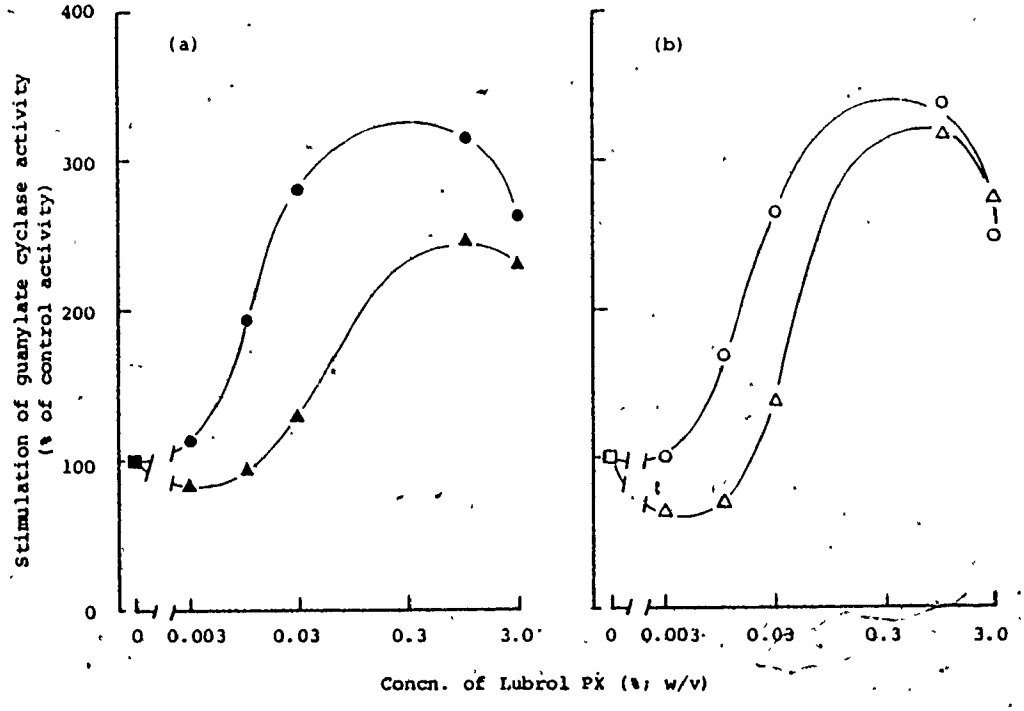


Fig. 4.4.3. *Effect of Lubrol PX on the specific activity of guanylate cyclase measured with different volumes of platelet lysate in the assay*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. Platelet lysate was prepared as described in Section 2.2.2. Approximately 10 min prior to assay, Lubrol PX was added to one portion of the platelet lysate to give a final concentration of 1.0% (w/v). The protein concentration of the platelet lysate (●) was 6.6 mg/ml, and that of the Lubrol PX-treated lysate (▲), 5.9 mg/ml. Increasing volumes of enzyme preparation were assayed by the addition of 10, 20, 50 and 100 µl to the assay mixtures, which were incubated for 20 min. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

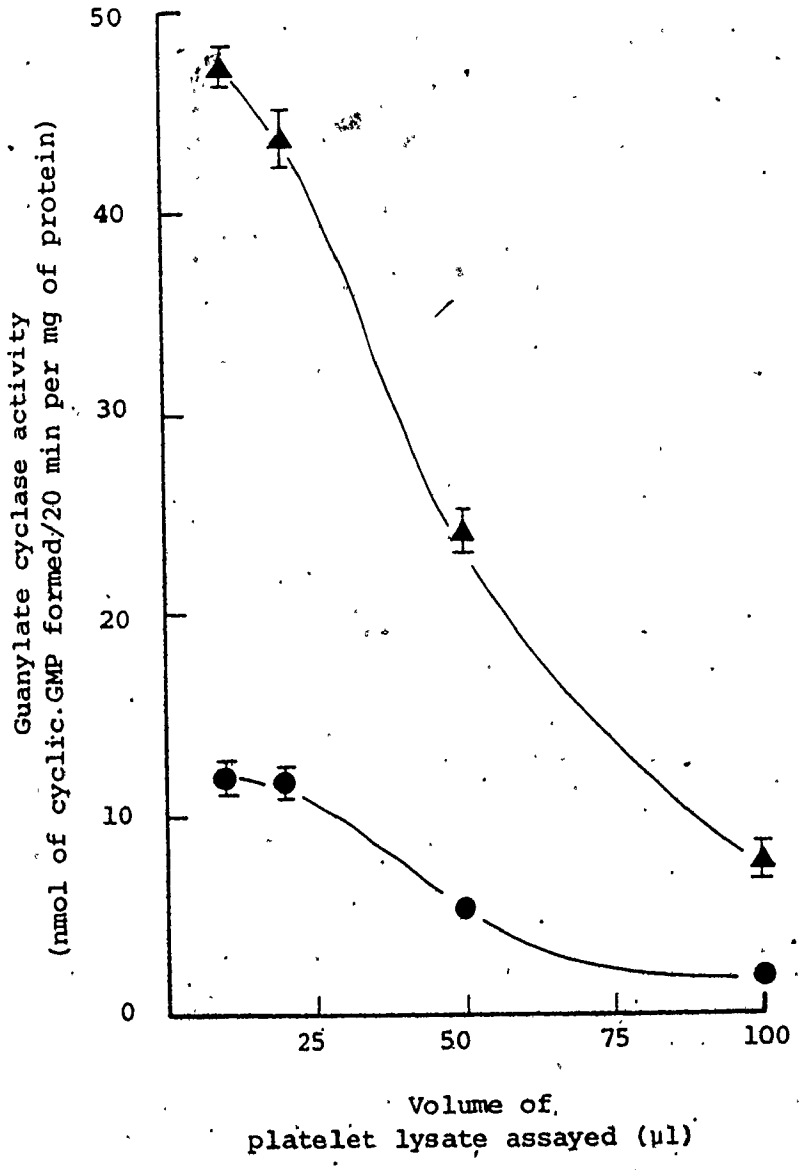


Table 4.4.1. *Effect of Lubrol PX on the guanylate cyclase activities of platelet lysate and of preincubated lysate assayed for different periods*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP- regenerating system was included in the assay mixtures. Platelet lysate was prepared and preincubated at 30°C as described in Section 2.2.2. Lubrol PX (1.0%, w/v - final concn. in the lysate) was added to the lysate at 0°C approximately 10 min prior to assay, or was preincubated at 30°C as indicated below. The protein concentration of the enzyme preparation was 4.0 mg/ml and 50 µl aliquots were assayed. Incubations were carried out for 5 or 20 min. Guanylate cyclase activity is expressed in terms of specific activity determined for the two sequential intervals: 0 to 5 min and 5 to 20 min. The ratio of the specific activities (5 to 20 min/0 to 5 min) reflects the acceleration of guanylate cyclase activity. The values given for guanylate cyclase activity are the means of triplicate determinations.

Enzyme preparation	Addition to the enzyme preparation	Preincubation at 30 °C with addition (min)	Guanylate cyclase activity				Ratio of activity (5 to 20 min/0 to 5 min)
			0 to 5 min		5 to 20 min		
			nmol cyclic GMP/ min per mg of protein	% of control activity	nmol cyclic GMP/ min per mg of protein	% of control activity	
Lysate	None	-	0.14	100	0.30	100	2.14
	Lubrol PX	-	0.92	657	1.02	340	1.11
Preincubated lysate	None	0	0.34	100	0.44	100	1.29
	Lubrol PX	0	0.86	253	0.88	200	1.02
	Lubrol PX	60	0.18	53	0.17	39	0.94

Table 4.4.2. *Effect of dithiothreitol-treatment of platelet lysate on the guanylate cyclase activities observed before and after preincubation*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was prepared as described in Section 2.2.2. and preincubated for 80 min at 30°C with and without dithiothreitol (5.0 mM-final concn. in the lysate). The protein concentration of the platelet lysate and the dithiothreitol-treated lysate was 3.4 mg/ml and 50 µl aliquots were assayed. Guanylate cyclase activity was assayed before and after preincubation both in the presence and absence of Lubrol PX added to the assay mixture at a final concentration of 1.0% (w/v). The values given are the means ± S.E.M. of triplicate determinations.

Enzyme preparation	Addition to the assay	Guanylate cyclase activity (nmol of cyclic GMP/10 min per mg of protein)		Ratio of guanylate cyclase activities (after/before preincubation)
		Before preincubation	After preincubation	
Lysate	None	8.64 ± 0.12	14.41 ± 0.18	1.67
	Lubrol PX	39.83 ± 0.65	42.00 ± 0.42	1.05
Dithiothreitol-treated lysate	None	2.49 ± 0.12	2.85 ± 0.06	1.14
	Lubrol PX	9.97 ± 0.24	9.26 ± 0.06	0.93

Fig. 4.4.4. *Effect of Lubrol PX on the guanylate cyclase activity of platelet lysate preincubated at 0° and at 37°C*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The platelet lysate was divided into two portions which were either kept on ice (circles) or preincubated (triangles) for 0, 15, 30, 60, 120 and 240 min at 37°C. The protein concentration of the platelet lysate and the preincubated lysate was 7.3 mg/ml and 25 µl aliquots were assayed. Lubrol PX was added to the assay mixtures at a final concentration of 1.0% (w/v) (closed symbols). Platelet lysate (○), platelet lysate assayed in the presence of Lubrol PX (●), preincubated lysate (△), preincubated lysate assayed in the presence of Lubrol PX (▲). The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. Unless otherwise indicated, the S.E.M. are within the limits of the symbols.

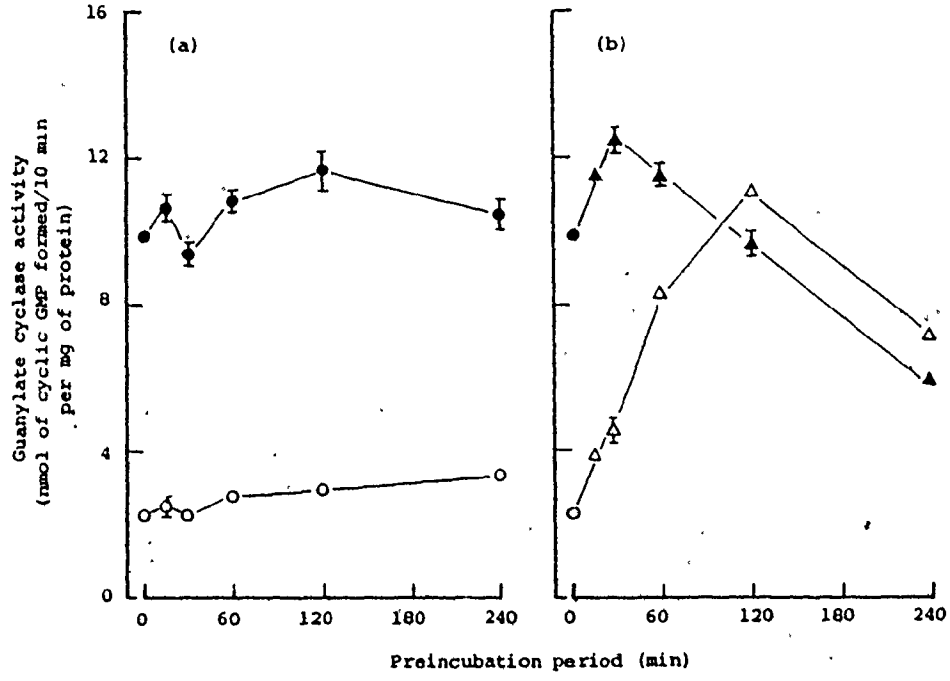


Table 4.4.3. *Effect of Lubrol PX on the guanylate cyclase activity of platelet lysate, tert-butylhydroperoxide-, sodium nitroprusside-, NaN_3- and N-ethylmaleimide-treated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. Lubrol PX (1.0%, w/v -final concn. in the lysate), *tert*-butylhydroperoxide (*t*-BHP) (1.0 mM-final concn. in the lysate), sodium nitroprusside (SNP) (1.0 mM-final concn. in the lysate), NaN_3 (10.0 mM-final concn. in the lysate) or *N*-ethylmaleimide (*N*-EM) (0.1 mM-final concn. in the lysate) was added to a portion of the lysate approximately 10 min prior to assay. Guanylate cyclase activity is expressed as a percentage of control activity. The values given are the means of data from two experiments. *Indicates values from only one experiment.

Enzyme preparation	Addition to the assay mixture	Concn.	Guanylate cyclase activity	
			% of control activity	% change in activity due to Lubrol PX
Lysate	None	-	100	-
	Lubrol PX	1.0%	233	+133
Lubrol PX-treated lysate	None	-	249	+149
N-EM-treated lysate	None	-	54	-
	Lubrol PX	1.0%	128	+149
Lubrol PX-treated lysate	*N*-EM	0.1 mM	142.	+166
t-BHP-treated lysate	None	-	155	-
	Lubrol PX	1.0%	249	+61
Lubrol PX-treated lysate	*t*-BHP	1.0 mM	192	+24
SNP-treated lysate	None	-	579	-
	Lubrol PX	1.0%	255	-55
Lubrol PX-treated lysate	SNP	1.0 mM	243	-52
NaN_3-treated lysate	None	-	444*	-
	Lubrol PX	1.0%	211*	-52
Lubrol PX-treated lysate	NaN_3	10.0 mM	158*	-64

Table 4.4.4. *Effect of Lubrol PX on the bivalent cation requirements of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3., and platelet lysate was prepared as described in Section 2.2.2. Lubrol PX (1.0%, w/v -final concn. in the lysate) was added to one portion of the lysate approximately 10 min prior to assay. The protein concentration of the enzyme preparations were as follows: (a)Experiment 1, 6.9 mg/ml, 20 µl aliquots were assayed; and, (b)Experiment 2, 6.2 mg/ml, 25 µl aliquots were assayed. The values for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. In the presence of 4.0 mM-$MnCl_2$, Lubrol PX stimulated the guanylate cyclase activity of platelet lysate by an average of 279% in the two experiments shown; whereas in the presence of 10.0 mM-$MgCl_2$, Lubrol PX stimulated enzyme activity by an average of 74%.

Exp. no.	Enzyme preparation	Addition to the assay mixture	Bivalent cation present in assay	Guanylate cyclase activity: nmol cyclic GMP/ 20 min per mg protein	Guanylate cyclase activity: % stimulation
1	Lysate	None	$MnCl_2$ (4.0 mM)	5.77 ± 0.07	-
	Lubrol PX-treated lysate	None		26.05 ± 0.14	352
	Lysate	None	$MgCl_2$ (10.0 mM)	1.80 ± 0.30	-
	Lubrol PX-treated lysate	None		2.89 ± 0.07	61
2	Lysate	None	$MnCl_2$ (4.0 mM)	7.83 ± 0.25	-
		Lubrol PX (1.0%)		23.90 ± 0.65	205
	Lysate	None	$MgCl_2$ (10.0 mM)	1.08 ± 0.05	-
		Lubrol PX (1.0%)		2.02 ± 0.02	87

4.5. *Results: Effects of fatty acids*

The effects of inducers of platelet aggregation on the guanylate cyclase activity of platelet lysate were tested. In contrast with 5-hydroxytryptamine (10 μM) and *l*-epinephrine (20 μM), which were without effect, and with ADP (100 μM), which inhibited enzyme activity by about 30% (measured in the absence of phosphocreatine and creatine phosphokinase), arachidonate stimulated the activity of platelet guanylate cyclase (Table 4.5.1.). These agents had similar effects on the guanylate cyclase activity in supernatant and particulate fractions of platelet lysate (Table 4.5.2.). The specificity and relevance of the effect of arachidonate on the guanylate cyclase activity of platelet lysate were studied further.

At an optimal concentration of 1.0 mM-fatty acid, the guanylate cyclase activity of platelet lysate was stimulated by 245 ± 28% (mean ± S.E.M. of four experiments) by arachidonate and by 206 ± 20% (mean ± S.E.M. of seven experiments) by oleate, whereas palmitate was almost inactive (see for example, Table 4.5.3.). Similar results were obtained with supernatant and particulate fractions of platelet lysate. Maximum stimulation of guanylate cyclase activity by arachidonate or oleate was dependent on the presence of bovine serum albumin in the assay mixture (as in Fig. 4.5.1.). When assays were carried out in the presence of fatty acid-free albumin (Fig. 4.5.1.b.) or albumin with fatty acid (*i.e.* typical assay conditions)(Fig. 4.5.1.c.), arachidonate stimulated enzyme activity to a much greater extent than oleate at all concentrations of fatty acid tested. However, without added albumin (Fig. 4.5.1.a.), oleate stimulated guanylate

cyclase activity somewhat more than arachidonate at concentrations greater than 0.1 mM. Furthermore, at lower concentrations (1 to 10 μM) at which oleate had no significant effect, arachidonate stimulated activity by a small percentage (*e.g.* about 60% with 4 μM)(Fig. 4.5.1.a.).

To investigate the physiological relevance of the stimulation of guanylate cyclase activity by arachidonate, the effect of indomethacin, a fatty acid cyclo-oxygenase inhibitor, was studied. Table 4.5.3. shows that indomethacin had no effect on either the basal or fatty acid-stimulated guanylate cyclase activities measured in the presence of albumin; thus the observed effect of arachidonate cannot be attributed to its metabolism to prostaglandin endoperoxides.

The effects of arachidonate, oleate and palmitate on preincubated and Lubrol PX-treated lysates were also studied. In percentage terms, the guanylate cyclase activity of preincubated lysate was stimulated only slightly less by either arachidonate or oleate than the untreated lysate (Table 4.5.3.). Thus, the actual increase in enzyme activity caused by preincubation of the lysate and subsequent addition of these fatty acids was greater than the sum of the individual treatments (Table 4.5.3.). Oleate increased the guanylate cyclase activity of preincubated lysate to values far higher than observed on treatment with Lubrol PX alone. In contrast, all three fatty acids exerted an inhibitory effect on the activity of Lubrol PX-treated lysate. Arachidonate was the most effective, at 1.0 mM-concentration it decreased the enzyme activity below that of untreated lysate in the absence of arachidonate.

