Biochem. J. (1991) 279, 657–664 (Printed in Great Britain)

Dual effects of guanosine 5′-[γ-thio]triphosphate on secretion by electroporated human neutrophils

James E. SMOLEN,*†‖ Sally Jo STOEHR,* Barbara KUCZYNSKI,* Eun Kyu KOH* and Geneva M. OMANN‡§
Departments of *Pediatrics, †Pathology, ‡Surgery and §Biological Chemistry, University of Michigan Medical Center, Ann Arbor, MI 48109, U.S.A.

It is generally believed that G-proteins play stimulatory roles on cell activation. In contrast, we found that guanosine 5′-[γ-thio]triphosphate (GTP[S]) was a potent inhibitor of Ca^{2+}-induced secretion from specific granules (as monitored by vitamin B-12-binding protein). GTP[S] inhibition of specific-granule release occurred in the presence or absence of adenine nucleotides, required Mg^{2+} (1–3 mM), and was half-maximal at 30 μM-GTP[S]. The dual stimulatory and inhibitory effects of GTP[S] could be readily observed and differentiated when degranulation was monitored over a range of Ca^{2+} concentrations. Inhibition of specific-granule release by GTP[S] was observed at low Ca^{2+} concentrations and resulted from shifting the Ca^{2+} dose–response curves to the right. In contrast, GTP[S] promoted azurophil-granule secretion at relatively high concentrations of Ca^{2+} and appeared to be due to a general enhancement at all Ca^{2+} concentrations. A series of hydrolysable and non-hydrolysable nucleotides did not mimic GTP[S] or block its action. Inhibition by GTP[S] occurred in cells which were sensitized with a protein kinase C agonist, suggesting that inhibition of secretion took place distal to this enzyme. However, the inhibitory effects of GTP[S] on specific-granule secretion were reversed by cytochalasin D, which prevents new microfilament formation; this compound also enhanced the stimulation of azurophil-granule release by GTP[S]. We also found that GTP[S] greatly increased the F-actin content of permeabilized neutrophils, whereas Ca^{2+} (to a lesser extent) decreased F-actin. These data are consistent with the hypothesis that at least two G-proteins are involved in regulating secretion: one which has been previously described as stimulating Ca^{2+}-induced secretion (particularly from azurophil granules) and a second, possibly involved in promoting microfilament assembly, which inhibits the discharge of specific granules.

INTRODUCTION

Permeabilized cell systems have been used to study intracellular signal transduction in a variety of cell types. The ability to introduce GTP and its analogues directly into the cytosol of permeabilized cells has been instrumental for implicating G-proteins in the pathways of signal transduction. The reigning paradigm, developed with these and other techniques, is that cell-surface receptors interact with and activate G-proteins. G-proteins can also be activated in permeabilized cells or broken-cell extracts by guanosine 5′-[γ-thio]triphosphate (GTP[S]), a poorly hydrolysable derivative of GTP. The activated G-proteins are believed to stimulate polyphosphoinositide-specific phospholipase C, which generates $InsP_3$ and diacylglycerol. $InsP_3$ causes the release of Ca^{2+} from intracellular stores which, along with diacylglycerol, stimulates protein kinase C (PKC).

In neutrophils, permeabilization with digitonin has been employed to study the interactions between G-proteins and cell-surface chemotactic-peptide receptors (Sklar *et al.*, 1987). Permeabilization has also been used to show that G-proteins stimulated with GTP[S] can activate phospholipase C (Bradford & Rubin, 1986). Guanine nucleotides have been shown to enhance O_2 consumption, actin polymerization and protein phosphorylation in neutrophils permeabilized by electroporation (Nasmith *et al.*, 1989; Therrien & Naccache, 1989; Downey *et al.*, 1989). Another focus for permeabilized-neutrophil studies is degranulation. The main stimulus for this process is Ca^{2+}, which, at micromolar concentrations, promotes secretion (Prentki *et al.*, 1984; Smolen & Stoehr, 1985; Smolen *et al.*, 1986; Smolen & Sandborg, 1990). In neutrophils permeabilized with digitonin, this secretory process is not affected by Mg^{2+} or ATP (Smolen *et al.*, 1986, 1989), but is enhanced by GTP[S] and a variety of other guanine nucleotides (Smolen & Stoehr, 1986). In addition, guanine nucleotides can stimulate secretion in the absence of Ca^{2+} (Smolen & Stoehr, 1986; Barrowman *et al.*, 1986). Similar findings have been made in HL60 cells (Stutchfield & Cockcroft, 1988) and mast cells (Koffer & Gomperts, 1989; Koopmann & Jackson, 1990), leading some investigators to speculate that an additional exocytosis-linked G-protein is involved in the secretory process (Smolen & Stoehr, 1986; Barrowman *et al.*, 1986; Gomperts *et al.*, 1986; Howell *et al.*, 1987; Stutchfield & Cockcroft, 1988).