To determine whether the stimulation of guanylate cyclase activity by arachidonate and oleate was dependent on the presence of Mn^{2+} for the

expression of maximum activity, assays were carried out with 10.0 mM-$MgCl_2$ instead of with 4.0 mM-$MnCl_2$. Table 4.5.4. shows that in the presence of $MnCl_2$, arachidonate stimulated enzyme activity by about 200%; whereas in the presence of $MgCl_2$, activity was inhibited by about 40%. Although the stimulatory effect of oleate was markedly decreased in the presence of $MgCl_2$, guanylate cyclase activity was not inhibited by this fatty acid.

Table 4.5.1. *Effect of aggregating agents on the guanylate cyclase activity of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phsophocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 6.6 mg/ml and 50 µl aliquots were assayed. Aggregating agents were added to the assay mixtures at the final concentrations indicated. The values given are the means ± S.E.M. of triplicate determinations.

Aggregating agent	Concn. (µM)	Guanylate cyclase activity	
		nmol of cyclic GMP/20 min per mg of protein	% of control
None	-	4.67 ± 0.05	100
5-Hydroxytryptamine	10	4.77 ± 0.27	102
l-epinephrine	20	4.93 ± 0.04	106
ADP	100	3.26 ± 0.07	70
Arachidonate	100	12.49 ± 0.14	268

Table 4.5.2. *Effects of aggregating agents on the guanylate cyclase activities in the supefnatant and particulate fractions of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixture. Supernatant and particulate fractions of platelet lysate were prepared as described in Section 2.2.2. The protein concentration of the supernatant fraction was 4.4 mg/ml and that of the particulate fraction was 3.8 mg/ml. In each case, 50 µl aliquots of enzyme preparations was assayed. Aggregating agents were added to the assay mixtures at the final concentrations indicated. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations.

Enzyme preparation	Aggregating agent	Concn. (µM)	Guanylate cyclase activity	
			nmol cyclic GMP/ 20 min per mg protein	% of control
Supernatant	None	-	8.77 ± 0.13	100
	5-Hydroxytryptamine	10	8.81 ± 0.02	101
	l-epinephrine	20	8.69 ± 0.22	99
	ADP	100	6.16 ± 0.06	70
	Arachidonate	100	22.32 ± 0.44	255
Particulate	None	-	3.35 ± 0.07	100
	5-Hydroxytrptamine	10	3.20 ± 0.07	96
	l-epinephrine	20	3.38 ± 0.13	101
	ADP	100	2.27 ± 0.10	68
	Arachidonate	100	12.49 ± 0.23	268

Table 4.5.3. *Effects of fatty acids on the guanylate cyclase activities of untreated platelet lysate, indomethacin-treated lysate, preincubated lysate and Lubrol PX-treated lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that no phosphocreatine/creatine phosphokinase GTP-regenerating system was included in the assay mixtures. Platelet lysate was prepared and preincubated at 30°C as described in Section 2.2.2. Approximately five min prior to assay, one portion of the platelet lysate was incubated at 30°C with indomethacin (20 µM-final concentration in the lysate; 4 µM-final concentration in the assay mixture). Approximately 10 min prior to assay, one portion of the lysate was treated with Lubrol PX (1.0%, w/v -final concentration in the lysate). The protein concentration of the enzyme preparations was 3.6 mg/ml and 50 µl aliquots were assayed. Fatty acids were added to the assay mixtures at the final concentrations indicated. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations.

Enzyme preparation	Fatty acid	Concn. (mM)	Guanylate cyclase activity: nmol of cyclic GMP/ 20 min per mg of protein	Guanylate cyclase activity: % of control activity
Lysate	None	-	7.52 ± 0.15	100
	Palmitate	0.1	8.43 ± 0.13	112
		1.0	8.68 ± 0.34	116
	Oleate	0.1	12.99 ± 0.45	173
		1.0	25.32 ± 0.92	337
	Arachidonate	0.1	12.44 ± 0.23	165
		1.0	15.59 ± 0.55	208
Indomethacin-treated lysate	None	-	7.55 ± 0.23	100
	Palmitate	0.1	8.55 ± 0.02	113
		1.0	8.35 ± 0.22	111
	Oleate	0.1	13.84 ± 1.00	182
		1.0	21.62 ± 0.98	284
	Arachidonate	0.1	12.33 ± 0.12	162
		1.0	15.96 ± 0.38	211
Preincubated lysate	None	-	12.03 ± 0.39	100
	Palmitate	0.1	12.25 ± 0.35	103
		1.0	13.25 ± 0.45	111
	Oleate	0.1	19.27 ± 0.41	161
		1.0	36.99 ± 1.43	308
	Arachidonate	0.1	18.50 ± 0.07	154
		1.0	22.30 ± 0.36	186
Lubrol PX-treated lysate	None	-	21.72 ± 0.23	100
	Palmitate	0.1	18.26 ± 0.16	84
		1.0	15.54 ± 0.39	71
	Oleate	0.1	20.27 ± 0.05	94
		1.0	13.51 ± 0.35	62
	Arachidonate	0.1	14.09 ± 0.98	65
		1.0	5.61 ± 0.58	26

Fig. 4.5.1. *Effect of arachidonic and oleic acids on the guanylate cyclase activity of platelet lysate assayed in the absence of bovine serum albumin, in the presence of fatty-acid free albumin or in the presence of albumin containing fatty acids*

Guanylate cyclase activity was assayed as described in Section 2.2.3. with the exception that a phosphocreatine/creatine phosphokinase GTP-regenerating system was not included in the assay mixtures. Platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 4.5 mg/ml and 25 µl aliquots were assayed. Arachidonic (●) or oleic acid (▲) was added at the final concentrations indicated on the abcissa, to assay mixtures containing no albumin (a), fatty-acid-free albumin (b) or albumin containing fatty acids (c). The guanylate cyclase activity of the platelet lysate assayed in the absence of fatty acids (■) was as follows for each assay mixture: (a)6.44 ± 0.09; (b) 6.25 ± 0.11; and, (c)6.10 ± 0.06 nmol of cyclic GMP formed/20 min per mg of protein (mean ± S.E.M. of 6 control determinations). Guanylate cyclase activity is expressed as a percentage of control activity. The values given are the means of triplicate determinations. This experiment has been carried out once.

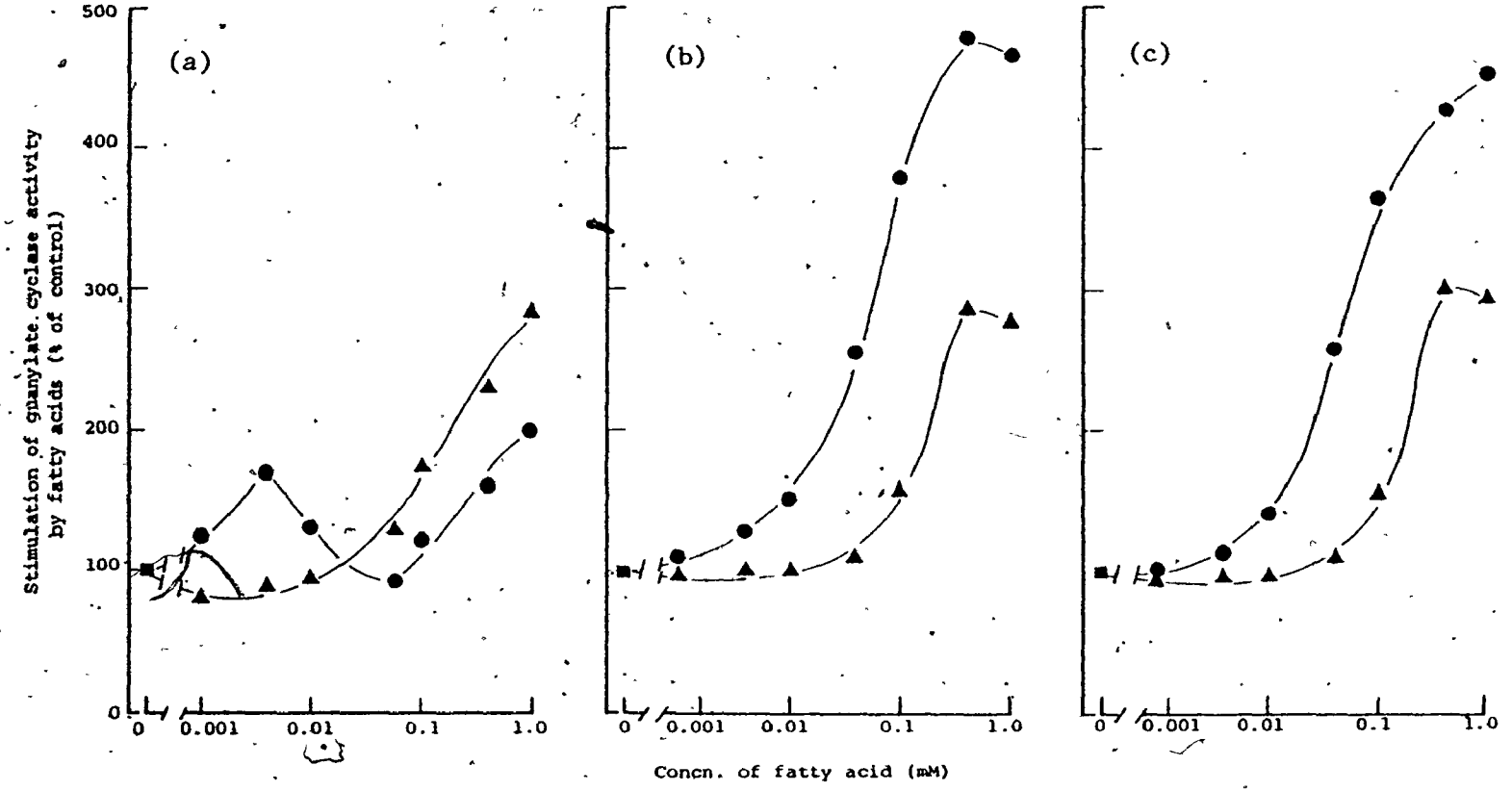


Table 4.5.4. *Effect of fatty acids on the bivalent cation requirements of platelet lysate*

Guanylate cyclase activity was assayed as described in Section 2.2.3. and platelet lysate was prepared as described in Section 2.2.2. The protein concentration of the platelet lysate was 6.0 mg/ml and 25 µl aliquots were assayed. The values given for guanylate cyclase activity are the means ± S.E.M. of triplicate determinations. This experiment has been carried out once.

Bivalent cation present in assay	Addition to the assay mixture	Concn. (mM)	Guanylate cyclase activity	
			nmol cyclic GMP/ 20 min per mg protein	% of control activity
$MnCl_2$ (4.0 mM)	None	-	7.83 ± 0.25	100
	Arachidonate	0.01	11.42 ± 0.26	146
		1.0	23.67 ± 0.63	302
	Oleate	0.01	6.88 ± 0.34	89
		1.0	19.62 ± 2.05	251
$MgCl_2$ (10.0 mM)	None	-	1.08 ± 0.05	100
	Arachidonate	0.01	0.89 ± 0.04	82
		1.0	0.63 ± 0.15	58
	Oleate	0.01	0.82 ± 0.04	76
		1.0	1.67 ± 0.34	155

4.6. *Discussion: Activation of platelet guanylate cyclase*

4.6.1. Preincubation

Incubation of platelet lysate for 60 min at 30°C or at 37°C increased its guanylate cyclase activity on average by 120% and 225%, respectively (Scheme 1). A similar increase in activity was found on preincubation of the supernatant fraction of platelet lysate; whereas the activity in the particulate fraction was unaffected by preincubation. A spontaneous time- and temperature-dependent activation of guanylate cyclase has also been observed in human platelet homogenates and supernatants by Böhme *et al.* (1974, 1978) and Glass *et al.* (1977*a*), as well as in soluble, but not particulate, fractions of rat lung (Chrisman *et al.*, 1975; White *et al.*, 1976), guinea pig splenic cells (Haddox *et al.*, 1978; Goldberg *et al.*, 1978) and rat uterus (Křaska *et al.*, 1977). Other workers studying guanylate cyclase in human platelets have not reported this phenomenon (Barber, 1976; Rodan & Feinstein, 1976; Hidaka & Asano, 1977*a*,*b*; Weiss *et al.*, 1978).

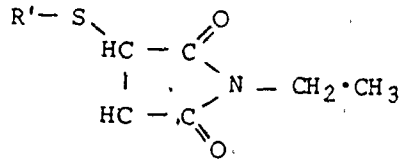
The failure of enzyme in particulate fractions from platelets and other tissues to undergo spontaneous activation may be attributable to the presence of inhibitory factors and/or the absence of factors necessary for the activation process. This explanation is supported by the observation that guanylate cyclase activity in particulate fractions prepared from preincubated platelet lysate was increased by about the same extent as that in whole lysate. Although it is also possible that enzyme in particulate enzyme cannot become activated under any circumstances, there is no evidence to date in favour of this suggestion.

The findings of the present study show that the increase in guanylate cyclase activity during preincubation cannot be attributed to a decrease in the inhibitory activity of ultrafilterable factors present in the lysate or to a decrease in the sensitivity of the enzyme to inhibition by these factors. Instead, the stimulatory effect of preincubation appears to be the result of an activation of the enzyme. Since sulfhydryl groups are known to play an important role in the regulation of activity of many enzymes (Webb, 1966), the effects of thiol-containing reducing agents such as dithiothreitol and mercaptoethanol on the preincubation process have been studied in several laboratories in order to gain insight into the mechanism of activation of guanylate cyclase. These compounds have been shown to prevent the activation of guanylate cyclase by preincubation in all tissues studied (Böhme *et al.*, 1974, 1978; Chrisman *et al.*, 1975; White *et al.*, 1976; Glass *et al.*, 1977*a*; Haddox *et al.*, 1978; Adams & Haslam, 1978). Since mercaptoethanol and dithiothreitol act as sulfhydryl-protecting reagents (Cleland, 1964; Webb, 1966), these observations suggested that activation by preincubation may be the result of the oxidation of specific sulfhydryl groups involved in enzyme regulation. Although mercaptoethanol will not reverse activation once it has occurred (White *et al.*, 1976), dithiothreitol has been found to reduce spontaneously enhanced guanylate cyclase activity to its basal level (Haddox *et al.*, 1978) or below (this thesis; Adams & Haslam, 1978). While this effect of dithiothreitol suggests that activation can be fully reversed by the reduction of protein disulfide groups, this interpretation is complicated by the fact that this agent also markedly inhibits the basal activity of guanylate cyclase from human platelets (Böhme *et al.*, 1974, 1978; Adams & Haslam, 1978), guinea pig

splenic cells (Haddox *et al.*, 1978; Goldberg *et al.*, 1978), as well as from various rat tissues, including lung (Böhme *et al.*, 1978). In the present study, dithiothreitol inhibited the basal and preincubation-activated guanylate cyclase activities of platelet lysate by about the same percentage, thus even in the presence of this compound, the preincubation effect could still be detected (Scheme 1). The inhibition of basal guanylate cyclase activity by dithiothreitol suggests that some degree of reversible activation may have occurred during the preparation and storage of the enzyme. Furthermore, the failure of both mercaptoethanol and dithiothreitol to reverse the stimulatory effect of preincubation does not rule out the possibility that activation is the result of oxidation of enzyme sulfhydryl groups since even oxidation to disulfide groups is not necessarily reversed by thiols if steric factors prevent reduction, and oxidation to higher oxidation states such as the sulfenate (SO^-), sulfinate (SO_2^-) or sulfonate (SO_3^-) state would not be expected to be reversed (Webb, 1966). In any case, the results of this study as well as those recently reported by Böhme *et al.* (1978), indicate that the enzyme obtained by dithiothreitol-treatment of the activated enzyme is different from the dithiothreitol-stabilized form of the enzyme.

Further investigations of the role of sulfhydryl groups in the regulation of guanylate cyclase activity were carried out by studying the effects of *N*-ethylmaleimide on the basal and preincubation-activated activities of platelet lysate. In contrast to the action of dithiothreitol, which maintains protein sulfhydryl groups in the reduced state, *N*-ethylmaleimide acts by alkylating accessible sulfhydryl groups which thereby permanently alters the reacted protein (Webb, 1966). Although the

reactivity of *N*-ethylmaleimide is not confined to sulfhydryl groups, demonstration of amino reactivity has required high concentrations of the reagent and prolonged incubation periods (Webb, 1966). Thus, under physiological conditions and in the absence of excess reagent, it can be assumed that *N*-ethylmaleimide reacts quickly and specifically with available sulfhydryl groups of proteins yielding products of the following type (Webb, 1966):



The findings of the present study show that *N*-ethylmaleimide shares with dithiothreitol the capacity to prevent activation of guanylate cyclase by preincubation thereby supporting the view that the activation process involves the oxidation of sulfhydryl groups. In contrast to the effect of dithiothreitol, low concentrations of *N*-ethylmaleimide inhibited guanylate cyclase activity of the preincubated lysate significantly less than that of the control lysate. The decrease in the sensitivity of the activated enzyme to inhibition by *N*-ethylmaleimide can be attributed to a decrease in either the number or accessibility of sulfhydryl groups and/or a conformational change in the enzyme. The marked decrease in the ability of the preincubated enzyme to utilize Mg^{2+} as a substitute for Mn^{2+} suggests that the properties of activated guanylate cyclase are clearly different from the control enzyme. In contrast, Böhme *et al.* (1978) have recently reported that activation of platelet guanylate cyclase by preincubation did not significantly change the ratio of activities measured with Mn^{2+} or Mg^{2+}.

Insight into the mechanism of activation by preincubation initially was provided by White *et al.* (1976) who demonstrated that both oxygen and

copper were required for activation of guanylate cyclase in rat lung supernatants. A similar oxygen requirement has since been reported for the activation of the enzyme from splenic cells (Haddox *et al.*, 1978; Goldberg *et al.*, 1978) and platelets (Böhme *et al.*, 1978). The observation of Böhme *et al.* (1978) that millimolar concentrations of EDTA retarded the activation of platelet guanylate cyclase, suggested that bivalent cations are involved in the activation process. This finding may be related to the observation by White *et al.* (1976) that Cu^{2+} is required for activation since EDTA binds Cu^{2+} much more effectively than other bivalent cations (Dawson *et al.*, 1969).

While it is relatively well established that spontaneous activation is an oxygen and perhaps Cu^{2+}-dependent process, the mechanism whereby these factors interact to cause oxidation of protein sulfhydryl groups is not yet understood. White *et al.* (1976) have hypothesized that guanylate cyclase in rat lung supernatants is ultimately activated by H_2O_2 generated non-enzymically by the interaction of oxyhemoglobin with a proton donor such as ascorbic acid. Although further evidence in support of this hypothesis has not yet been reported, the finding of Mittal & Murad (1977*b*) that superoxide dismutase markedly enhanced the activity of guanylate cyclase from rat liver has in effect confirmed and extended the observations of White *et al.* (1976), since H_2O_2 is generated by the dismutation of superoxide anion and also because superoxide dismutase is a cupri-zinc enzyme in eukaryotes (Fridovich, 1976). Mittal & Murad (1977*b*) found that both superoxide anion (O_2^-) and H_2O_2 were required for activation of guanylate cyclase by superoxide dismutase, and that activation was inhibited by hydroxyl radical (OH•) scavengers. On the basis of these findings, Mittal & Murad (1977*b*) proposed

that hydroxyl radicals which are formed by the Haber-Weiss reaction (Haber & Weiss, 1934) from superoxide anion and H_2O_2, are responsible for the activation of guanylate cyclase by superoxide dismutase. Thus, one general mechanism of activation of guanylate cyclase may involve the formation of free hydroxyl radicals and subsequent oxidative attack on sulfhydryl or other groups on the enzyme. Although it is not yet known whether guanylate cyclase is ultimately activated by hydroxyl radicals during preincubation, the available evidence does not exclude the possiblity of such a mechanism.

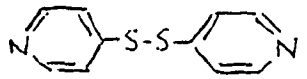
4.6.2. Oxidants

While many enzymes contain critical sulfhydryl groups which, if oxidized or chemically modified result in a loss of catalytic activity (Webb, 1966), the catalytic activity of guanylate cyclase appears to be enhanced by the oxidation of enzyme-associated sulfhydryls. The role of sulfhydryl groups in the regulation of guanylate cyclase was investigated further by studying the effects of several oxidants of increasing strength on the activity of this enzyme in platelet lysates.

In general, disulfides are considered to be the most specific oxidants of protein sulfhydryl groups; however, these agents have certain disadvantages that limit their use in enzyme studies (Webb, 1966). Firstly, it has been shown that the reaction of a thiol with a disulfide is not a simple oxidation-reduction but an exchange reaction involving a two-step ionic displacement, often with the formation of mixed disulfides, as follows:

$$X-SH + Y-S-S-Y \rightleftarrows X-S-S-Y + Y-SH$$
$$X-S-S-Y + X-SH \rightleftarrows X-S-S-X + Y-SH$$

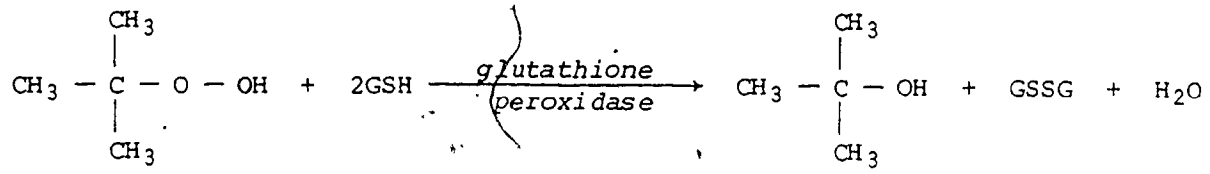
(Webb, 1966). Secondly, the reaction of enzymes with disulfides has been found to proceed very slowly in many cases (Webb, 1966). Thirdly, the

environment of the enzyme sulfhydryl groups may be unfavourable for the approach of a disulfide or may affect the redox potential in such a manner as to deter the interaction (Webb, 1966). Combined, these considerations make it difficult to interpret the effect of disulfides on enzyme activity. Of the two disulfides tested in the present study, *i.e.* oxidized glutathione and 4,4'-dithiodipyridine  , only the latter was found to significantly stimulate platelet guanylate cyclase activity. However, the small stimulatory effect of 4,4'-dithiodipyridine (*i.e.* 40% stimulation) was highly concentration-dependent, since a dramatic inhibition of enzyme activity was observed with concentrations only slightly higher than the optimum of 0.04 mM. On the basis of the available evidence, it is not possible to determine whether this biphasic effect of 4,4'-dithiodipyridine is due to the formation of inhibitory mixed disulfides or to the complete oxidation of enzyme-associated sulfhydryls. However, in view of the extremely slow reactivity of this disulfide with protein sulfhydryl groups (Grassetti & Murray, 1967), it is unlikely that either the stimulatory or inhibitory effect could be due to the complete oxidation of all available sulfhydryl groups on the enzyme.

The stimulatory effect of diamide (diazenedicarboxylic acid *bis*-(*N*,*N*-dimethylamide)){$(CH_3)_2NCON = NCON(CH_3)_2$} was more than two-fold greater than that of 4,4'-dithiodipyridine (Scheme 1). Diamide was originally introduced by Kosower *et al.* (1969) as a specific reagent for the rapid

and complete oxidation of glutathione within erythrocytes. However, in addition to oxidizing glutathione, diamide has been reported to react with protein-bound sulfhydryl groups as well as with reduced pyridine nucleotides, thereby causing extensive cellular damage (Harris & Bigelow, 1972). Srivastava *et al.* (1974) subsequently found that in spite of the indiscriminate, chemical oxidation of thiols and protein sulfhydryl groups by diamide, not all sulfhydryl-containing enzymes are affected by the reagent. Similarly, glutathione reductase, which has adjacent sulfhydryls in its active site, is not affected by diamide presumably because the sulfhydryl groups are protected with a flavin ring (Jones & Williams, 1975). From these observations, Rebhun *et al.* (1976) postulated that only certain proteins containing sulfhydryl groups will be affected by diamide, and that these are proteins that contain adjacent sulfhydryl groups that are not in the active site of an enzyme. Thus, the stimulation of guanylate cyclase activity by diamide supports the hypothesis that this enzyme can be activated by the oxidation of specific sulfhydryl groups. Although the effect of diamide on the concentration of glutathione in the platelet was not determined, it is unlikely that the stimulatory effect of this reagent can be attributed to the oxidation of endogenous glutathione as it has been shown that the concentration of non-protein thiol (presumably glutathione) in the complete assay mixtures is too low, by several orders of magnitude, to have any effect on enzyme activity (see Section 3.2.). Further evidence in favour of a mechanism involving the oxidation of enzyme sulfhydryl residues is provided by the observation that either preincubation or *N*-ethylmaleimide-treatment of the lysate markedly decreased or abolished the stimulatory effect of diamide.

Tert-butylhydroperoxide is a stable organic hydroperoxide which has been used as a specific oxidant of glutathione. Since this compound can serve as a substrate for the enzyme, glutathione peroxidase, glutathione is enzymically oxidized by the following mechanism (Srivastava *et al.*, 1974):



Although *tert*-butylhydroperoxide was found to oxidize nearly all the non-protein thiol present in the platelet lysate, the marked stimulation of guanylate cyclase activity by this agent was unrelated to its effect on non-protein thiol levels. Instead, the data indicate that the activation of guanylate cyclase by *tert*-butylhydroperoxide was the result of an oxidation of specific enzyme-associated sulfhydryls. In view of the fact that *tert*-butylhydroperoxide decomposes in aqueous solution by the following mechanism (Pryor, 1976):

$$2t\text{-BuOOH} \longrightarrow t\text{-BuO}\cdot + H_2O + t\text{-BuOO}\cdot$$

it is possible to speculate that the attacking species would be the *tert*-butoxy radicals generated from this reaction since they are extremely powerful oxidants.

The results of the present study suggest that the stimulation of guanylate cyclase by 4,4'-dithiodipyridine, diamide or *tert*-butylhydroperoxide is roughly proportional to the redox potential of these agents (Scheme 1). However, it is not known whether the magnitude of the stimulatory effect observed with these agents is a function of the number of sulfhydryl groups that react, a function of the state to which they are oxidized, or

a combination of both. The finding that sodium periodate could stimulate platelet guanylate cyclase activity by about 16-fold (Adams & Haslam, unpublished results), indicates that oxidation to higher oxidation states activated the enzyme to a much greater extent. In any case, the results also show that the properties of guanylate cyclase activated by oxidative processes (*i.e.* preincubation or *tert*-butylhydroperoxide) are similar to each other, but different from the control enzyme in two respects. Firstly, preincubation abolished and *tert*-butylhydroperoxide markedly reduced the inhibition of guanylate cyclase activity observed with 50 or 100 µl of lysate in the assay. Secondly, enzyme in both the preincubated and *tert*-butylhydroperoxide-treated lysates exhibited a marked decrease in ability to utilize Mg^{2+} as a substitute for Mn^{2+}. These observations support the view that the stimulatory effects of preincubation and of *tert*-butylhydroperoxide are qualitatively similar. Although there are no previous studies of the effects of the oxidants described in this thesis on guanylate cyclase activity in any other tissues, Haddox *et al.* (1978) and Goldberg *et al.* (1978) have recently reported that the oxidant, dehydroascorbic acid (5.0 mM) can stimulate the activity of the soluble and particulate forms of guanylate cyclase from guinea pig splenic cells by about 200 to 300%. In contrast to the effects of diamide and *tert*-butylhydroperoxide described in the present study, these workers found that the stimulatory effect of dehydroascorbic acid appeared to be additive with the spontaneous air activation of the soluble form of the enzyme. On the basis of this observation, Goldberg *et al.* (1978) suggested that the site of dehydroascorbic acid activation is separate from the site affected through the spontaneous process. Evidence that oxidation of enzyme sulfhydryls was involved in the dehydroascorbic

acid-induced stimulation of splenic cell guanylate cyclase was provided by the finding that *N*-ethylmaleimide was effective in preventing this activation. Furthermore, both dithiothreitol and glutathione were able to prevent as well as reverse the activation by dehydroascorbic acid (Haddox *et al.*, 1978; Goldberg, 1978). Thus, the activation of splenic cell guanylate cyclase by dehydroascorbic acid appears comparable to the activation of platelet guanylate cyclase by diamide or *tert*-butylhydroperoxide with respect to oxidation of enzyme sulfhydryl groups by these agents.

Recently, Vesely & Levey (1978) reported that butadiene diepoxide, a representative of the epoxide class of chemical carcinogens, markedly stimulated (*i.e.* two- to 17-fold) guanylate cyclase activity in several rat tissues. Although these workers did not determine whether activation by butadiene diepoxide was an oxidative process, the oxidative properties of this carcinogen (Mudd, 1976) indicate that such a mechanism is highly probable.

Other oxidizing agents such as methylene blue, H_2O_2 and $K_3Fe(CN)_6$ were found to have no significant effect on basal guanylate cyclase activity (Arnold *et al.*, 1977; Katsuki *et al.*, 1977; White *et al.*, 1976).

4.6.3. Sodium azide and sodium nitroprusside

The activation of guanylate cyclase by sodium azide in some but not in all tissues was first reported by Kimura *et al.* (1975a). By mixing azide-responsive and -non-responsive preparations, these workers demonstrated that some factors are required for activation ("azide activator factor") while other factors exist that prevent the effect (Kimura *et al.*, 1975a; Mittal *et al.*, 1975, 1977, 1978). Thus, the tissue specificity of the azide effect is a function of the presence and/or absence of activating

and inhibiting factors. The "azide activator factor" in supernatant fractions from liver has been purified, characterized and is or closely resembles catalase (Mittal *et al.*, 1975, 1977, 1978). However, peroxidase, cytochrome b_2 and cytochrome c reductase have been found to be effective substitutes for the activator requirement (Mittal & Murad, 1977*a*, Mittal *et al.*, 1977, 1978; Murad *et al.*, 1978). These enzymes are known to convert azide and another activating agent, hydroxylamine (Deguchi, 1977), to nitric oxide (Keilin & Hartree, 1954). Since nitric oxide has been found to activate guanylate cyclase from most tissues tested (Arnold *et al.*, 1977*a*,*b*; Katsuki *et al.*, 1977; Murad *et al.*, 1978), these workers have hypothesized that the stimulation of enzyme activity by sodium azide is indirectly due to the effect of nitric oxide. Some of the inhibitory factors (Kimura *et al.*, 1975*a*) have also been purified and characterized, and have been identified as hemoglobin and myoglobin (Miki *et al.*, 1977*a*,*b*; Mittal & Murad, 1977*a*; Mittal *et al.*, 1978; Murad *et al.*, 1978).

The present study shows that platelet guanylate cyclase can be activated by sodium azide, which implied that platelets contain the necessary activating factor. This finding has recently been confirmed by Weiss *et al.* (1978). The comparatively small stimulatory effect of sodium azide on the guanylate cyclase activity in particulate fractions of platelet lysate can be attributed to the selective distribution of catalase or other azide activating enzymes in the supernatant fraction.

Activation of platelet guanylate cyclase by sodium azide resembled that in other tissues in being time- and temperature-dependent. The results suggested that there were two components to this process, one that can be interpreted as due to the slow formation of an active species from

sodium azide (*i.e.* nitric oxide) and another that appeared to involve a 'conditioning' of a component of the lysate by preincubation, which enabled sodium azide activation of guanylate cyclase to occur more rapidly.

In addition to sodium azide, many other nitrogen containing materials have been found to stimulate guanylate cyclase activity. These include $NaNO_2$, phenylhydrazine (Kimura *et al.*, 1975), hydrazine (Vesely & Levey, 1977), nitrosamines (DeRubertis & Craven, 1976*a*,*b*, 1977*a*,*b*), nitrosoureas (Vesely *et al.*, 1977), nitroglycerin and nitroprusside (Katsuki *et al.*, 1977*a*,*b*; Mittal *et al.*, 1977; Schultz *et al.*, 1977; Böhme *et al.*, 1978), which can be converted to nitric oxide under the appropriate conditions (Mittal & Murad, 1977*a*; Murad *et al.*, 1978). Since activation by nitric oxide or sodium nitroprusside has no requirement for an activator factor such as catalase (Katsuki *et al.*, 1977*a*,*b*; Mittal & Murad, 1977*a*; Arnold *et al.*, 1977*a*,*b*), it is believed that any nitro- or nitroso-compound or amine that can form nitric oxide could activate guanylate cyclase (Mittal & Murad, 1977*a*; Murad *et al.*, 1978). Although it is not presently known whether nitric oxide interacts directly with guanylate cyclase to produce activation or whether another intermediate product is involved (Murad *et al.*, 1978), the thiol reactivity of nitroxide radicals (Schultz & McCalla, 1969; Morrisett & Drott, 1969; Schoental & Rive, 1969; Lawley & Thatcher, 1970; Mirvish *et al.*, 1970; Nagata *et al.*, 1973; Scribner & Naimy, 1975) suggests that activation may ultimately be the result of the oxidation of enzyme-associated sulfhydryls. This possibility was investigated by studying the effects of sodium nitroprusside, which is known to be a potent sulfhydryl group oxidant (Grunert & Phillips, 1951; Leussing *et al.*, 1960) as well as a smooth muscle relaxant (Kreye *et al.*, 1975), on platelet guanylate

cyclase activity.

The findings of the present study, which have recently been confirmed (Böhme *et al.*, 1978; Weiss *et al.*, 1978), show that platelet guanylate cyclase can be activated by sodium nitroprusside. This compound was found to have a substantially greater stimulatory effect on enzyme activity than any other reagent tested in this study (Scheme 1). In contrast to its effect on guanylate cyclase activity in whole platelet lysate or in supernatant fractions, sodium nitroprusside had only a small stimulatory effect on the activity in particulate fractions of platelet lysate. This finding cannot be attributed to a decrease in the availability of catalase in the particulate fraction since activation with sodium nitroprusside has no requirement for an activator factor. Although the concentration of hemoglobin in platelet lysate was not measured, it would be found in the supernatant and not the particulate fraction and as such could not account for the decreased effect of sodium nitroprusside. These considerations suggest that other factors are responsible for the apparent suppression of the stimulatory effect of sodium nitroprusside on particulate enzyme activity or induction of its action on supernatant activity.

Whereas preincubation of platelet lysate greatly enhanced activation by sodium azide, it had an inhibitory effect on activation by sodium nitroprusside. In fact, the results showed that the stimulatory effects of preincubation and sodium nitroprusside were mutually exclusive, which suggested that the oxidation of specific sulfhydryl groups during preincubation blocks their oxidation or other modification by sodium nitroprusside. Böhme *et al.* (1978) similarly found that preincubation could prevent activation of platelet guanylate cyclase by sodium nitroprusside. Activation

by sodium nitroprusside was also markedly supressed with lysate that had been pretreated with *tert*-butylhydroperoxide. The inhibitory effect of other oxidizing agents such as methylene blue, $K_3Fe(CN)_6$ and H_2O_2 on the activation of guanylate cyclase by sodium azide, sodium nitroprusside (Katsuki *et al.*, 1977*a*,*b*) or nitric oxide (Arnold *et al.*, 1977*a*,*b*) has been attributed to the conversion of nitric oxide to less effective higher oxides of nitrogen such as nitrogen dioxide; however, in contrast to *tert*-butylhydroperoxide, these agents do not stimulate basal activity Although an interaction between *tert*-butylhydroperoxide and sodium nitroprusside cannot be ruled out, an alternative interpretation is that the inhibitory effect of *tert*-butylhydroperoxide is comparable to that of preincubation. In any case, the much larger stimulatory effect of sodium nitroprusside on guanylate cyclase activity compared to that of preincubation or *tert*-butylhydroperoxide indicates that activation by this agent is unlikely to be due to a similar oxidation of sulfhydryl groups at the same reactive site on or associated with the enzyme.