In electroporated neutrophils, a substantially different picture of secretion is seen, probably owing to the smaller size of the pores produced, which results in greater retention of intracellular components. In this system, Ca^{2+} is again the primary stimulus for secretion, but Mg^{2+} is now an important promoter (Smolen & Sandborg, 1990). The effect of Mg^{2+} is further enhanced by the presence of ATP and a wide variety of other hydrolysable and stable nucleotides (Smolen & Sandborg, 1990). All of these nucleotides appear to promote secretion by interacting with a poorly selective receptor, possibly located on the cell surface, eventually leading to PKC activation. However, one of our most surprising findings was that GTP[S] did not always stimulate secretion as expected; rather, it sometimes proved to be a potent inhibitor of degranulation (Smolen & Sandborg, 1990). In the work reported here, we have further investigated the response of

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; ADP[S], adenosine 5′-[β-thio]diphosphate; PMA, phorbol myristate acetate; PT, pertussis toxin; PKC, protein kinase C; NEM, *N*-ethylmaleimide.

‖ To whom reprint requests should be addressed, at: University of Michigan, Department of Pediatrics, Room 7510C MSRB I, Box 0684, Ann Arbor, MI 48109-0684, U.S.A.

electroporated neutrophils to GTP[S]. We found that both inhibitory and stimulatory effects of this nucleotide on secretion can be obtained, depending on the concentration of GTP[S], the presence or absence of Mg^{2+}, the concentration of Ca^{2+}, and the granule type being measured. These results suggest that at least two G-proteins are involved in regulating secretion and that these proteins might be coupled differently with the several granule types found in neutrophils.

EXPERIMENTAL

Reagents

GTP, guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG), guanosine 5′-[β-thio]diphosphate (GDP[S]), ATP, adenosine 5′-[β-thio]diphosphate (ADP[S]), XTP, UTP, ferricytochrome *c*, cytochalasin B, cytochalasin D and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Guanosine 5′-[γ-thio]triphosphate (GTP[S]) was obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A. Pertussis toxin (PT) and cholera toxin were obtained from List Biologicals, Campbell, CA, U.S.A. NBD-phallacidin was obtained from Molecular Probes, Eugene, OR, U.S.A. All other materials were reagent grade.

Preparation of cell suspensions

Heparinized (10 units/ml) venous blood was obtained from healthy adult donors. Purified preparations of neutrophils were isolated from this blood by means of Hypaque/Ficoll gradients (Boyum, 1968), followed by standard techniques of dextran sedimentation and hypo-osmotic lysis of erythrocytes (Zurier *et al.*, 1973). This allowed studies of cell suspensions containing 98 ± 2 % neutrophils with few contaminating platelets or erythrocytes. The cells were washed and finally suspended in phosphate-buffered saline, consisting of 138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4 and 1.5 mM-KH_2PO_4. For permeabilization studies, the cells were instead washed once and resuspended in KCl/Hepes buffer (100 mM-KCl, 20 mM-NaCl, 1 mM-EGTA and 30 mM-Hepes, pH 7.0).

Neutrophil permeabilization

Electropermeabilization was conducted essentially as described by Grinstein & Furuya (1988) with some variations (Smolen & Sandborg, 1990). In essence, neutrophils were washed and then resuspended in ice-cold KCl/Hepes buffer. The cells, at a concentration of 5×10^7/ml, were transferred to a chilled Bio-Rad Pulsar cuvette and subjected to three discharges of 5 kV/cm from a 25 μF capacitor (Bio-Rad Gene Pulsar) with gentle stirring between each pulse. The capacitor discharged with a time constant of 0.5–0.7 ms under these conditions. All permeabilization operations were conducted at 4 °C. Viability, as assessed by Trypan Blue exclusion, was consistently greater than 85 %, in accord with Grinstein & Furuya (1988).

Lysosomal enzyme release

After permeabilization, neutrophils were used with or without washing, as indicated. Portions of the ice-cold permeabilized-cell suspensions (100 μl, containing 5×10^6 cells) were added to 900 μl of pre-warmed KCl/Hepes buffer containing the indicated additions along with the desired concentration of free Ca^{2+} (determined for the buffer with a Ca^{2+} electrode; Bers, 1982). The cells were then incubated at 37 °C for 7 min; cells incubated without Ca^{2+} and intact neutrophils (in KCl/Hepes buffer) were employed as routine controls. The cell suspensions were then centrifuged at 750 ***g*** for 5 min. Samples of the supernatants were taken for standard determinations of β-glucuronidase (an enzyme found exclusively in azurophil granules) (Brittinger *et al.*, 1968), lysozyme (an enzyme found in both specific and azurophil granules) (Worthington Enzyme Manual, 1972; pp. 100–101) and vitamin B-12-binding protein (a component of specific granules alone) (Smith & Peters, 1982).

Toxin treatment

Neutrophils (50×10^6/ml) were resuspended in Krebs Buffer Mix (118 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-Mg_2SO_4, 5.5 mM-glucose and 25 mM-Hepes, pH 7.0), supplemented with 6.3 mg of cytochrome *c*/ml, as described by Omann & Porasik-Lowes (1991). PT hydrated in high-salt buffer (200 μg of PT/ml, 0.5 M-NaCl, 0.1 M-sodium phosphate, pH 7.0), was added to 2 ml samples of the cell suspensions to yield a final concentration of PT of 5 μg/ml. The samples were constantly and gently rotated at 37 °C. At 1 h intervals, samples of control cells or PT-treated cells were removed, washed, and resuspended in the appropriate buffers for electropermeabilization or analysis of *N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe)-stimulated responses [actin polymerization (Sklar *et al.*, 1985) or O_2^- generation (Goldstein *et al.*, 1977)]. Cell suspensions in ice-cold phosphate-buffered saline, which were washed and resuspended in the appropriate buffers, served as the zero-time controls for the experiments.

F-actin determination

Permeabilized neutrophils were incubated with Ca^{2+} and GTP[S] as detailed above for 'Lysosomal enzyme release', except that the cell suspensions were fixed after 1 min with 3.7 % formaldehyde. The fixed cell suspensions were then stained with NBD-phallacidin and assayed for F-actin content as previously described (Omann *et al.*, 1989).

RESULTS

We previously reported that micromolar Ca^{2+} alone is sufficient to induce secretion by electroporated cells (Smolen & Sandborg, 1990). In general, release of specific granules requires less Ca^{2+} than does release of azurophil granules. Mg^{2+} promotes Ca^{2+}-induced secretion, particularly in the presence of adenine nucleotides. Stimulation by adenine nucleotides can be mimicked by a variety of other nucleotides, suggesting that a weakly specific purine nucleotide receptor is also involved. Several laboratories, including our own, have reported that guanine nucleotides can enhance Ca^{2+}-induced degranulation from permeabilized cells (Smolen & Stoehr, 1986; Barrowman *et al.*, 1986; Stutchfield & Cockcroft, 1988; Koffer & Gomperts, 1989; Koopmann & Jackson, 1990). Yet one of our most intriguing findings was that the poorly hydrolysable guanine nucleotide GTP[S] inhibits secretion of specific granules under certain circumstances (Smolen & Sandborg, 1990). In this work, we examine more closely the stimulatory and inhibitory effects of this nucleotide.

Inhibition of specific-granule secretion by GTP[S]

Our earlier experiments showed that GTP[S] (30 μM) inhibited Ca^{2+}-induced secretion of specific granules from electroporated neutrophils in the presence of Mg^{2+} (Smolen & Sandborg, 1990). The dose–response characteristics of this inhibition are shown in Fig. 1. In these, as well as in most subsequent experiments, we used a relatively low (5 μM) concentration of Ca^{2+} and 1 mM-Mg^{2+}, conditions which were optimal for detecting the discharge of specific granules, monitored by the release of vitamin B-12-binding protein (Smolen & Sandborg, 1990). These conditions evoked very little release of azurophil-granule constituents, which are consequently not shown. As shown in Fig. 1, secretion from cells stimulated with 5 μM-Ca^{2+} alone was unaffected by GTP[S]

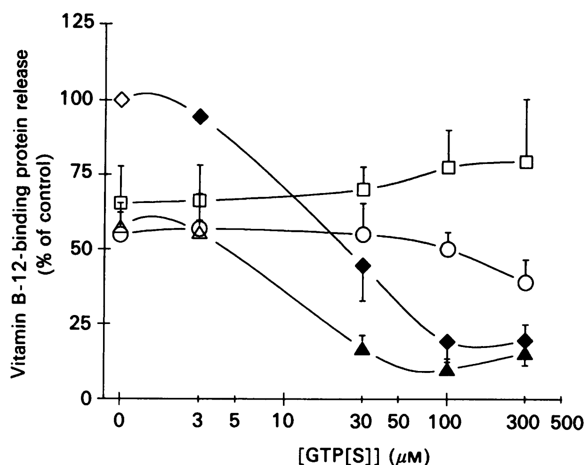

Fig. 1. Inhibitory effects of GTP[S], ATP and Mg^{2+} on specific-granule release

Human neutrophils were isolated and permeabilized as described in the Experimental section. Portions of the cell suspension were added to prewarmed tubes in KCl/Hepes buffer containing the indicated concentrations of GTP[S], along with 5 μM free Ca^{2+}. These incubations also contained ATP (0.3 mM) and Mg^{2+} (1.0 mM) where indicated. The cells were incubated for 7 min at 37 °C and then centrifuged. Specific-granule release was monitored by discharge of vitamin B-12-binding protein. Results are given as percentages (mean ± S.E.M.) of control release, which was obtained with Mg^{2+}-ATP in the absence of GTP[S]; this control value was 32.5 ± 4.2% ($n = 4$) of the total cellular complement. Black symbols denote conditions in which GTP[S] statistically ($P < 0.05$, using paired Student t test statistics) promoted secretion (compared with no GTP[S]). Background levels were 4.3 ± 1.4% of total (10% of control). Key: ○, no addition; □, ATP; △, Mg^{2+}; ◇, Mg-ATP.