The finding reported in this study, as well as by Böhme *et al.* (1978), that pretreatment of the platelet enzyme with dithiothreitol markedly enhanced, in percentage terms, the magnitude of the stimulatory effect of sodium nitroprusside indicated that in the reduced state, this enzyme is more susceptible to the effects of this agent. However, the relative inability of *N*-ethylmaleimide (0.1 mM) to block activation by sodium nitroprusside suggests that these sulfhydryl groups may not be directly involved in the activation process. DeRubertis & Craven (1977*a*) found that high concentrations (*i.e.* 0.5 to 1.0 mM) of *N*-ethylmaleimide were required to inhibit nitroprusside activation of guanylate cyclase in rat liver

homogenates. Similar results might have been obtained had higher concentrations of *N*-ethylmaleimide been used in the present study. The finding that the inhibitory effect of 0.1 mM-*N*-ethylmaleimide was substantially reduced in lysate treated with sodium nitroprusside suggests that the availability of reactive sulfhydryl groups may be decreased in the nitroprusside-activated enzyme.

Although the precise mechanism whereby sodium azide and sodium nitroprusside activate guanylate cyclase in platelets or in other tissues is not presently known, it has been shown that activation of guanylate cyclase by nitric oxide or by agents that generate nitric oxide results in a marked change in certain properties of the enzyme (Kimura *et al.*, 1975a, 1976; Mittal *et al.*, 1977; Mittal & Murad, 1977*a*,*b*; Murad *et al.*, 1978). While there is no significant change in the molecular size of the enzyme, the bivalent cation specificity is altered as a result of activation. Kimura *et al.* (1975a, 1976) first reported that the activated enzyme can effectively use either Mg^{2+} or Mn^{2+} as sole cation. Moreover, after activation, soluble rat liver guanylate cyclase has no apparent requirement for free Mn^{2+} but retains its requirement for free Mg^{2+} (Kimura *et al.*, 1976). In the present study it was found that activation by sodium azide or sodium nitroprusside markedly increased the effectiveness of Mg^{2+} as sole cation, thereby decreasing the dependence of this enzyme on Mn^{2+} for the expression of maximum activity. An alteration such as this may be of particular importance in view of the extremely low concentrations of Mn^{2+} normally present in tissues. Kimura *et al.* (1976) also found that after activation of soluble liver enzyme with azide or other agents, Ca^{2+} either had no effect or was somewhat inhibitory. Although inhibition of guanylate cyclase by ATP

has been found to be similar with native or azide-activated guanylate cyclase (Mittal *et al.*, 1977; this thesis), activated enzyme can form significant amounts of cyclic AMP from ATP (Mittal & Murad, 1977*a*).

4.6.4. Non-ionic detergents

It is shown in the present study that the non-ionic detergents, Triton X-100 and Lubrol PX, activate platelet guanylate cyclase. There are no previous studies of the effect of Lubrol PX on the platelet enzyme, but Triton X-100 has variously been reported to have an inhibitory effect (Böhme *et al.*, 1974), a slight stimulatory effect (Barber, 1976; Glass *et al.*, 1977*a*) or no effect at all (Rodan & Feinstein, 1976). Although the reasons for these discrepancies are not clear, in the present studies Triton X-100 was substantially less effective than Lubrol PX in stimulating guanylate cyclase activity, particularly at the relatively low concentrations used by other workers. Lubrol PX was as effective in increasing the guanylate cyclase activity in 100 000*g* supernatant from lysed platelets as in whole lysate, suggesting that the detergent activates soluble enzyme rather than solubilizes particulate enzyme. Comparable results have been obtained with soluble guanylate cyclase from several mammalian tissues (Hardman *et al.*, 1973; Kimura & Murad, 1974*a*; Chrisman *et al.*, 1975). Neer & Sukiennik (1975) have shown that Lubrol PX activates the soluble form of guanylate cyclase by changing its conformation, possibly as a result of a small amount of detergent binding to specific hydrophobic sites on the enzyme.

Addition of Lubrol PX to a preincubated lysate resulted in a further increase in guanylate cyclase activity; however, since the activity of this preparation was roughly equivalent to that of a Lubrol PX-treated

lysate that had not been preincubated, the stimulatory effects of these two treatments were not additive, and appeared to be mutually exclusive (Scheme 1). Both Chrisman *et al.* (1975) and White *et al.* (1976) observed that the maximum increases in activity obtained by preincubation or by treatment of rat lung supernatant with Triton X-100 were approximately the same and that addition of Triton X-100 to a preincubation-activated lysate had no measurable effect on guanylate cyclase activity. The results of the present study indicate that the state of activation of guanylate cyclase after preincubation is different from that induced by treatment with Lubrol PX. Evidence in support of this conclusion is provided by the observations that neither dithiothreitol nor *N*-ethylmaleimide was effective in preventing activation by Lubrol PX as well as by the marked similarity between the effects of Lubrol PX on preincubated and tert-butylhydroperoxide-treated lysates (Scheme 1). It is therefore unlikely that activation of platelet guanylate cyclase by Lubrol PX is in any way attributable to an oxidation of sulfhydryl groups on the enzyme or on a related component. Instead, it is more likely that Lubrol PX activates guanylate cyclase by binding to specific hydrophobic sites and subsequently inducing a conformational change. A conformational change could also explain the observation that Lubrol PX was equally effective in preventing and reversing activation of platelet guanylate cyclase by sodium azide or by sodium nitroprusside. Although Kimura *et al.* (1975*a*,*b*) first reported that Triton X-100 could prevent as well as reverse activation by sodium azide, these workers provided no explanation for this phenomenon. The marked decrease in the capacity of Lubrol PX-activated enzyme to utilize Mg^{2+} as a substitute for Mn^{2+}, suggests that in addition to altering bivalent cation requirements, activation by

Lubrol PX may affect other kinetic properties of the enzyme.

4.6.5. Fatty acids

The effects of fatty acids on the activity of platelet guanylate cyclase have attracted interest (Barber, 1976; Glass *et al.*, 1977a; Hidaka & Asano, 1977*a*,*b*; Goldberg *et al.*, 1978) because of the specific role in platelet function of arachidonate and its metabolism to prostaglandin endoperoxides and derivatives (Hamberg & Samuelsson, 1974; Hamberg *et al.*, 1975). However, oleate was found to be almost as effective as arachidonate in activating guanylate cyclase in platelet lysates. This finding is in accord with the results reported by Barber (1976), in suggesting that activation of platelet guanylate cyclase by fatty acids is a relatively non-specific effect, perhaps attributable to the surfactant properties of the fatty acids. In contrast, Glass *et al.* (1977a) claim that platelet guanylate cyclase is specifically activated by arachidonate and closely related polyunsaturated fatty acids through a specific hydrophobic binding site for these fatty acids. Hidaka & Asano (1977a) believe that the effect of arachidonate depends on its conversion into the hydroperoxy derivative by lipoxygenase which thereby activates guanylate cyclase by oxidizing sulfhydryl groups on the enzyme. Whether the activation of guanylate cyclase by arachidonate is structurally specific or not, the physiological significance of this effect is doubtful. Since the induction of platelet aggregation and the release reaction by exogenous arachidonate and the associated increase in the intracellular concentration of cyclic GMP are all blocked by indomethacin, a potent inhibitor of fatty acid cyclooxygenase, it appears that in intact platelets all the effects of arachidonate are dependent on its metabolism by the cyclooxygenase and not the lipoxygenase pathway (Davies *et al.*, 1976;

Glass *et al.*, 1977b). In contrast, activation of guanylate cyclase by arachidonate in platelet lysates was not blocked by indomethacin (the present study) nor aspirin (Glass *et al.*, 1977a). Thus, the *in vitro* activation of guanylate cyclase by arachidonate is unlikely to be relevant to the action of this fatty acid in platelet function.

Much higher concentrations of fatty acids were required to activate guanylate cyclase in the experiments reported in this thesis (*i.e.* 0.1 to 1.0 mM) than in those reported by Barber (1976) or Glass *et al.* (1977a). This difference may be attributable to the inclusion of bovine serum albumin in the assay mixtures in the present study. A small stimulatory effect of micromolar concentrations of arachidonate but not of oleate was observed when albumin was omitted. Although the stimulatory effect of oleate was not affected by the omission of albumin from the assay mixtures, the magnitude of the stimulatory effect of millimolar concentrations of arachidonate was substantially less. Since arachidonate increased guanylate cyclase activity by approximately the same extent in the presence of defatted or control albumin, it is unlikely that this effect of arachidonate is due to displacement of other fatty acids from binding sites on the albumin. Instead, it is more likely that the albumin enhanced the solubility of arachidonate and/or the interaction of arachidonate with hydrophobic sites on the enzyme.

The stimulatory effect of oleate or arachidonate was more than additive with the increase in activity caused by preincubation, whereas these fatty acids inhibited the activity of Lubrol PX-treated lysate (Scheme 1). These experiments suggest that the fatty acids may exert their stimulatory effects on guanylate cyclase by a mechanism that is distinct

from those responsible for the effects of preincubation and of Lubrol PX, though in the latter case formation of micelles of mixed composition could have influenced the results. The finding that the stimulatory effect of oleate was markedly reduced, whereas arachidonate inhibited enzyme activity when assays were carried out with Mg^{2+} in place of Mn^{2+} as the sole bivalent cation, suggests that activation of guanylate cyclase by fatty acids alters this property of the enzyme. In contrast to enzyme activated by sodium azide and sodium nitroprusside, activation of guanylate cyclase by preincubation, *tert*-butylhydroperoxide, Lubrol PX and unsaturated fatty acids resulted in a marked decrease in the ability of the enzyme to utilize Mg^{2+} as a substitute for Mn^{2+}. Thus, activation by these agents results in markedly different bivalent cation requirements than activation by either sodium azide or sodium nitroprusside.

Wallach & Pastan (1976*b*) have reported somewhat different results with membrane-bound guanylate cyclase from fibroblasts. This enzyme was stimulated by a variety of highly amphiphilic fatty acids, including palmitate and oleate, in the presence of Mg^{2+} and certain concentrations of Lubrol PX, which were substantially lower than required for optimal activation of the enzyme in platelet lysates. However, these authors also concluded that fatty acids and Lubrol PX act by different mechanisms.

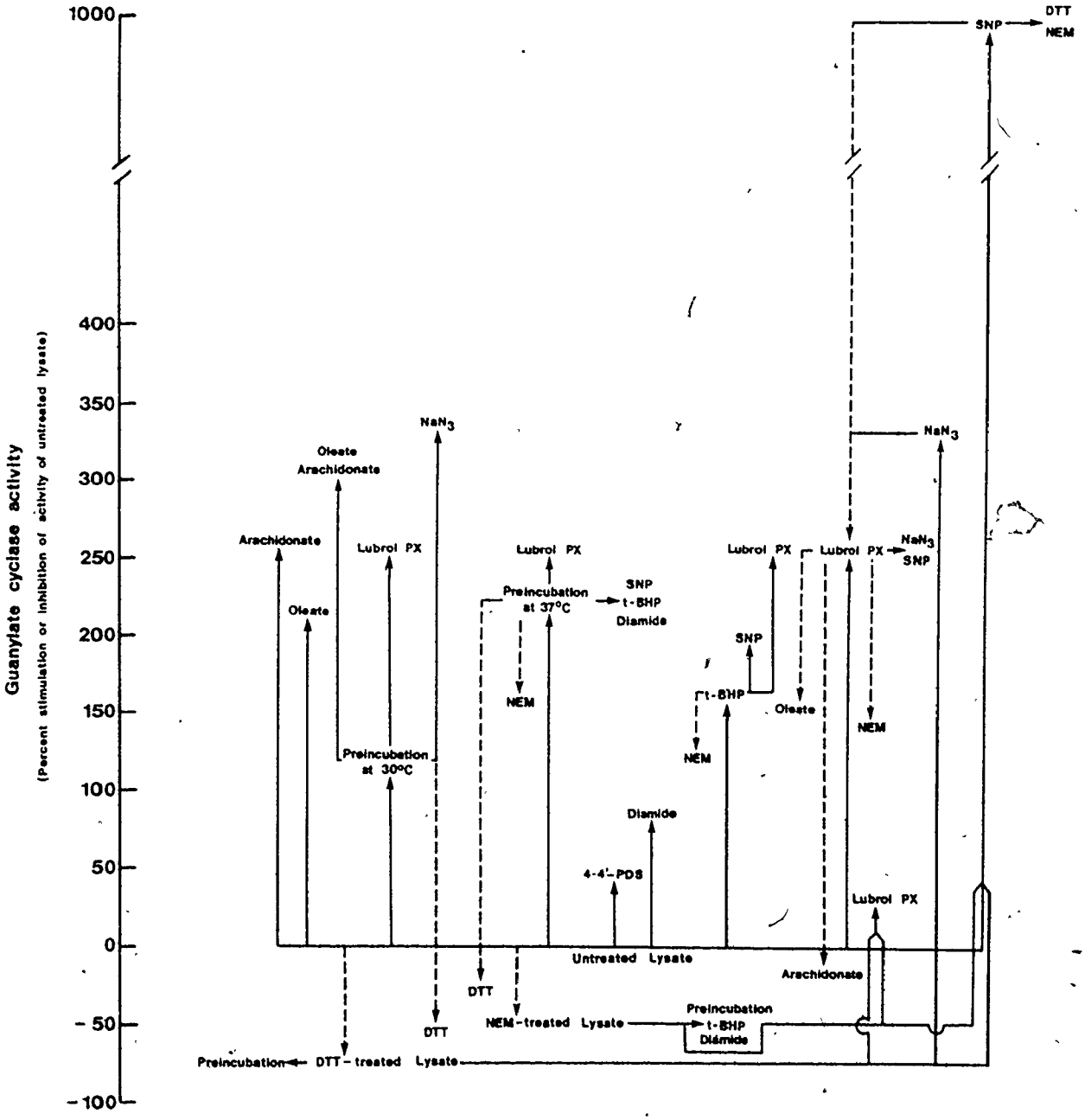
Recently, Goldberg *et al.* (1978) reported that the soluble form of splenic cell guanylate cyclase could be activated by micromolar concentrations of the prostaglandin endoperoxides, PGG_2 and PGH_2, as well as by the fatty acid endoperoxides, 15-OOH-20:4, 12-OOH-20:4 and 13-OOH-18:2. The finding that reducing agents such as dithiothreitol and glutathione were effective not only in preventing but also in reversing the activation

once it had occurred, suggested to these workers that the activation of guanylate cyclase by the endoperoxy- and/or hydroperoxy-containing fatty acids was the result of an oxidation of sulfhydryl functions associated with a hydrophobic site on the enzyme or on a closely related component. Goldberg *et al.* (1978) also found that the activation of soluble splenic cell guanylate cyclase induced by PGG_2 was somewhat greater when Mg^{2+} was used in place of Mn^{2+} as sole cation. This effect of PGG_2 contrasts with the effect of unsaturated fatty acids on the bivalent cation requirements of platelet guanylate cyclase.

Although the role of sulfhydryl groups on or associated with the enzyme was not investigated with respect to the activation of platelet guanylate cyclase by arachidonate or oleate, it is unlikely that high concentrations of unsaturated fatty acids activate the enzyme by the same mechanism as low concentrations of their endoperoxy- or hydroperoxy- derivatives. Instead, it is more reasonable to suggest that unsaturated fatty acids act as surfactants by binding to hydrophobic sites on the enzyme that are distinct from those occupied by Lubrol PX, but induce a conformational change analogous to that induced by non-ionic detergents.

Scheme 1. *Summary of the relative effects of different combinations of factors that affect platelet guanylate cyclase activity*

The approximate guanylate cyclase activities after the various treatments are indicated in terms of the percent stimulation or inhibition of the activity of untreated lysate. Stimulatory effects are indicated by solid lines, and inhibitory effects by broken lines. The sequence of exposure to each agent or treatment is shown. Depending on the experiment, agents were either added directly to untreated or preincubated lysate or included in the assay mixtures; however, the final concentrations of the agents to which the enzyme was exposed were as follows: arachidonate (1.0 mM); oleate (1.0 mM); Lubrol PX (1.0%,w/v); 4,4'-dithiodipyridine (4,4'-PDS) (0.04 mM); diamide (0.4 mM); *tert*-butylhydroperoxide (*t*-BHP)(1.0 mM); sodium azide (NaN_3)(10.0 mM); sodium nitroprusside (SNP)(1.0 mM); dithiothreitol (DTT)(5.0 mM) and *N*-ethylmaleimide (NEM)(0.1 mM).


Guanylate cyclase activity
(Percent stimulation or inhibition of activity of untreated lysate)
1000
400
350
300
250
200
150
100
50
0
-50
-100
Arachidonate
Oleate
Oleate
Arachidonate
Lubrol PX
Preincubation at 30°C
NaN3
DTT
Lubrol PX
Preincubation at 37°C
SNP
t-BHP
Diamide
NEM
Diamide
4-4'-PDS
Untreated Lysate
DTT
NEM-treated Lysate
Preincubation
t-BHP
Diamide
Preincubation
DTT-treated Lysate
Lubrol PX
Lubrol PX
SNP
t-BHP
NEM
Oleate
Lubrol PX
Lubrol PX
NaN3
SNP
NEM
Arachidonate
SNP
DTT
NEM
NaN3
Lubrol PX

Chapter 5

General Discussion

A hypothetical model of the *in vitro* activation and inhibition of platelet guanylate cyclase activity is proposed in order to explain the observations summarized in Scheme 1. The relationship of this model to those recently proposed by other workers as well as the relevance of the findings of this thesis to the regulation of guanylate cyclase activity in platelets are discussed in the following sections.

5.1. Hypothetical models for the in vitro regulation of guanylate cyclase activity

In the model illustrated in Scheme 2, it is proposed that the regulation of guanylate cyclase activity is dependent on the oxidative state of sulfhydryl groups located at two sites, A and B, that are on the enzyme itself and/or on other components involved in the control of enzyme activity. The sulfhydryl groups of Site A are more accessible and therefore more susceptible to oxidation than those of Site B. Site A functions as an inhibitor of guanylate cyclase when in the reduced state, but has no effect on enzyme activity when the sulfhydryl groups are oxidized to the disulfide state. In addition to stimulating enzyme activity, oxidation of Site A results in an increase in the accessibility and susceptibility of the sulfhydryl groups of Site B to oxidative attack. Oxidation of Site B increases enzyme activity by a much greater extent than oxidation of Site A, thus Site B appears to function as an activator of guanylate cyclase when in an oxidized state.

According to the proposed model, Site A would be maintained in the reduced or inhibitory state by the high concentrations of glutathione normally present in intact platelets (*i.e.* 4.0 to 5.0 mM); however, it is

suggested that this site is spontaneously oxidized to the disulfide state during the preparation of platelet lysate because of the marked dilution (*i.e.* approximately 60- to 100-fold) of the platelet cytosol and hence of endogenous glutathione. Thus, the so-called basal guanylate cyclase activity assayed *in vitro* actually reflects the activity of enzyme that has been partially activated. The observed inhibition of basal activity by dithiothreitol can be attributed to the reduction of the disulfide bond at Site A and restoration of its inhibitory effect. In contrast, inhibition of basal activity by *N*-ethylmaleimide could not involve Site A, but instead would be due to alkylation of the available sulfhydryl groups on Site B (see Scheme 2).

As it has been assumed that Site A is oxidized in platelet lysate, the stimulation of guanylate cyclase activity by oxidants such as 4,4'-dithiodipyridine, diamide and *tert*-butylhydroperoxide can be attributed to the oxidation of Site B. The ability of *N*-ethylmaleimide to block the stimulatory effect of diamide and *tert*-butylhydroperoxide indicates that the sulfhydryl groups of Site B, but not of Site A, are necessary for activation to occur. On the other hand, if Site A were not already oxidized, as would be expected in intact platelets, these agents could also activate the enzyme by oxidizing Site A first, and then Site B; whereas *N*-ethylmaleimide would block activation by reacting only with Site A (see Scheme 2). To account for the marked differences in the magnitude of the stimulatory effects of these oxidants, it is suggested that each agent oxidizes the sulfhydryl groups of Site B to a different extent (see Section 4.6.2.). Thus, the relatively small stimulatory effect of 4,4'-dithiodipyridine (*i.e.* 40% stimulation) could be due to the slow formation of mixed

disulfides, whereas the much larger effect of diamide (*i.e.* approximately 90% stimulation) could result from a more effective oxidation of enzyme sulfhydryl groups to the disulfide state. Since *tert*-butylhydroperoxide is an extremely powerful oxidant, the marked stimulation of guanylate cyclase activity caused by this agent (*i.e.* 165% stimulation) could be due to the oxidation of enzyme sulfhydryl groups to higher oxidation states such as the sulfenate (SO^-), sulfinate (SO_2^-) or sulfonate (SO_3^-) state.

In addition to oxidants, it is suggested that the stimulation of guanylate cyclase activity by preincubation of platelet lysate (*i.e.* 225% stimulation) is due to the oxidation of Site B. Although it is not yet established that guanylate cyclase is ultimately activated by hydroxyl radicals during preincubation, it is assumed that some powerful oxidant such as the hydroxyl radical, which could be generated in platelet lysate, is the attacking species. Since hydroxyl radicals are generated from *tert*-butylhydroperoxide, it is suggested that this agent and preincubation activate guanylate cyclase by the same mechanism - *i.e.* oxidation of Site B to the sulfenate, sulfinate or sulfonate state. This conclusion is supported by the following observations: the stimulatory effects of *tert*-butylhydroperoxide and preincubation are quantitatively similar, their effects are not additive, and both treatments decreased the ability of the enzyme to utilize Mg^{2+} as a substitute for Mn^{2+}. According to the proposed model, pretreatment of platelet lysate with dithiothreitol indirectly blocks activation by preincubation by restoring and maintaining Site A in the reduced or inhibitory state, which thereby renders the sulfhydryl groups of Site B inaccessible to oxidative attack. On the other hand, *N*-ethylmaleimide directly blocks activation by alkylating the available sulfhydryl groups

on Site B since Site A is assumed to be oxidized in platelet lysate (see Scheme 2). To explain why the stimulatory effect of preincubation is still detectable in the presence of dithiothreitol, it is suggested that this agent acts on Site A, but not on Site B since thiols cannot reverse oxidation past the disulfide state. Thus, the marked inhibition of preincubation-activated enzyme by dithiothreitol can be attributed to the reduction of the disulfide bond at Site A and subsequent restoration of its inhibitory capacity.

Murad *et al.* (1978) have recently suggested that the marked stimulation of guanylate cyclase activity by sodium nitroprusside may be attributable to the effects of nitric oxide, which could be spontaneously generated from nitroprusside. Although the mechanism by which sodium nitroprusside (nitric oxide) stimulates enzyme activity is not known, the findings of this thesis indicate that the activation of platelet guanylate cyclase by this agent is both quantitatively and qualitatively different from that caused by preincubation or *tert*-butylhydroperoxide. Firstly, the stimulatory effect of sodium nitroprusside (*i.e.* almost 1000% stimulation) is substantially larger than that of *tert*-butylhydroperoxide or preincubation. Secondly, pretreatment of platelet lysate with *tert*-butylhydroperoxide markedly reduced the stimulatory effect of sodium nitroprusside, while preincubation abolished it. Thirdly, an excess of 4.0 mM-dithiothreitol failed to inhibit the activity of enzyme pretreated with sodium nitroprusside. Finally, activation of guanylate cyclase by sodium nitroprusside resulted in a marked increase in the capacity of the enzyme to utilize Mg^{2+} as sole bivalent cation, whereas activation by *tert*-butylhydroperoxide or preincubation had the opposite effect. To explain these

findings according to the proposed model, it is suggested that the activation of platelet guanylate cyclase by sodium nitroprusside involves an unique oxidation by nitric oxide of the sulfhydryl groups of Site B and that this oxidation induces a conformational change in the enzyme that abolishes the regulatory function of Site A and alters the properties of the substrate and/or bivalent cation binding sites. Furthermore, Site B cannot be oxidized by nitric oxide unless the sulfhydryl groups are in the reduced state, which accounts for the failure of sodium nitroprusside to activate preincubated or *tert*-butylhydroperoxide-treated lysates (see Scheme 2).

To explain the finding that Lubrol PX results in approximately the same level of guanylate cyclase activity (*i.e.* 255% stimulation) regardless of whether it is added to untreated lysate or to lysate pretreated by preincubation, *tert*-butylhydroperoxide, sodium azide or sodium nitroprusside, it is proposed that this non-ionic detergent binds to Site B and induces a conformational change which abolishes the regulatory influence of the sulfhydryl groups located at this site, thus neither prior nor subsequent oxidation of this site can result in activation. Although in percentage terms, the stimulatory effect of Lubrol PX is not affected by pretreatment of lysate with dithiothreitol or *N*-ethylmaleimide, the final level of guanylate cyclase activity attained by these preparations is markedly lower than that observed with untreated or activated enzyme. In the case of dithiothreitol, inhibition is attributed to the reduction of the disulfide bond at Site A and restoration of its inhibitory function. Thus, Lubrol PX-activated enzyme appears to remain susceptible to the regulatory influence of Site A, but not of Site B. In the case of

N-ethylmaleimide, alkylation of the available sulfhydryl groups of Site B may irreversibly maintain the inhibitory influence of this site, which cannot be superceded by Lubrol PX, even though activation can still occur (see Scheme 2).

In summary, it has been proposed that the *in vitro* modulation of guanylate cyclase activity may be a function of the redox state of sulfhydryl groups located at two sites on the enzyme itself or on components involved in the control of enzyme activity.

In addition to the model described in this thesis, two other models have recently been proposed by Böhme *et al.* (1978) and by Goldberg *et al.* (1978) for the regulation of soluble guanylate cyclase from platelets and splenic cells, respectively. Although each of these models is based on the concept that the regulation of guanylate cyclase activity involves at least two oxidizable sites, probably sulfhydryl groups, that are either on the enzyme or on associated components, other aspects of these models are not directly comparable because somewhat different agents were tested in each study. As none of the evidence reported in this study or by Böhme *et al.* (1978) or Goldberg *et al.* (1978) is contradictory, differences in the proposed models can be attributed entirely to the interpretation of the data available in each case. The primary advantage of the model proposed in this thesis is that it is based on the interpretation of the effects and interactions of a much wider range of treatments and agents on guanylate cyclase activity.

The model proposed by Böhme *et al.* (1978) is based solely on results obtained from studies of the time- and oxygen-dependent activation and of the sodium nitroprusside-induced activation of guanylate cyclase in

human platelets. Of the two sites thought to be involved in the control of enzyme activity, one is assumed to be more reactive and susceptible to modifications than the other. Oxidation of this site during time- and oxygen-dependent activation, or other modification by sodium nitroprusside is believed to cause an increase in enzyme activity. Maintenance of this site in the reduced state with dithiothreitol blocks activation by preincubation but not by sodium nitroprusside. Thus, in both this model and in the model proposed in the present study, the same site is believed to be affected by activation by preincubation and by sodium nitroprusside. However, in contrast to the model described above, Böhme *et al.* (1978) believe that oxidation or other modification of the less reactive site results in decreased guanylate cyclase activity. This latter site is also believed to dominate with respect to effective regulation of enzyme activity. Although the model proposed by these workers can explain the effects of preincubation, sodium nitroprusside and dithiothreitol on the activity of platelet guanylate cyclase, the effects of other compounds on enzyme activity decribed in this thesis are inconsistent with their hypothesis.

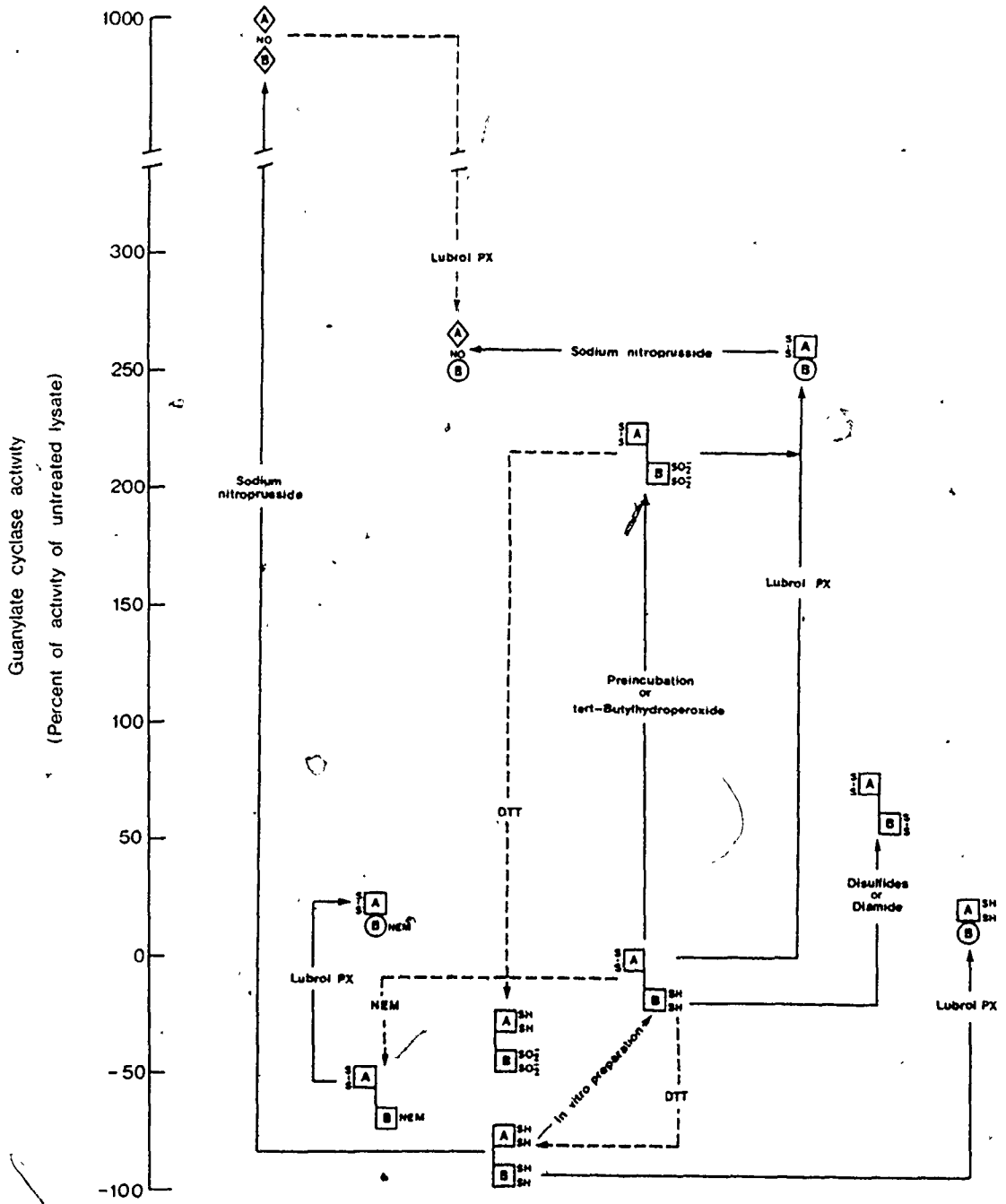
The possibility that cellular events involving oxidation and reduction may represent a general mechanism for the regulation of guanylate cyclase activity was first suggested by Haddox *et al.* (1976) who found that the concentration of cyclic GMP in splenic cells could be increased by oxidants and decreased by reducing agents. The results of subsequent studies (Haddox *et al.*, 1978; Goldberg *et al.*, 1978; Graff *et al.* 1978) of the effects of dehydroascorbic acid, fatty acid hydroperoxides, prostaglandin endoperoxides and of dithiothreitol and *N*-ethylmaleimide on the activity of guanylate cyclase from splenic cells led to the suggestion

(Goldberg *et al.*, 1978) that the enzyme and/or a related component exhibits separate regulatory sites for hydrophilic and hydrophobic oxidants which promote a sulfhydryl-disulfide interconversion at these sites, thereby causing an activation of the enzyme. The presence of separate hydrophilic and hydrophobic ligand sites was indicated by the observation that the stimulatory effects of the two classes of oxidants were additive (Goldberg *et al.*, 1978). Although this model is not directly comparable to the model proposed in this thesis or by Böhme *et al.* (1978), investigation of the effects of prostaglandin endoperoxides and fatty acid hydroperoxides on the activity of platelet guanylate cyclase would indicate whether differences between the models reflect tissue differences or differences in the activating agents studied.

Scheme 2. *Hypothetical model for the in vitro regulation of platelet guanylate cyclase activity*

In this model it is proposed that the regulation of guanylate cyclase activity is dependent on the oxidative state of sulfhydryl groups located at two sites, A and B, that are on the enzyme itself and/or on other components involved in the control of enzyme activity. The sulfhydryl groups of Site A are more accessible and therefore more susceptible to oxidation than those of Site B. Site A functions as an inhibitor of guanylate cyclase when in the reduced state, but has no effect on enzyme activity when the sulfhydryl groups are oxidized to the disulfide state. In addition to stimulating enzyme activity, oxidation of Site A results in an increase in the accessibility and susceptibility of the sulfhydryl groups of Site B to oxidative attack. Oxidation of Site B increases enzyme activity by a much greater extent than oxidation of Site A; thus Site B appears to function as an activator of guanylate cyclase when in an oxidized state.

The approximate guanylate cyclase activities after the various treatments are indicated in terms of the percent stimulation or inhibition of the activity of untreated lysate. Stimulatory effects are indicated by solid lines, and inhibitory effects by broken lines. The sequence of exposure to each agent or treatment is shown. Dithiothreitol (DTT), *N*-ethylmaleimide (NEM).


Guanylate cyclase activity
(Percent of activity of untreated lysate)
1000
300
250
200
150
100
50
0
-50
-100
Sodium nitroprusside
Lubrol PX
Sodium nitroprusside
Lubrol PX
Preincubation
or
tert-Butylhydroperoxide
DTT
Disulfides
or
Diamide
Lubrol PX
NEM
Lubrol PX
In vitro preparation
DTT

5.2. Physiological relevance of the in vitro activation and inhibition of platelet guanylate cyclase

Although the specific activity of platelet guanylate cyclase *in vitro* is higher than in almost all other mammalian tissues, the results of the present study indicate that the combined effects of inhibitory factors such as ATP and glutathione and of suboptimal ionic conditions are likely to lower the guanylate cyclase activity in intact platelets to almost negligible values in the absence of activating factors. Thus, the net effect of low levels of guanylate cyclase activity and high levels of cyclic GMP phosphodiesterase activity in platelets (*e.g.* >250 pmoles/min per mg of protein with 1 μM cyclic GMP)(Haslam *et al.*, 1975) would be likely to result in a low steady-state concentration of cyclic GMP in platelets, which has variously been estimated to be from 0.3 to 4 pmoles/10^9 platelets (for review see Haslam *et al.*, 1978a)(*n.b.* it is likely that the higher values are in error due to the use of activated platelets or methodological problems in the assays)(Haslam *et al.*, 1978a).