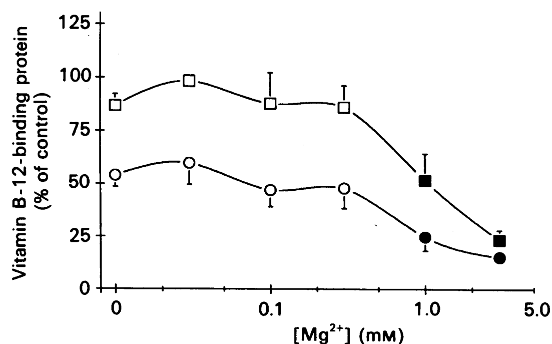

Fig. 2. Mg^{2+} requirements for inhibition by GTP[S]

Permeabilized human neutrophils were incubated with the indicated concentrations of Mg^{2+} along with 5 μM-Ca^{2+} and 100 μM-GTP[S]; these incubations also were done in the absence (○) or presence (□) of ATP (0.3 mM). The results are given as percentages of control release, which was determined in the presence of 1 mM-Mg^{2+}, 0.3 mM-ATP and 5 μM-Ca^{2+} (no guanine nucleotide). This control value was 34.9 ± 1.2% of the total cellular vitamin B-12-binding protein ($n = 5$). Black symbols denote those concentrations of Mg^{2+} which significantly inhibited ($P < 0.05$) degranulation (compared with no Mg^{2+}). See the Experimental section and the legend to Fig. 1 for further details.

(○). When 0.3 mM-ATP (no Mg^{2+}) was present, GTP[S] did not significantly affect secretion (□). In contrast, when Mg^{2+} was present, inhibition was observed, in both the absence (△) and the presence (◇) of ATP. Mg-ADP[S] could substitute for Mg-ATP (results not shown), indicating that ATP was not being used for metabolic energy (Smolen & Sandborg, 1990). From these data, we concluded that GTP[S] produced half-maximal inhibition at approx. 30 μM and required Mg^{2+}.

Mg^{2+} requirements for inhibition by GTP[S]

The data in Fig. 1 showed that Mg^{2+} was required for inhibition by GTP[S]. The effects of various concentrations of Mg^{2+} on Ca^{2+}-induced secretion in the presence of GTP[S] are shown in Fig. 2. A moderately high concentration of GTP[S] (100 μM) was used in order to support both inhibition and activation (should they be present). The results showed that in both the presence and the absence of ATP, inhibition of secretion by GTP[S] was obtained at Mg^{2+} concentrations of 1 mM and above. Since Mg^{2+} itself did not inhibit secretion (Fig. 1), this inhibition could only be attributed to GTP[S] acting in concert with the bivalent cation. At low concentrations of Mg^{2+}, there was occasional stimulation of release of vitamin B-12-binding protein; however, this was not consistently observed, and did not prove to be statistically significant.

Ca^{2+} requirements

Our data in Figs. 1 and 2 were all obtained at Ca^{2+} concentrations appropriate for the release of specific granules only. Since azurophil and specific granules have different Ca^{2+} requirements for release (Smolen & Sandborg, 1990), it was important to examine this factor in detail. Consequently, we performed extensive Ca^{2+}-dose–response experiments in the absence and presence of GTP[S] and Mg^{2+}. The results shown in Fig. 3 indicated that both granule type and Ca^{2+} concentration were critical parameters. In Fig. 3, black symbols denote those conditions for which the presence of GTP[S] was statistically different from its absence (all white symbols). Fig. 3(*a*) shows that release of the specific-granule marker (vitamin B-12-binding protein) usually required only relatively low Ca^{2+} concentrations (○). In the absence of Mg^{2+}, GTP[S] inhibited marginally, albeit significantly (□). However, it was in the presence of Mg^{2+} that the most interesting results were seen. By itself, Mg^{2+} synergistically enhanced vitamin B-12-binding protein release at low, but not high, Ca^{2+} concentrations (△). It was also at these low Ca^{2+} concentrations that GTP[S] had its greatest effects, virtually abolishing Mg^{2+}-enhanced secretion (◆); at higher Ca^{2+} concentrations, GTP[S] was without effect.

A different picture was seen with azurophil-granule secretion, as indicated by β-glucuronidase (Fig. 3*b*). Considerably higher concentrations of Ca^{2+} were required to induce release of this component. The Ca^{2+}-dose–response curve was enhanced, but not shifted, by Mg^{2+}. In the absence of Mg^{2+}, GTP[S] statistically enhanced secretion of β-glucuronidase. Mg^{2+} was also stimulatory over the entire Ca^{2+} range, and the combination of Mg^{2+} plus GTP[S] provoked additional degranulation, but did not shift the Ca^{2+}-dose–response curve. Thus the effects of GTP[S] on azurophil-granule release were qualitatively different from those seen from specific-granule release and are in accord with previous observations of enhancement by guanine nucleotides (Smolen & Stoehr, 1986; Barrowman *et al.*, 1986; Stutchfield & Cockcroft, 1988; Koffer & Gomperts, 1989; Koopmann & Jackson, 1990). As might be expected, a mixed pattern was seen with lysozyme, an enzyme found in both of the granule types (results not shown). At low Ca^{2+} concentrations the inhibitory effects of GTP[S] prevailed, whereas at the higher Ca^{2+} concentrations GTP[S] was stimulatory, as with β-glucuronidase.

Other nucleotides

It was decided to see whether other nucleotides could mimic the stimulatory action of ATP or the inhibitory effect of GTP[S], and how these nucleotides might interact. Consequently, we

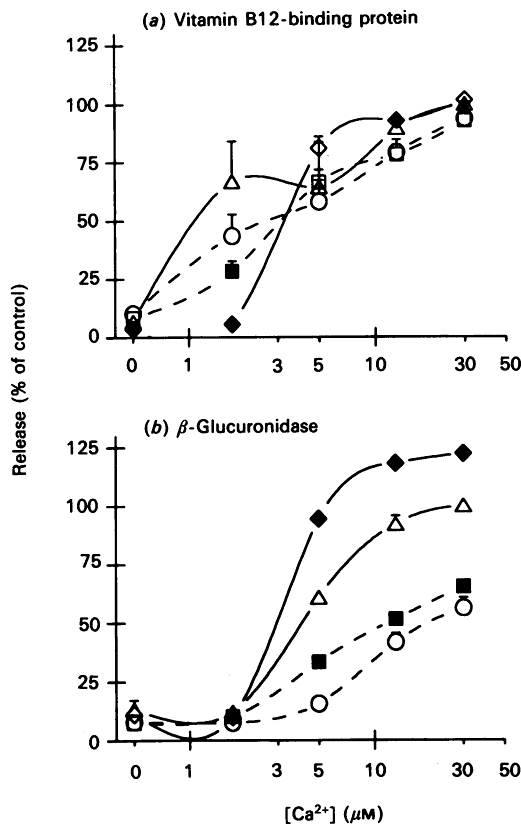

Fig. 3. Effects of GTP[S] and Mg^{2+} on Ca^{2+} dose–response curves

Permeabilized neutrophils were incubated with 0.3 mM-ATP and the indicated concentration of free Ca^{2+}; GTP[S] (100 μM) and/or Mg^{2+} (1 mM) were also present, as indicated. Supernatants were analysed for release of vitamin B-12-binding protein (*a*), β-glucuronidase (*b*) and lysozyme (an enzyme found in both specific and azurophil granules; results not shown). The data are expressed as percentages of control samples, namely those incubated with 30 μM-Ca^{2+} with Mg^{2+} and ATP. These control samples constituted 52.9±7.5% the total cellular vitamin B-12-binding protein, 18.3±1.5% of the total β-glucuronidase and 28.9±5.0% of the total lysozyme (*n* = 4). Black symbols denote those Ca^{2+} concentrations at which release in the presence and absence of GTP[S] were statistically different. Symbols: ○, no addition; □, GTP[S]; △, Mg^{2+}; ◇, Mg^{2+} + GTP[S].

Table 1. Effects of various nucleotides on inhibition of specific-granule release by GTP[S]

Permeabilized human neutrophils were incubated with 3.5–5 μM-Ca^{2+} and Mg^{2+} (1 mM), and with 100 μM-GTP[S] and/or 300 μM-nucleotide, as indicated. The results are a compilation of six experiments for control and ATP samples, with three experiments for each of the other nucleotides. The results are presented as percentages of those for control samples, released by Ca^{2+} and Mg^{2+} in the presence of ATP, which constituted 43.5±7.5% of the total cellular vitamin B-12-binding protein. Except for GDP[S], the results obtained in the presence of GTP[S] were statistically different (*$P < 0.05$) from those obtained in the absence of the nucleotide.

	Release (%)	
	No GTP[S]	+GTP[S]
Control	77.1±9.2	8.3±1.6*
ATP	(100)	17.0±2.5*
ADP[S]	94.7±2.4	15.8±2.6*
XTP	95.0±2.7	16.8±4.5*
UTP	85.2±6.2	12.5±2.1*
GTP	98.6±9.4	17.7±2.0*
GDP[S]	63.5±11.1	16.8±5.6
p[NH]ppG	78.5±11.7	28.6±6.9*

tested a variety of nucleotides, both hydrolysable and non-hydrolysable, on specific-granule secretion (using vitamin B-12-binding protein) in both the absence and presence of GTP[S] (see Table 1). The top line shows that Ca^{2+}-induced secretion (in the presence of Mg^{2+}) was inhibited by GTP[S]. As shown above and on the second line, Mg-ATP generally enhanced secretion, which was inhibitable by GTP[S]. In the presence of Mg^{2+} (but not in its absence; results not shown), ATP could be replaced by ADP[S], XTP, UTP and GTP to augment vitamin B-12-binding protein secretion (Smolen & Sandborg, 1990); none of the nucleotides inhibited degranulation like GTP[S]. The data show that GTP[S] antagonized secretion augmented by each of the nucleotides, regardless of its nature. Only one nucleotide, p[NH]ppG, seemed to antagonize the action of GTP[S] (last row), and this antagonism was only partial.

Inhibitors and G-protein toxins

Inhibition of secretion by GTP[S], such as we found for specific granules at low Ca^{2+} concentrations, has not been widely reported. However, in other cell systems where inhibition has been noted, a number of proteins involved in intracellular membrane fusion have been reported, some of which are sensitive to *N*-ethylmaleimide (NEM) (Mayorga *et al.*, 1989; Weidman *et al.*, 1989; Rothman & Orci, 1990). Therefore, we wished to see whether or not degranulation was also NEM-sensitive. In experiments not shown, specific-granule secretion (in the presence of Mg^{2+}) was highly sensitive to inhibition by NEM. For azurophil-granule release, similar NEM-sensitivity was obtained. The data were consistent with the hypothesis that secretion under these conditions had components sensitive to both GTP[S] and NEM. These data suggested that enhancement of azurophil-granule secretion by this guanine nucleotide did not involve a uniquely NEM-sensitive component.