The marked increases in platelet cyclic GMP that occur in response to aggregating stimuli (White *et al.*, 1973; Haslam & McClenaghan, 1974; Haslam, 1975, 1978*a*,*b*; Jacobs *et al.*, 1975; Chiang *et al.*, 1975; Davies *et al.*, 1976; Glass *et al.*, 1977*b*; Weiss *et al.*, 1978) are believed to occur as a result of an activation of guanylate cyclase rather than as a result of an inhibition of cyclic nucleotide degradation; however, the mechanism whereby extracellular stimuli activate guanylate cyclase in platelets or in other tissues is only beginning to be understood. The fact that the platelet

enzyme appears to be entirely soluble, together with the finding that aggregating agents that increase platelet cyclic GMP levels did not, with the exception of arachidonate, increase the guanylate cyclase activity of whole platelet lysate, support the view that indirect mechanisms may exist by which physiological and pharmacological stimuli can activate the soluble enzyme in the cytosol. The findings of the present study combined with those of other workers (Goldberg *et al.*, 1978; Murad *et al.*, 1978; Böhme *et al.*, 1978; Graff *et al.*, 1978) indicate that processes involving oxidation and reduction may represent a general mechanism for the regulation of guanylate cyclase activity *in vivo*. It is therefore possible to speculate that upon stimulation by hormones or other physiological agents that increase the intracellular concentration of cyclic GMP, specific 'intermediary oxidants' are generated which couple the signal at the cell membrane with the activation of guanylate cyclase. Thus, the prostaglandin endoperoxides, PGG_2 and PGH_2 , which are generated from arachidonic acid during thrombin or collagen-induced platelet aggregation (Hamberg *et al.*, 1974, 1975; Samuelsson *et al.*, 1976), may serve as the 'intermediary oxidants' that activate guanylate cyclase in intact platelets. This possibility is suggested by the observation that PGG_2 can increase the concentration of cyclic GMP in intact platelets (Glass *et al.*, 1977*b*) together with the finding that PGG_2 or PGH_2 can stimulate soluble guanylate cyclase from splenic cells by an oxidative mechanism (Goldberg *et al.*, 1978; Graff *et al.*, 1978). Although the effect of the prostaglandin endoperoxides on platelet guanylate cyclase has not yet been specifically tested, there is sufficient evidence to believe that activation would occur, thereby distinguishing the platelet as the only system in which a naturally occurring effector of guanylate

cyclase can be identified.

References

Adam, H. (1965) Adenosine-5'-triphosphate. (Determination with phosphoglycerate kinase), IN *Methods of Enzymatic Analysis* (Bergmeyer, H. -U.,ed) p.p. 539-543 Academic Press, New York

Adam, H. (1965) Adenosine-5'-diphosphate and adenosine-5'-monophosphate. IN *Methods of Enzymatic Analysis* (Bergmeyer, H. -U.,ed) p.p. 573-577 Academic Press, New York

Adams, A.F. & Haslam, R.J. (1978) Factors affecting the activity of guanylate cyclase in lysates of human blood platelets. *Biochem. J.* 174, 23-35

Agarwal, K.C. & Parks, R.E. Jr. (1975) Adenosine analogues and human platelets. Effects on nucleotide pools and the aggregation phenomenon. *Biochem. Pharmacol.* 24, 2239-2248

Amer, M.S. (1974) Cyclic guanosine 3',5'-monophosphate and gall bladder contraction. *Gasteroenterol.* 67, 333-337

Amer, M.S. & McKinney, G.R. (1973) The possible role of cyclic GMP in gall bladder contraction and gastric acid secretion in rabbits. *Pharmacologist* 15, 157 (abs)

Arnold, W.P., Mittal, C.K., Katsuki, S, & Murad, F. (1977*a*) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic nucleotide monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. U.S.A.* 74, 3203-3207

Arnold, W.P., Aldred, R. & Murad, F. (1977*b*) Cigarette smoke activates guanylate cyclase and increases guanosine 3'5'-monophosphate in tissues. *Science* 198, 934-936

Asakawa, T., Scheinbaum, I. & Ho, R. -J. (1976) Stimulation of guanylate cyclase by several fatty acids. *Biochem. Biophys. Res. Commun.* 73, 141-148

Asano, T. & Hidaka, H. (1977) Purification of guanylate cyclase from human platelets and the effects of arachidonic acid peroxide. *Biochem. Biophys. Res. Commun.* 78, 910-918

Ashman, D.F., Lipton, R., Melicow, M.M. & Price, T.D. (1963) Isolation of adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate from rat urine. *Biochem. Biophys. Res. Commun.* 11, 330-334

Aster, R.H. & Jandl, J.H. (1964) Platelet sequestration in man, I. Methods. *J. Clin. Invest.* 43, 843-855

Ball, J.H., Kaminsky, N.I., Broadus, A.E., Hardman, J.G., Sutherland, E.W. & Liddle, G.W. (1970) Effects of catecholamines and adrenergic-blocking agents on cyclic nucleotides in human plasma. *Clin. Res.* 18, 336 (abs)

Ball, J.H., Kaminsky, N.I., Hardman, J.G., Broadus, A.E., Sutherland, E.W. & Liddle, G.W. (1972) Effects of catecholamines and adrenergic-blocking agents on plasma and urinary cyclic nucleotides in man. *J. Clin. Invest.* 51, 2124-2129

Barber, A.J. (1976) Cyclic nucleotides and platelet aggregation. Effect of aggregating agents on the activity of cyclic nucleotide -metabolizing enzymes. *Biochim. Biophys. Acta* 444, 579-595

Bensinger, R.E., Fletcher, R.T. & Chader, G.J. (1974) 'Piggyback' chromatography: assay for guanylate cyclase in retina and other neural tissue. *J. Neurochem.* 22, 1131-1134

Bergmeyer, H. -U., Bernt, E. & Hess, B. (1965) Lactic Dehydrogenase. IN *Methods of Enzymatic Analysis*. (Bergmeyer, H. -U., ed) p.p. 736-743 Academic Press, New York

Berridge, M.J. (1975) The interaction of cyclic nucleotides and calcium in the control of cellular activity. *Adv. Cyclic Nucleotide Res.* 6, 2-98

Blosser, J.C. & Appel, S.H. (1978) Properties and distribution of mammalian skeletal muscle guanylate cyclase. Alterations in denervated and dystrophic muscle. *J. Biol. Chem.* 253, 3088-3093

Böhme, E. (1970) Guanyl-cyclase. Bildung von guanosin-3':5'-monophosphat in niere und anderen geweben der ratte. *Eur. J. Biochem.* 14, 422-429

Böhme, E., Jung, R. & Mechler, I. (1974) Guanylate cyclase in human platelets. *Methods in Enzymol.* 38C, 199-202

Böhme, E., Graf, H. & Schultz, G. (1978) Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. *Adv. Cyclic Nucleotide Res.* 9, 131-143

Born, G.V.R. (1970) Observations on the shape change of blood platelets brought about by adenosine diphosphate. *J. Physiol.* (Lond.) 209, 487-511

Broadus, A.E., Kaminsky, N.I., Northcutt, R.C., Hardman, J.G., Sutherland, E.W. & Liddle, G.W. (1970) Effects of glucagon on adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in human plasma and urine. *J. Clin. Invest.* 49, 2237-2245

Broadus, A.E., Hardman, J.G., Kaminsky, N.I., Ball, J.H., Sutherland, E.W. & Liddle, G.W. (1971) Extracellular cyclic nucleotides. *Ann. N.Y. Acad. Sci.* 185, 50-66

Caldwell, P.C. (1970) Calcium chelation and buffers. IN *Calcium and Cellular Function, A Symposium* (Cuthbert, A.W., ed) p.p. 10-16 St. Martin's Press Inc., New York

Catalan, R.E., Castillon, M.P. & Municio, A.M. (1976) Cyclic nucleotide cyclase variation during development of the insect *Ceratis capitata*. *Biochem. Biophys. Res. Commun.* 69, 914-919

Charo, I.F., Feinman, R.D. & Detwiler, T.C. (1976) Inhibition of platelet secretion by an antagonist of intracellular calcium. *Biochem. Biophys. Res. Commun.* 72, 1462-1467

Chiang, T.M., Beachley, E.H. & Kang, A.H. (1975) Interaction of a chick skin collagen fragment (α1-CB5) with human platelets. Biochemical studies during the aggregation and release reaction. *J. Biol. Chem.* 250, 6916-6922

Chiang, T.M., Dixit, S.N. & Kang, A.H. (1976) Effect of cyclic 3',5'-guanosine monophosphate on human platelet function. *J. Lab. Clin. Med.* 88, 215-221

Chrisman, T.D., Garbers, D.L., Parks, M.A. & Hardman, J.G. (1975) Characterization of particulate and soluble guanylate cyclases from rat lung. *J. Biol. Chem.* 250, 374-381

Claesson, H. -E. & Malmsten, C. (1977) On the interrelationship of prostaglandin endoperoxide G_2 and cyclic nucleotides in platelet function. *Eur. J. Biochem.* 76, 277-284

Clark, V.L. & Bernlohr, R.W. (1972) Guanyl cyclase of *Bacillus licheniformis*. *Biochem. Biophys. Res. Commun.* 46, 1570-1575

Clyman, R.I., Blacksin, A.S., Manganiello, V.C. & Vaughan (1975) Oxygen and cyclic nucleotides in human umbilical artery. *Proc. Natl. Acad. Sci. U.S.A.* 72, 3883-3887

Craven, P.A. & DeRubertis, F.R. (1976) Properties and subcellular distribution of guanylate cyclase activity in rat renal medulla: Correlation with tissue content of guanosine 3',5'-monophosphate. *Biochem.* 15, 5131-5137

Craven, P.A. & DeRubertis, F.R. (1977) Inhibition by retinol and butylated hydroxy anisole of carcinogen-mediated increases in guanylate cyclase activity and guanosine 3',5'-monophosphate accumulation. *Cancer Res.* 37, 4088-4097

Criss, W.E., Murad, F. & Kimura, H. (1976*a*) Properties of guanylate cyclase from rat kidney cortex and transplantable kidney tumors. *J. Cyclic Nucleotide Res.* 2, 11-19

Criss, W.E., Murad, F., Kimura, H. & Morris, H.P. (1976*b*) Properties of guanylate cyclase in adult rat liver and several Morris hepatomas. *Biochim. Biophys. Acta* 445, 500-518

Cotzias, G.C. (1972) Manganese IN *Mineral Metabolism* (Comar, C.L. & Bronner, F. eds) Vol. II, Part B p.p. 404-442 Academic Press, New York

Daniel, W.W. (1974) *Biostatistics: A Foundation for Analysis in Health Sciences* John Wiley & Sons, Inc., New York 387 pp

Davies, T., Davidson, M.M.L., McClenaghan, M.D., Say, A. & Haslam, R.J. (1976) Factors affecting platelet cyclic GMP levels during aggregation induced by collagen and by arachidonic acid. *Thromb. Res.* 9, 387-405

Deguchi, T. (1977) Activation of guanylate cyclase in cerebral cortex of rat by hydroxylamine. *J. Biol. Chem.* 252, 596-601

Deguchi, T., Amano, E. & Nakane, M. (1976) Subcellular distribution and activation by non-ionic detergents of guanylate cyclase in cerebral cortex of rat. *J. Neurochem.* 27, 1027-1034

DeJonge, H.R. (1975*a*) The localization of guanylate cyclase in rat small intestinal epithelium. *F.E.B.S. Lett.* 53, 237-242

DeJonge, H.R. (1975*b*) Properties of guanylate cyclase and levels of cyclic GMP in rat small intestinal villous and crypt cells. *F.E.B.S. Lett.* 55, 143-152

DeRubertis, F.R. & Craven, P.A. (1976*a*) Properties of the guanylate cyclase-guanosine 3':5'-monophosphate system of rat renal cortex. Activation of guanylate cyclase and calcium-independent modulation of tissue guanosine 3':5'-monophosphate by sodium azide. *J. Biol. Chem.* 251, 4651-4658

DeRubertis, F.R. & Craven, P.A. (1976*b*) Calcium independent modulation of cyclic GMP and activation of guanylate cyclase by nitrosamines. *Science* 193,897-899

DeRubertis, F.R. & Craven, P.A. (1977*a*) Activation of hepatic guanylate cyclase by N-methyl-N'-nitro-N-nitrosoguanidine. Effects of thiols, N-ethylmaleimide, and divalent cations. *J. Biol. Chem.* 252, 5804-5814

DeRubertis, F.R. & Craven, P.A. (1977*b*) Activation of the renal cortical and hepatic guanylate cyclase-guanosine 3',5'-monophosphate systems by nitrosoureas. Divalent cation requirements and relationship to thiol reactivity. *Biochim. Biophys. Acta* 499, 337-351

Deviller, R., Y. Cille & Betuel, H. (1975) Guanyl cyclase activity of human blood lymphocytes. *Enzyme* 19, 300-313

Durham, J.P. (1976) Guanylate cyclase: Assay and properties of the particulate and supernatant enzymes in mouse parotid. *Eur. J. Biochem.* 61, 535-544

Earp, H.S., Smith, P., Ovig, S.H. & Steiner, A.L. (1977) Regulation of hepatic nuclear guanylate cyclase. *Proc. Natl. Acad. Sci. U.S.A.* 74, 946-950

Estensen, R., Hadden, E.M., Touraire, F., Touraire, J.L., Haddox, M.K. & Goldberg, N.D. (1974) Phorbol myristate acetate: Effects of a tumor promotor on intracellular cyclic GMP in mouse fibroblasts and as a mitogen on human lymphocytes. IN *The Cold Spring Harbor Symposium on Cell Proliferation in Animal Cells* (Clarkson, B. & Baserga, R., eds) p.p. 627-634 Cold Spring Harbor Laboratory, New York

Fain, J.N. & Butcher, F.R. (1976) Cyclic guanosine 3':5'-monophosphate and the regulation of lipolysis in rat fat cells. *J. Cyclic Nucleotide Res.* 2, 71-78

Feinman, R.D. & Detwiler, T.C. (1974) Platelet secretion induced by divalent cation ionophores. *Nature* 249, 172-173

Feinstein, M.B., Fiekers, J. & Fraser, C. (1976) An analysis of the mechanism of local anaesthetic inhibition of platelet aggregation and secretion. *J. Pharmacol. Exp. Ther.* 197, 215-228

Ferrendelli, J.A., Steiner, A.L., McDougal, D.B. Jr. & Kipnis, D.M. (1970) The effect of oxotremorine and atropine on cyclic GMP and cyclic AMP levels in mouse cerebral cortex and cerebellum. *Biochem. Biophys. Res. Commun.* 41, 1061-1067

Ferrendelli, J.A., Kinscherf, D.A. & Kipnis, D.M. (1972) Effects of amphetamine, chlorpromazine and reserpine on cyclic GMP and cyclic AMP levels in mouse cerebellum. *Biochem. Biophys. Res. Commun.* 46, 2114-2120

Ferrendelli, J.A., Kinscherf, D.A. & Chang, M.M. (1973) Regulation of levels of guanosine 3',5'-monophosphate in the central nervous system: Effects of depolarizing agents. *Mol. Pharmacol.* 9, 445-454

Fridovich, I. (1975) Superoxide dismutases. *Ann. Rev. Biochem.* 44, 145-159

Fridovich, I. (1976) Oxygen radicals, hydrogen peroxide and oxygen toxicity. IN *Free Radicals in Biology* (Pryor, W.A., ed) Vol. I, p.p. 239-277 Acdemic Press, New York

Fujimoto, M. & Okabayaski, T. (1975) Proposed mechanisms of stimulation and inhibition of guanylate cyclase with reference to the actions of chlorpromazine, phospholipases and Triton X-100. *Biochem. Biophys. Res. Commun.* 67, 1332-1336

Garbers, D.L. (1976) Sea urchin sperm guanylate cyclase. Purification and loss of cooperativity. *J. Biol. Chem.* 251, 4071-4077

Garbers, D.L. & Hardman, J.G. (1975) Effects of metals and nucleotides on the inactivation of sea urchin sperm guanylate cyclase by heat and *N*-ethylmaleimide. *J. Biol. Chem.* 250, 2482-2486

Garbers, D.L., Hardman, J.G. & Rudolf, F.B. (1974) Kinetic analysis of sea urchin sperm guanylate cyclase. *Biochem.* 13, 4166-4171

Garbers, D.L., Dyer, E.L. & Hardman, J.G. (1975*a*) Effects of cations on guanylate cyclase of sea urchin sperm. *J. Biol. Chem.* 250, 374-381

Garbers, D.L., Chrisman, T.D., Suddath, J.L. & Hardman, J.G. (1975*b*) Formation of pyrophosphate by soluble guanylate cyclase from rat lung. *Arch. Biochem. Biophys.* 166, 135-138

George, W.J., Polson, J.B., O'Toole, A.G. & Goldberg, N.D. (1970) Elevation of guanosine 3',5'-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proc. Natl. Acad. Sci. U.S.A.* 66, 398-403

Glass, D.B., White, J., Haddox, M.K. & Goldberg, N.D. (1973) Epinephrine- and collagen-induced accumulation of cyclic GMP in human platelets. *Pharmacologist* 15, 156 (abs)

Glass, D.B., White, J.G. & Goldberg, N.D. (1974) Rapid elevation in cyclic GMP levels with epinephrine-induced platelet aggregation. *Fed. Proc.* 33, 611 (abs)

Glass, D.B., Frey, W. II, Carr, D.W. & Goldberg, N.D. (1977*a*) Stimulation of human platelet guanylate cyclase by fatty acids. *J. Biol. Chem.* 252, 1279-1285

Glass, D.B., Gerrard, J.H., Townsend, D., Carr, D.W., White, J.G. & Goldberg, N.D. (1977*b*) The involvement of prostaglandin endoperoxide formation in the elevation of cyclic GMP levels during platelet aggregation. *J. Cyclic Nucleotide Res.* 3, 37-44