It was decided to see whether or not the dual positive and negative effects of GTP[S] were mediated by conventional G-proteins. To this end, we designed experiments in which cells were incubated with PT and cholera toxin, to modify G_i-like and G_s-like proteins respectively. Cells were incubated for 3 h with PT, conditions which both abolished fMet-Leu-Phe-stimulated O_2^- production and substantially inhibited fMet-Leu-Phe-stimulated changes in right-angle light scatter (Sklar *et al.*, 1989). This criterion is substantially more stringent than that generally used and has been shown to be necessary for full inactivation of G-proteins (Osmann & Porasik-Lowes, 1991). For cholera toxin, no unambiguous positive control was available; O_2^- generation was eliminated, but this could have been due to either cyclic AMP accumulation or G_s activation.

We found that the greatest effect on Ca^{2+}-induced secretion was that produced by the 3 h incubation period at 37 °C before permeabilization (results not shown). After the cells were incubated for 3 h in buffer alone, the functional properties of the cells were altered. In particular, far less secretion could be elicited by low levels of Ca^{2+}, and the inhibition due to GTP[S] was no longer evident. Compared with this sham incubation, neither cholera toxin nor PT had any significant effect on secretion enhanced by GTP[S]. This was also true for discharge of azurophil-granule constituents.

Phorbol myristate acetate (PMA)

Because nucleotides in the presence of Mg^{2+} stimulate the PKC pathway in permeabilized neutrophils (Smolen & Sandborg,

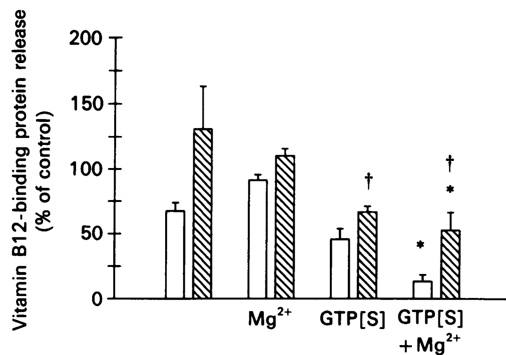

Fig. 4. Effects of PMA and GTP[S] on specific-granule secretion

Permeabilized human neutrophils were incubated in the presence of 0.3 mM-ATP and with Ca^{2+} (1.5 or 30 μM), Mg^{2+} (1 mM), GTP[S] (100 μM) or PMA (10 ng/ml) as indicated. The results are presented as percentages of control samples, which were incubated with 30 μM-Ca^{2+} and Mg-ATP. These control values constituted 41.0 ± 1.2 % of the total cellular vitamin B-12-binding protein ($n = 3$). See the Experimental section and the legends to Figs. 1–3 for further details. Key: □, 1.5 μM free Ca^{2+}; ▧, 1.5 μM free Ca^{2+} + PMA; *$P < 0.05$ for difference with and without GTP[S]; †$P < 0.05$ for difference with and without PMA.

Table 2. Effects of PMA and GTP[S] on azurophil-granule secretion

Permeabilized human neutrophils were incubated in the presence of 0.3 mM-ATP and with Ca^{2+} (1.5 or 30 μM), Mg^{2+} (1 mM), GTP[S] (100 μM) or PMA (10 ng/ml), as indicated. The results are presented as percentages of those control samples, which were incubated with 30 μM-Ca^{2+} and Mg-ATP. These control values constituted 12.4 ± 1.1 % of the total β-glucuronidase ($n = 3$). See the Experimental section and the legends to Figs. 1–3 for further details. *Significant difference ($P < 0.05$) compared with corresponding sample without GTP[S]. †Significant difference ($P < 0.05$) compared with corresponding sample without PMA.

[Ca^{2+}] (μM)	Mg^{2+}	GTP[S]	β-Glucuronidase release (%) −PMA	+PMA
1.5			10.2 ± 0.5	39.7 ± 7.0
1.5	+		13.2 ± 1.3	119.0 ± 25.5
1.5		+	20.5 ± 3.0*	39.5 ± 4.0
1.5	+	+	12.2 ± 1.2	52.0 ± 13.7†
30			55.1 ± 1.2	99.0 ± 7.8
30	+		(100)	173.4 ± 17.6
30		+	70.0 ± 9.8	107.5 ± 14.1
30	+	+	134.9 ± 14.9	164.6 ± 24.0†

1990), it was desirable to see if a PKC agonist, such as PMA, was able to reverse the effects of GTP[S]. Such reversal might be expected if PKC acted at a more distal step in the signal-transduction pathway than did the guanine nucleotide (as in the simplistic serial signal-transduction model of GTP[S] interacting with a traditional G-protein which activates phospholipase C, producing diacylglycerol, stimulating PKC). Under this scheme, direct activation of PKC should overcome the block by GTP[S]. If PKC agonists could not overcome the block, then GTP[S] would probably be acting downstream of PKC, as has been suggested previously (Smolen & Stoehr, 1986; Barrowman *et al.*, 1986; Koffer & Gomperts, 1989). Experiments designed to test this possibility are shown in Fig. 4. Specific-granule release was stimulated by low concentrations of Ca^{2+}, with or without Mg^{2+} and GTP[S]. The expected inhibition of secretion by GTP[S] (in

Table 3. Cytochalasin D reverses inhibition by GTP[S]

Permeabilized human neutrophils were incubated in the presence of 1 mM-Mg^{2+}, 0.3 mM-ATP, and with Ca^{2+} (3 or 30 μM), GTP[S] (100 μM) or cytochalasin D (Cyto D; 2 μg/ml) as indicated. The results are presented as percentages of those for control samples, which were incubated with 30 μM-Ca^{2+} and Mg-ATP. These control values constituted 35.4 ± 6.0 % of the total cellular vitamin B-12-binding protein and 16.9 ± 9.0 % of the total β-glucuronidase ($n = 3$). See the Experimental section and the legends to Figs. 1–3 for further details. *Significant difference ($P < 0.05$) with and without GTP[S]; †significant difference ($P < 0.05$) with and without Cyto D.

	Vitamin B-12-binding protein (%) No Cyto D	+Cyto D
3 μM-Ca^{2+}	69.8 ± 19.4	58.1 ± 18.1†
3 μM-Ca^{2+} + GTP[S]	19.7 ± 11.9*	62.6 ± 14.3†
30 μM-Ca^{2+}	(100)	97.8 ± 0.8
30 μM-Ca^{2+} + GTP[S]	115.6 ± 11.8	101.2 ± 7.4
	β-Glucuronidase (%)	
3 μM-Ca^{2+}	32.7 ± 10.4	32.2 ± 9.8
3 μM-Ca^{2+} + GTP[S]	28.9 ± 7.0	108.5 ± 33.4*
30 μM-Ca^{2+}	(100)	85.6 ± 6.2
30 μM-Ca^{2+} + GTP[S]	148.4 ± 9.3	166.5 ± 2.1*

the presence of Mg^{2+}) can be seen by comparing conditions 2 and 4, open bars. GTP[S] also significantly inhibited this degranulation in the presence of PMA (again compare condition 2 with condition 4, striped bars). Thus PMA did not negate the effects of GTP[S], suggesting that GTP[S] acted at some site distal to PKC. Under conditions of high Ca^{2+}, neither GTP[S] nor PMA had any significant effect on secretion of vitamin B-12-binding protein (results not shown).

For azurophil-granule release (Table 2), both GTP[S] and PMA would be expected to be stimulatory in the presence of high Ca^{2+} (from the above results and Smolen & Sandborg, 1990). The effects of these two agents were not additive, perhaps because all the mobilizable granules were discharged under either condition. Under low-Ca^{2+} conditions, GTP[S] (plus Mg^{2+}) inhibited PMA-enhanced degranulation. Overall, the data indicate that inhibition of secretion by GTP[S] occurs distal to PKC activation; similar conclusions regarding activation of azurophil-granule release by the guanine nucleotide cannot be made.

Cytochalasin D

One possible mechanism for the inhibition of secretion afforded by GTP[S] was suggested by the observation that this nucleotide promotes microfilament assembly in permeabilized neutrophils (Therrien & Naccache, 1989; Downey *et al.*, 1989; Bengtsson *et al.*, 1990). It is possible that the microfilament network can physically block translocation of granules to the plasma membrane and that blocking of microfilament assembly might be one of the means by which Ca^{2+} promotes secretion (Downey *et al.*, 1990). Similarly, promotion of microfilament assembly by GTP[S] under low-Ca^{2+} conditions might serve to inhibit secretion. This hypothesis was tested by directly blocking GTP[S]-induced microfilament assembly with cytochalasin D, which had been shown to block microfilament assembly in intact neutrophils (Howard & Oresajo, 1985). As shown in the top half of Table 3, cytochalasin D did not stimulate specific-granule secretion on its own, but completely reversed inhibition by GTP[S] (at low Ca^{2+} concentrations); the alkaloid had no effect at high Ca^{2+} concen-

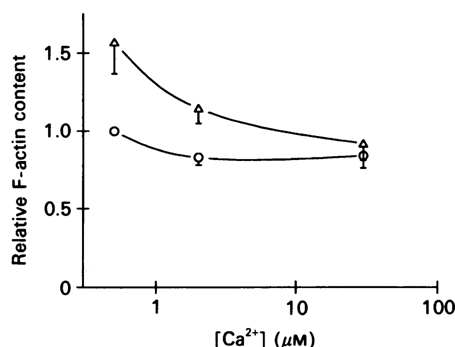

Fig. 5. Effect of Ca^{2+} and GTP[S] on F-actin content

Permeabilized human neutrophils were incubated for 1 min at 37 °C in the presence of 1 mM-Mg^{2+}, 0.3 mM-ATP, and with Ca^{2+} (0, 3 or 30 μM) or GTP[S] (100 μM), as indicated. The cells were then fixed with 3.7% (v/v) formaldehyde, stained with NBD-phallacidin, and assayed for F-actin content. The results are presented as percentages (mean ± S.E.M., $n = 6$) of control samples, which were incubated with Mg-ATP in the absence of Ca^{2+}. See the Experimental section for further details. Key: ○, control; △, +GTP[S].

trations. These data are thus in accord with the hypothesis that GTP[S] inhibited degranulation by promoting microfilament assembly. When azurophil-granule secretion was measured (Table 3, lower half), cytochalasin D was again without effect on its own; however, in the presence of the alkaloid, GTP[S] was highly stimulatory. Thus inhibition of microfilament assembly seemed to uncover a latent amplifying mechanism for the guanine nucleotide. Similar results were obtained with cytochalasin B (results not shown).