Glusa, M., Markwardt, F. & Stürzebecher, J. (1974) Effects of sodium nitroprusside and other pentacyanonitrosyl complexes on platelet aggregation. *Hemostasis* 3, 249-256

Goldberg, N.D., Dietz, S.B. & O'Toole, A.G. (1969) Cyclic guanosine 3',5'-monophosphate in mammalian tissues and urine. *J. Biol. Chem.* 244, 4458-4466

Goldberg, N.D., Lust, W.D., O'Dea, R.F., Wei, S. & O'Toole, A.G. (1970) A role of cyclic nucleotides in brain metabolism. *Adv. Biochem. Psychopharmacol.* (Costa, E. & Greengard, P., eds) Vol. 3, p.p. 67-87 Raven Press, New York

Goldberg, N.D., Haddox, M.K., Hartle, D.K. & Hadden, J.W. (1973a) The biological role of cyclic 3',5'-guanosine monophosphate. IN *Pharmacology and the Future of Man. Proc. 5th Int. Cong. Pharmacol., San Francisco. 1972.* Vol. 5, p.p. 146-169. Karger, Basal

Goldberg, N.D., O'Dea, R.F. & Haddox, M.K. (1973b) Cyclic GMP. *Adv. Cyclic Nucleotide Res.* 3, 155-223

Goldberg, N.D., Haddox, M.K., Durham, E., Lopes, C. & Hadden, J.W. (1974) The Yin-Yang hypothesis of biological control: Opposing influences of cyclic GMP and cyclic AMP in the regulation of cell proliferation and other biological processes. IN *The Cold Spring Harbor Symposium on the Regulation of Proliferation in Animal Cells* (Clarkson, B. & Basaga, R., eds) p.p. 609-626 Cold Spring Harbor, New York, Cold Spring Harbor Laboratories

Goldberg, N.D., Haddox, M.K., Nicol. S.E., Glass, D.B., Sanford, C.H., Kuehl, F.A. Jr. & Estensen, R.D. (1975) Biologic regulation through opposing influences of cyclic GMP and cyclic AMP: The Yin-Yang hypothesis. *Adv. Cyclic Nucleotide Res.* 5, 307-330

Goldberg, N.D. & Haddox, M.K, (1977) Cyclic GMP metabolism and involvement in biological regulation. *Ann. Rev. Biochem.* 46, 823-896

Goldberg, N.D., Graff, G., Haddox, M.K., Stephenson, J.H., Glass, D.B. & Mosel, M.E. (1978) Redox modulation of splenic cell soluble guanylate cyclase activity: Activation by hydrophilic and lydrophobic oxidants represented by ascorbic and dehydroascorbic acids, fatty acid hydroperoxides, and prostaglandin endoperoxides. *Adv. Cyclic Nucleotide Res.* 9, 101-130

Goridis, C. & Morgan, I.G. (1973) Guanyl cyclase in rat brain subcellular fractions. *F.E.B.S. Lett.* 34, 71-73

Goridis, C. & Reutter, W. (1975) Plasma membrane-associated increase in guanylate cyclase activity in regenerating rat liver. *Nature* 257, 698-700

Goridis, C., Virmaux, N., Urban, P.F. & Mandel, P. (1973) Guanyl cyclase in a mammalian photoreceptor. *F.E.B.S. Lett.* 30, 163-166

Goridis, C., Massarelli, R., Sensenbrenner, M. & Mandel, P. (1974) Guanyl cyclase in chick embryo brain cell cultures: Evidence of neuronal localization. *J. Neurochem.* 23, 135-138

Gorman, R.R., Bunting, S. & Miller, O.V. (1977) Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins* 13, 377-388

Graff, G., Stephenson, J.H., Glass, D.B., Haddox, M.K. & Goldberg, N.D. (1978) Activation of soluble splenic cell guanylate cyclase by prostaglandin endoperoxides and fatty acid hydroperoxides. *J. Biol. Chem.* 253, 7662-7676

Grassetti, D.R. & Murray, J.F. Jr. (1967) Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch. Biochem. Biophys.* 119, 41-44

Gray, J.P., Hardman, J.G., Bibring, T. & Sutherland, E.W. (1970) High guanyl cyclase activity in sea urchin spermatozoa. *Fed. Proc.* 29, 608 (abs)

Gray, J.P., Drummond, G.I. & Luk, D.W. (1976) Enzymes of cyclic nucleotide metabolism in invertebrate and vertebrate sperm. *Arch. Biochem. Biophys.* 172, 20-30

Grunert, R.R. & Phillips, P.H. (1951) A modification of the nitroprusside method of analysis for glutathione. *Arch. Biochem. Biophys.* 30, 217-225

Haber, F. & Weiss, J. (1934) *Proc. R. Soc. Edinb. Sect. A* 147, 332-351

Haddox, M.K., Stephenson, J.H. & Goldberg, N.D. (1974) Cyclic GMP in meristematic and elongating regions of bean root. *Fed. Proc.* 33, 522 (abs)

Haddox, M.K., Furcht, L.T., Gentry, S.R., Moser, M.E., Stephenson, J.H. & Goldberg, N.D. (1976) Periodate-induced increase in cyclic GMP in mouse and guinea pig splenic cells in association with mitogenesis. *Nature* 262, 146-148

Haddox, M.K., Furcht, L.T., Gentry, S.R., Moser, S.R., Stephenson, J.H. & Goldberg, N.D. (1977) Oxidative-reductive modulation of guinea pig splenic cell guanylate cyclase activity. IN *Cyclic Nucleotides and the Regulation of Cell Growth* (Abou-Sabe, M., ed) p.p. 207-224 Dowden, Hutchinson & Ross, Pennsylvania

Haddox, M.K., Stephenson, J.H., Moser, M.E. & Goldberg, N.D. (1978) Oxidative-reductive modulation of guinea pig splenic cell guanylate cyclase activity. *J. Biol. Chem.* 253, 3154-3152

Hamberg, M. & Samuelsson, B. (1974) Prostaglandin endoperoxides: Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U.S.A.* 71, 3400-3404

Hamberg, M., Svensson, J., Wakabayashi, T. & Samuelsson, B. (1974) Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 71, 345-349

Hamberg, M., Svensson, J. & Samuelsson, B. (1975) Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.* 72, 2994-2998

Hamet, P., Fraysee, J. & Franks, D.J. (1978) Cyclic nucleotides and aggregation in platelets of spontaneously hypertensive rats. *Circulation Res.* 43, 583-591

Hardman, J.G. & Sutherland, E.W. (1969) Guanyl cyclase, an enzyme catalyzing the formation of guanosine 3',5'-monophosphate from guanosine triphosphate. *J. Biol. Chem.* 244, 6363-6370

Hardman, J.G., Davis, J.W. & Sutherland, E.W. (1966) Measurement of guanosine 3',5'-monophosphate and other cyclic nucleotides. *J. Biol. Chem.* 241, 4812-4815

Hardman, J.G., Beavo, J.A., Gray, J.P., Chrisman, T.D., Patterson, W.D. & Sutherland, E.W. (1971) The formation and metabolism of cyclic GMP. *Ann. N.Y. Acad. Sci.* 185, 27-35

Hardman, J.G., Chrisman, T.D., Gray, J.B., Suddath, J.L. & Sutherland, E.W. (1973) Guanylate cyclase: Alteration of apparent subcellular distribution and activity by detergents and cations. IN *Cellular Mechanisms Proc. 5th Int. Cong. Pharmacol. San Francisco, 1972.* (Maxwell, R.A. & Acheson, G.H., eds) *Pharmacology and the Future of Man* Vol. 5, p.p. 134-145 Karger, Basal

Harker, L.A. (1974) *Hemostasis Manual* (Harker, L.A., ed) 81 pp F.A. Davis Co., Philadelphia

Harris, J.W. & Bigelow, J.E. (1972) Non-specific reactions of the glutathione oxidant "diamide" with mammalian cells. *Biochem. Biophys. Res. Commun.* 46, 1743-1749

Haslam, R.J. (1973) Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser. Haematol.* 6, 333-350

Haslam, R.J. (1975) Roles of cyclic nucleotides in platelet function. IN *Biochemistry and Pharmacology of Platelets,* Ciba Foundation Symposium 35 (new series) p.p. 121-151 Elsevier/Excerpta Medica/ North-Holland, Amsterdam

Haslam, R.J. (1978) Cyclic nucleotides in platelet function. IN *Platelet Function Testing, Proceedings from Workshop on Platelets, Philadephia, 18-19 October, 1976* (Day, H.J., Holmsen, H. & Zucker, M.B., eds) p.p. 487-503 U.S. Department of Health, Education and Welfare, DHEW Publication No. (NIH) 78-1087

Haslam, R.J. & Lynham, J.A. (1972) Activation and inhibition of blood platelet adenylate cyclase by adenosine or by 2-chloroadenosine. *Life Sci.* 11, Part II, 1143-1154

Haslam, R.J. & Rosson, G.M. (1972) Aggregation of human blood platelets by vasopressin. *Am. J. Physiol.* 223, 958-967

Haslam, R.J. & McClenaghan, M.D. (1974) Effects of collagen and of aspirin on the concentration of guanosine 3':5'-cyclic monophosphate in human blood platelets: measurement by a prelabelling technique. *Biochem. J.* 138, 317-320

Haslam, R.J. & Lynham, J. (1976) Increased phosphorylation of specific blood platelet proteins in association with the release reaction. *Biochem. Soc. Trans.* 4, 694-697

Haslam, R.J., McClenaghan, M.D. & Adams, A. (1975) Depression of cGMP in blood platelets by acetylsalicylic acid (ASA) and related drugs. *Adv. Cyclic Nucleotide Res.* 5, 821 (abs)

Haslam, R.J., Davidson, M.M.L., Davies, T., Lynham, J.A. & McClenaghan, M.D. (1978*a*) Regulation of blood platelet function by cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* 9, 533-551

Haslam, R.J., Davidson, M.M.L., Fox, J.E.B. & Lynham, J.A. (1978*b*) Cyclic nucleotides in platelet function. IN *Platelets: Intracellular Control Mechanisms, 5th Int. Cong. Thrombosis Haemostasis, Philadelphia 1977. Thromb. Haem.* 40, 232-240

Helenius, A. & Simons, K. (1975) Solubilization of membranes by detergents. *Biochim. Biophys. Acta* 415, 29-79

Helwig, J.J., Bollack, C., Mandel, P. & Goridis, C. (1975) Renal cortex guanylate cyclase: Preferential enrichment in glomerular membranes. *Biochim. Biophys. Acta* 377, 463-472

Hidaka, H. & Asano, T. (1977*a*) Stimulation of human platelet guanylate cyclase by unsaturated fatty acid peroxides. *Proc. Natl. Acad. Sci. U.S.A.* 74, 3657-3661

Hidaka, H. & Asano, T. (1977*b*) Purification of guanylate cyclase from human platelets and effect of arachidonic acid peroxide. *Biochem. Biophys. Res. Commun.* 78, 910-918

Holmsen, H. & Day, H.J. (1971) Adenine nucleotides and platelet function. *Ser. Haematol.* 4, 28-58

Holmsen, H., Day, H.J. & Stormorken, H. (1969) The blood platelet release reaction. *Scand. J. Haematol. Suppl.*, 8, 1-29

Howard, P.L. (1976) *Basic Liquid Scintillation Counting* American Soc. Clin. Pathol. Pub. Chicago 86 p.p.

Howell, S.L. & Montague, W. (1974) Regulation of guanylate cyclase in guinea-pig Islets of Langerhans. *Biochem. J.* 142, 379-384

Ichihara, K., Larner, J., Kimura, H. & Murad, F. (1977) Activation of liver guanylate cyclase by bile salts and contamination in crude secretin and pancreozymin preparations. *Biochim. Biophys. Acta* 481, 734-740

Ignarro, L.J. (1973) Neutral protease release from human leukocytes regulated by neurohormones and cyclic nucleotides. *Nature (London) New Biol.* 245, 151-154

Ignarro, L.J., Lint, T.F. & George, W.J. (1974) Hormonal control of lysosomal enzyme release from human neutrophils. Effects of autonomic agents on enzyme release, phagocytosis and cyclic nucleotide levels. *J. Exp. Med.* 139, 1395-1414

Ishikawa, E., Ishikawa, S., Davis, J.W. & Sutherland, E.W. (1969) Determination of guanosine 3',5'-monophosphate in tissues and of guanyl cyclase in rat intestine. *J. Biol. Chem.* 244, 6371-6376

Jones, E.T. & Williams, C.H. Jr. (1975) The sequence of amino acid residues around the oxidation-reduction active disulfide in yeast glutathione reductase. Homology with the analogous region in lipoamide dehydrogenase. *J. Biol. Chem.* 250, 3779-3784

Kaliner, M., Orange, R.P. & Austen, K.F. (1973) Immunological release of histamine and slow reacting substance of anaphylaxis from human lung. IV Enhancement by cholinergic and alpha adrenergic stimulation. *J. Exp. Med.* 136, 556-567

Kaminsky, N.I., Ball, J.H., Broadus, A.E., Hardman, J.G., Sutherland, E.W. & Liddle, G.W. (1970) Hormonal effects on extracellular cyclic nucleotides in man. *Trans. Assoc. Amer. Physicians* 83, 235-243

Käser-Glanzman, R., Jakábová, M., George, J.N. & Lilsher, E.F. (1977) Stimulation of calcium uptake in platelet membrane vesicles by adenosine 3',5'-monophosphate and protein kinase. *Biochim. Biophys. Acta* 466, 429-440

Katagiri, T., Terao, T. & Osawa, T. (1976) Activation of mouse splenic lymphocyte guanylate cyclase by calcium ion. *J. Biochem.* 79, 849-852

Katsuki, S., Arnold, W., Mittal, C.K. & Murad, F. (1977*a*) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic Nucleotide Res.* 3, 23-35

Katsuki, S., Arnold, W.P. & Murad, F. (1977*b*) Effects of sodium nitroprusside, nitroglycerin and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. *J. Cyclic Nucleotide Res.* 3, 239-247

Keilin, D. & Hartree, E.F. (1954) Reactions of methhaemoglobin and catalase with peroxides and hydrogen donors. *Nature* 173, 720-723

Kimura, H. & Murad, F. (1974*a*) Evidence for two different forms of guanylate cyclase in rat heart. *J. Biol. Chem.* 249, 6910-6916

Kimura, H. & Murad, F. (1974*b*) Non-enzymatic formation of guanosine 3':5'-monophosphate from guanosine triphosphate. *J. Biol. Chem.* 249, 329-331

Kimura, H. & Murad, F. (1975*a*) Two forms of guanylate cyclase in mammalian tissues and possible mechanisms for their regulation. *Metabolism* 24, 439-445

Kimura, H. & Murad, F. (1975*b*) Subcellular localization of guanylate cyclase. *J. Biol. Chem.* 250, 2675-2681

Kimura, H. & Murad, F. (1975*c*) Two forms of guanylate cyclase in several rat tissues. *Adv. Cyclic Nucleotide Res.* 5, 822 (abs)

Kimura, H. & Murad, F. (1975*d*) Localization of particulate guanylate cyclase in plasma membranes and microsomes of rat liver. *J. Biol. Chem.* 250, 4810-4817

Kimura, H. & Murad, F. (1975*e*) Increased particulate and decreased soluble guanylate cyclase activity in regenerating lever, fetal liver and hepatoma. *Proc. Natl. Acad. Sci. U.S.A.* 72, 1965-1969

Kimura, H., Mittal, C.K. & Murad, F. (1975*a*) Increases in cyclic GMP levels in brain and liver with sodium azide as an activator of guanylate cyclase. *Nature* 257, 700-702

Kimura, H., Mittal, C.K. & Murad, F. (1975*b*) Activation of guanylate cyclase from rat liver or other tissues by sodium azide. *J. Biol. Chem.* 250, 8016-8022

Kimura, H., Mittal, C.K. & Murad, F. (1976) Appearance of magnesium guanylate cyclase activity in rat liver with sodium azide activation. *J. Biol. Chem.* 251, 7769-7773

Kirschbaum, B.B. & Bosman, H.B. (1973) Lysolecithin enhancement of glycoprotein: glycosyltransferase activity. *F.E.B.S. Lett.* 34, 129-132

Knappe, J., Schacht, J., Mockel, W., Hopner, Tn., Vetter, H. Jr. & Edenharder, R. (1969) Pyruvate formate lyase reaction in *Escherichia coli*. *Eur. J. Biochem.* 11, 316-327

Kosower, N.S., Kosower, E.M. & Wertheim, B. (1969) Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem. Biophys. Res. Commun.* 37, 593-596

Kraska, R.C., Stephenson, J.H. & Goldberg, N.D. (1976) Spontaneous oxidative activation and inactivation of rat uterine guanylate cyclase. *Fed. Proc.* 36, 686 (abs)

Kreye, V.A.W., Baron, G.D., Lüth, J.B. & Schmidt-Gayk, H. (1975) Mode of action of sodium nitroprusside on vascular smooth muscle. *Naunyn. Schmiedebergs Arch. Pharmacol.* 288, 381-402

Kuo, J. -F., Lee, T. -P., Reyes, P.L., Walton, K.G., Donelly, T.E.Jr. & Greengard, P. (1972) Cyclic nucleotide-dependent protein kinases. X. An assay method for the measurement of guanosine 3',5'-monophosphate in various biological materials and a study of agents regulating its levels in heart and brain. *J. Biol. Chem.* 247, 16-22

Lawley, P.D. & Thatcher, C.J. (1970) Methylation of deoxyribonucleic acid in cultured mammalian cells by *N*-methyl-*N*'-nitr-*N*-nitrosoguanidine. *Biochem. J.* 116, 693-707

LeBreton, G.C., Dinerstein, R.J., Roth, L.J. & Feinberg, H. (1976) Direct evidence for intracellular divalent cation redistribution associated with platelet shape change. *Biochem. Biophys. Res. Commun.* 71, 362-370

Lee, T.P., Kuo, J.F. & Greengard, P. (1972) Role of muscarinic cholinergic receptors in regulation of guanosine 3',5'-cyclic monophosphate content in mammalian brain, heart muscle, and intestinal smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.* 69, 3287-3291

Limbird, L.E. & Lefkowitz, R.J. (1975) Myocardial guanylate cyclase: Properties of the enzyme and effects of cholinergic agonists *in vitro*. *Biochim. Biophys. Acta* 377, 186-196

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193, 265-275

Macchia, V., Varrone, S., Weissbach, H., Miller, D.L. & Pastan, I. (1975) Guanylate cyclase in *Escherichia coli*. Purification and properties. *J. Biol. Chem.* 250, 6214-6217

Massini, P. & Lüscher, E.F. (1976) On the significance of the influx of calcium ions into stimulated human blood platelets. *Biochim. Biophys. Acta* 436,652-663

Marks, F. (1973) The second messenger system of mouse epidermis. III. Guanyl cyclase. *Biochim. Biophys. Acta* 309, 349-356

Miki, N., Nagano, M. & Kuriyama, K. (1977*a*) Catalase activates cerebral guanylate cyclase in the presence of sodium azide. *Biochem. Biophys. Res. Commun.* 72, 952-959

Miki, N., Kawabe, Y. & Kuriyama, K. (1977*b*) Activation of cerebral guanylate cyclase by nitric oxide. *Biochem. Biophys. Res. Commun.* 75, 851-856

Mirvish, S.S., Wallcane, L., Eagen, M. & Shubik, P. (1972) Ascorbate-nitrite reaction: Possible means of blocking the formation of carcinogenic *N*-nitroso compounds. *Science* 177, 65-68

Mittal, C.K. & Murad, F. (1977*a*) Properties and oxidative regulation of guanylate cyclase. *J. Cyclic Nucleotide Res.* 3, 381-391

Mittal, C.K. & Murad, F. (1977*b*) Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: A physiological regulator of guanosine 3':5'-monophosphate formation. *Proc. Natl. Acad. Sci. U.S.A.* 74, 4360-4364

Mittal, C.K., Kimura, F. & Murad, F. (1975) Requirement for a macromolecular factor for sodium azide activation of guanylate cyclase. *J. Cyclic Nucleotide Res.* 1, 261-269

Mittal, C.K., Kimura, H. & Murad, F. (1977) Purification and properties of a protein required for sodium azide activation. *J. Biol. Chem.* 252, 4384-4390

Mittal, C.K., Arnold, W.P. & Murad, F. (1978) Characterization of protein inhibitors of guanylate cyclase activation from rat heart and bovine lung. *J. Biol. Chem.* 253, 1266-1271

Molnar, J. & Lorand, L. (1961) Studies on apyrases. *Arch. Biochem. Biophys.* 93, 353-363

Moncada, S. & Vane, J.R. (1978) Unstable metabolites of arachidonic acid and their role in haemostasis and thrombosis. *Br. Med. Bull.* 34, 129-135

Morrisett, J.D. & Drott, H.R. (1969) Oxidation of the sulfhydryl and disulfide groups by the nitroxyl radical. *J. Biol. Chem.* 244, 5083-5084

Murad, F. & Pak, C.Y. (1973) Clinical application of cyclic nucleotide levels. IN *Pharmacology and the Future of Man. Proc. 5th Int. Cong. Pharmacol., San Francisco. 1972* Vol. 5, p.p. 240-241 (abs) Karger, Basal

Mustard, J.F. & Packham, M.A. (1970) Factors influencing platelet function: Adhesion, release and aggregation. *Pharmacol. Rev.* 22, 97-187

Mustard, J.F., Kinlough-Rathbone, R.L. & Packham, M.A. (1974) Recent status of research in the pathogenesis of thrombosis. *Thromb. Diath. Haemorh. Suppl.* 59, k57-188

Nagata, C., Ioki, Y., Kodama, M., Tagashira, Y. & Nadadate, M. (1973) Free radical induced in rat liver by a chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Ann. N.Y. Acad. Sci.* 222, 1031-1047

Nakazawa, K. & Sano, M. (1974) Studies on guanylate cyclase: A new assay method for guanylate cyclase and properties of the cyclase from rat brain. *J. Biol. Chem.* 249, 4207-4211

Nakazawa, K., Sano, M. & Saito, T. (1976) Subcellular distribution and properties of guanylate cyclase in rat cerebellum. *Biochim. Biophys. Acta* 444, 563-570

Neer, E.J. & Sukiennik, E.A. (1975) Guanylate cyclase from the rat renal medulla. Physical properties and comparison with adenylate cyclase. *J. Biol. Chem.* 250, 7905-7909

O'Sullivan, W.J. (1969) Stability constants of metal complexes. IN *Data for Biochemical Research* (Dawson, R.M.C., Elliott, D.C., Elliott, W.H. & Jones, K.M., eds) p.p. 423-435 Oxford University Press, New York

Packham, M.A., Guccione, M.A., Chang, P. -L. & Mustard, J.F. (1973) Platelet aggregation and release: effects of low concentrations of thrombin or collagen. *Am. J. Physiol.* 255, 38-47

Pannbacker, R.G. (1973) Control of guanylate cyclase activity in rod outer segment. *Science* 182, 1138-1140

Pointer, R.H., Butcher, F.R. & Fain, J.N. (1976) Studies on the role of cyclic guanosine 3':5'-monophosphate and extracellular Ca^{2+} in the regulation of glycogenolysis in rat liver cells. *J. Biol. Chem.* 251, 2987-2992

Portzehl, H., Caldwell, P.C. & Ruegg, J.C. (1964) Dependence of contraction and relaxation of muscle fibers from the crab *Maia squenado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* 79, 581-591

Price, T.D., Ashman, D.F. & Melicow, M.M. (1967) Organophosphates of urine including adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate. *Biochim. Biophys. Acta* 138, 452-465

Pryor, W.A. (1976) Thr role of free radical reactions in biological systems. IN *Free Radicals in Biology* (Pryor, W.A., ed) Vol. 1 p.p. 1-49 Academic Press, New York

Quill, H. & Weiser, M.M. (1975) Adenylate and guanylate cyclase activities and cellular differentiation in rat small intestine. *Gasteroenterol.* 69, 470-478

Rasmussen, H. (1970) Cell communication, calcium ion and cyclic adenosine monophosphate. *Science* 170, 404-412

Raveed, D., Fleischman, D. & Rolnick, P. (1976) Ultrastructure, composition and guanylate cyclase activity of isolated bovine retinal rod cilia. *J. Cell Biol.* 70, A165 (abs)

Rebhun, L.I., Miller, M., Schnaitman, T.C., Nath, J. & Mellon, M. (1976) Cyclic nucleotides, thiodisulfide status of proteins, and cellular control processes. *J. Supramolec. Struct.* 5, 199-219

Rodan, G.A. & Feinstein, M.B. (1976) Interrelationships between Ca^{2+} and adenylate and guanylate cyclases in the control of platelet secretion and aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 73, 1829-1833

Salzman, E.W., Kenster, P.C. & Levine, L. (1973) Cyclic 3',5'-adenosine-monophosphate in human blood platelets. IV. Regulatory role of cyclic AMP in platelet function. *Ann. N.Y. Acad. Sci.* 201, 61-71

Samuelsson, B., Hamberg, M., Malmsten, C. & Svensson, J. (1976)

Adv. Prostaglandin Thromboxane Res. 2, 737-746

Sano, M. (1976) Subcellular localizations of guanylate cyclase and 3',5'-cyclic nucleotide phosphodiesterase in sea urchin sperm. *Biochim. Biophys. Acta* 428, 525-531

Schoental, R. & Rive, D.J. (1965) Interaction of *N*-alkyl-*N*-nitrosourethanes with thiols. *Biochem. J.* 97, 466-474

Schoepflin, G.S., Pickett, W., Austen, K.E. & Goetzl, E.J. (1977) Elevation of the cyclic GMP concentration in human platelets by sodium ascorbate and 5-hydroxytryptamine. *J. Cyclic Nucleotide Res.* 3, 355-365

Schultz, G. & Hardman, J,G, (1975) Regulation of cyclic GMP levels in the ductus deferens of the rat. *Adv. Cyclic Nucleotide Res.* 5, 353-374

Schultz, G., Böhme, E. & Munske, K. (1969) Guanyl cyclase. Determination of enzyme activity. *Life Sci.* 8, 1323-1332

Schultz, G., Hardman, J.G., Davis, J.W., Schultz, K. & Sutherland, E.W. (1972) Determination of cyclic GMP by a new enzymatic method. *Fed. Proc.* 31, 440 (abs)

Schultz, G., Hardman, J.G., Schultz, K., Baird, C.E., Parks, M.A., Davis, J.W. & Sutherland, E.W. (1973*a*) Cyclic GMP and cyclic AMP in ductus deferens and submaxillary gland of the rat. IN *Pharmacology and the Future of Man. Proc. 5th Int. Cong. Pharmacol., San Francisco. 1972* Vol. 5 p. 206 (abs) Karger, Basal

Schultz, G., Hardman, J.G., Schultz, K., Baird, C.E. & Sutherland, E.W. (1973*b*) The importance of calcium ions for the regulation of guanosine 3':5'-cyclic monophosphate levels. *Proc. Natl. Acad. Sci. U.S.A.* 70, 3889-3843

Schultz, K. -D., Schultz, K. & Schultz, G. (1977) Sodium nitroprusside and other smooth-muscle relaxants increase cyclic GMP levels in rat ductus deferens. *Nature* 265,750-751

Schultz, W. & McCalla, D.R. (1969) Reactions of cysteine with *N*-methyl-*N*-nitroso-*N*-toluene sulfonamide and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Can. J. Chem.* 47, 2021-2027

Scribner, J.D. & Naimy, N.K. (1975) Destruction of triplet nitrenium ion by ascorbic acid. *Experientia* 31, 470-471

Sedlak, J. & Lindsay, R.H. (1968) Estimation of total, protein-bound, and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25, 192-205

Shier, W.T. & Trotter, T. III. (1976) Stimulation of liver microsomal sialyl transferase activity by lysolecithin. *F.E.B.S. Lett.* 62, 165-168

Shier, W.T., Baldwin, J.H., Hamilton, M.N., Hamilton, R.T. & Thanassi, N.M. (1976) Regulation of guanylate and adenylate cyclase activities by lysolecithin. *Proc. Natl. Acad. Sci. U.S.A.* 73, 1586-1590

Siegel, M.I., Puca, G.A. & Cuatrecasas, P. (1976) Guanylate cyclase. Existence of different forms and their regulation by nucleotides in calf uterus. *Biochim. Biophys. Acta* 438, 310-323

Silverman, P.M. (1976) Regulation of guanylate cyclase activity during cytodifferentiation of *Blastocladiella emersoni*. *Biochem. Biophys. Res. Commun.* 70, 381-388

Smith, J.B., Ingerman, C., Kocsis, J.J. & Silver, M.J. (1973) Formation of prostaglandins during the aggregation of human blood platelets. *J. Clin. Invest.* 52, 965-969

Smith, J.B., Ingerman, C., Kocsis, J.J. Silver, M.J. (1974) Formation of an intermediate in prostaglandin biosynthesis and its association with the platelet release reaction. *J. Clin. Invest.* 53, 1468-1472

Spruill, W.A., Stoner, A.L. & Earp, H.S. (1977) Testicular guanylate cyclase: correlation between tissue cGMP levels and soluble guanylate cyclase activity (GCA). *Fed. Proc.* 36, 347 (abs)

Srivastava, S.K., Awasthi, Y.C. & Bentler, E. (1974) Useful agents for the study of glutathione metabolism in erythrocytes: Organic hydroperoxides. *Biochem. J.* 139, 289-295

Steiner, A.L., Parker, C.W. & Kipnis, D.M. (1970) The measurement of cyclic nucleotides by radioimmunoassay. *Adv. Biochem. Psycopharmacol.* 3, 89-111

Steiner, A.L., Pagliaro, A.S., Chase, L.R. & Kipnis, D.M. (1972) Radioimmunoassay for cyclic nucleotides. II. Adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in mammalian tissues and body fluids. *J. Biol. Chem.* 244, 1114-1120

Stibbe, J. & Holmsen, H. (1977) Effects of sodium azide on platelet function. *Thrombos. Haemostasis (Stuttg.)* 38, 1042-1053

Stoner, J., Manganiello, V.C. & Vaughan, M (1974) Guanosine cyclic 3',5'-monophosphate and guanylate cyclase activity in guinea pig lung: Effects of acetylcholine and cholinesterase inhibitors. *Molec. Pharmacol.* 10, 155-161

St. Louis, P. & Sulakhe, P.V. (1976) Adenylate cyclase, guanylate cyclase and cyclic nucleotide phosphodiesterases of guine-pig sarcolemma. *Biochem. J.* 158, 535-541

Sulahke, P.V., Sulahke, S.J., Leung, N.L. -K., St. Louis, P.J. & Hickie, R.A.. (1976) Properties of particulate, membrane-associated and soluble guanylate cyclase from cardiac muscle, skeletal muscle, cerebral cortex, and liver. *Biochem. J.* 157, 705-712

Sun, I.C., Shapiro, L. & Rosen, O.M. (1974) Purification and characterization of guanylate cyclase from *Caulobacter crescentus*. *Biochem. Biophys. Res. Commun.* 61, 193-203

Sutherland, E.W. & Rall, T.W. (1958) Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J. Biol. Chem.* 232, 1077-1091

Thiers, R.E. & Vallee, B.L. (1957) Distribution of metals in subcellular fractions of rat liver. *J. Biol. Chem.* 226, 911-920

Thompson, W.J., Williams, R.H. & Little, S.A. (1973a) Activation of guanyl cyclase and adenyl cyclase by secretin. *Biochim. Biophys. Acta* 302, 329-337

Thompson, W.J., Williams, R.H. & Little, S.A. (1973*b*) Studies on the assay and activities of guanyl and adenyl cyclase of rat liver. *Arch. Biochem. Biophys.* 159, 206-213

Thompson, W.J., Johnson, D.G., Lavis, V.R. & Williams, R.H. (1974) Effects of secretin on guanyl cyclase of various tissues. *Endocrinol.* 94, 276-278

Van Sande, J., Decoster, C. & Dumont, J.E. (1975) Control and role of cyclic 3',5'-guanosine monophosphate in the thyroid. *Biochem. Biophys. Res. Commun.* 62, 168-175

Vesely, D.L. & Levey, G.S. (1977) Hydrazine activation of guanylate cyclase: potential application to tobacco carcinogenesis. *Biochem. Biophys. Res. Commun.* 74, 780-784

Vesely, D.L. & Levey, G.S. (1978) Butadiene diepoxide activation of guanylate cyclase. *Enzyme* 23, 140-143

Vesely, D.L., Rovere, L.E. & Levey, G.S. (1977) Activation of guanylate cyclase by streptozotocin and 1-methyl-1-nitrosourea. *Cancer Res.* 37, 28-31

Virmaux, N., Nullans, G. & Goridis, C. (1976) Guanylate cyclase in vertebrate retina: evidence for specific association with rod outer segments. *J. Neurochem.* 26, 233-235

Walaas, O., Walaas, E. & Wick, A.N. (1969) The stimulatory effect of insulin on the incorporation of ^{32}P radioactive inorganic phosphate into intracellular inorganic phosphate, adenine nucleotides and guanine nucleotides of the intact isolated rat diaphragm. *Diabetologia* 5, 79-87

Wallach, D. & Pastan, I. (1976*a*) Stimulation of membranous guanylate cyclase by concentrations of calcium that are in the physiological range. *Biochem. Biophys. Res. Commun.* 72, 859-865

Wallach, D. & Pastan, I. (1976*b*) Stimulation of guanylate cyclase of fibroblasts by free fatty acids. *J. Biol. Chem.* 251, 5802-5809

Webb, J.L. (1966) *Enzyme and Metabolic Inhibitors* Vol. II Academic Press, New York

Weiss, A., Baenziger, N.L. & Atkinson, J.P. (1978) Platelet release reaction and intracellular cyclic GMP. *Blood* 52, 524-531

White, A.A. & Aurbach, G.D. (1969) Detection of guanyl cyclase in mammalian tissues. *Biochim. Biophys. Acta* 191, 686-697

White, A.A. & Lad, P.J. (1975) Activation of the soluble guanylate cyclase from rat lung by preincubation. *Fed. Proc.* 34, 232 (abs)

White, A.A., Aurbach, G.D. & Carlson, S.F. (1969) Identification of guanyl cyclase in mammalian tissues. *Fed. Proc.* 28, 473 (abs)

White, A.A., Crawford, K.M., Patt, C.S. & Lad, P.J. (1976) Activation of soluble guanylate cyclase from rat lung by incubation or by hydrogen peroxide. *J. Biol. Chem.* 251, 7304-7312

White, J.G., Goldberg, N.D., Estensen, R.D., Haddox, M.K. & Rao, G.H.R. (1973) Rapid increase in platelet cyclic 3',5'-guanosine monophosphate (cGMP) levels in association with irreversible aggregation, degranulation and secretion. *J. Clin. Invest.* 52, 89 (abs)

White, J.G., Rao, G.H.R. & Gerrard, J.M. (1974) Effects of the ionophore A 23187 on blood platelets. *Am. J. Pathol.* 77, 135-149

White, L.E., Ignarro, L.J. & George, W.J. (1973) Stimulation of rat cardiac guanyl cyclase by acetylcholine. *Pharmacologist* 15, 157 (abs)

Zwiller, J., Ciesielski-Treska, J. & Mandel, P. (1976) Effects of lysolecithin on guanylate and adenylate cyclase activities in neuroblastoma cells in culture. *F.E.B.S. Lett.* 69, 286-290