F-actin content

To determine directly whether or not Ca^{2+} and GTP[S] could modify polymerization of microfilaments, we measured F-actin content. Permeabilized neutrophils were incubated for 1 min at 37 °C with Mg-ATP and with or without GTP[S] (100 μM) and Ca^{2+}. As shown in Fig. 5, increasing Ca^{2+} concentrations alone (○) decreased F-actin content slightly (these and all other changes were statistically significant). GTP[S] itself produced a marked increase in F-actin content, which was antagonized by increased Ca^{2+} (△). Thus, conditions which affect degranulation also affect microfilament state.

DISCUSSION

Our novel finding was that GTP[S] both stimulated and inhibited secretion by electroporated human neutrophils. The exact effect of GTP[S] was highly dependent on the experimental conditions and the phenomenon being observed, as summarized in Table 4. Augmentation of secretion by GTP[S] was most readily seen by using azurophil-granule secretion, which was, by necessity, elicited by high free Ca^{2+} concentrations. The Mg^{2+} requirements for augmentation were not conclusive. Inhibition of secretion by GTP[S], in contrast, has been reported only rarely. We found that this phenomenon was most pronounced for the secretion of specific-granule constituents elicited by low free Ca^{2+}. The presence of millimolar Mg^{2+} was absolutely required for inhibition to be observed. Since these inhibitory and stimulatory aspects of GTP[S] are likely to be mediated by different mechanisms, the two phenomena will be discussed separately.

Table 4. Summary of the effects of GTP[S]

	Enhancement	Inhibition
Granule type	Azurophil	Specific
GTP[S] concentration	High	Low
Ca^{2+} concentration	High	Low
Mg^{2+} required	Unclear	Yes
Cytochalasin D reversal	No	Yes

Enhancement of degranulation

Stimulation of secretion by GTP[S] has been widely reported in permeabilized cells (Bittner *et al.*, 1986; Smolen & Stoehr, 1986; Gomperts *et al.*, 1986; Barrowman *et al.*, 1986, 1987; Knight & Scrutton, 1986, 1987; Vallar *et al.*, 1987; Howell *et al.*, 1987; Wollheim *et al.*, 1987; Sorimachi *et al.*, 1987; Stutchfield & Cockcroft, 1988). In a number of systems, including human neutrophils, GTP or its non-hydrolysable derivatives can elicit some secretion on their own, without additional Ca^{2+}. In some cases, but not all, low levels of Ca^{2+} act synergistically with guanine nucleotides to promote secretion. The enhancement of secretion which we observed with electroporated neutrophils bears a number of similarities to these studies, but also a number of differences. The Mg^{2+} requirements were not always evident, and appeared to be dependent not only on GTP[S] concentration, but also on the individual experiment. For augmentation of secretion to occur, GTP[S] concentrations of 100 μM or greater were required. This is somewhat higher than has been observed in some other secretory systems, but is not necessarily uncommon. Furthermore, the channels produced by electroporation admit nucleotides only slowly (J. E. Smolen, unpublished work), so the effective concentrations during the first 1 min of stimulation are substantially lower. Secretion induced by Ca^{2+} in both the presence and the absence of GTP[S] appeared to be similarly sensitive to NEM (results not shown); thus there was no evidence that stimulation of secretion by GTP[S] was mediated by an NEM-sensitive protein(s).

The mechanism by which GTP[S] enhances secretion is not well understood in either this or other cell systems. This effect of GTP[S] was not inhibited by preincubation with either cholera toxin or PT, which irreversibly modify G_s and G_i, respectively. In these experiments, we used particularly stringent incubation conditions for PT, since the abolition of different neutrophil responses may require differing extents of G-protein inactivation (Omann & Porasik-Lowes, 1991). Even under conditions (Sklar *et al.*, 1989) which virtually abolished the most refractory of neutrophil responses (right-angle light scatter), enhancement of Ca^{2+}-induced secretion by GTP[S] was normal compared with controls (results not shown). PT-insensitivity has been reported for GTP augmentation of secretion in digitonin-permeabilized neutrophils (Smolen & Stoehr, 1986) and other cell systems (Lindau & Nusse, 1987; Therrien & Naccache, 1989; Lu & Grinstein, 1989; Cockcroft & Stutchfield, 1988), as well as the G_e exocytosis-linked G-protein which has been postulated by Gomperts and co-workers (Barrowman *et al.*, 1986; Koffer & Gomperts, 1989). However, since PT treatment should merely uncouple G-proteins from receptors, these negative data do not necessarily eliminate a G_i-like moiety from consideration (Lapetina, 1986; Bokoch & Gilman, 1984). The only indication that we have as to the site at which GTP[S] intervenes in the stimulus–response coupling pathway is from our experiments with PMA (Fig. 4, Table 2) and cytochalasin D (Table 3). In the former studies, PMA further increased secretion in response to GTP[S] and Mg^{2+}, but the reverse (enhancement of the PMA response with GTP[S]) was not seen. This would be expected if PKC acted downstream from the locus of GTP[S] augmentation.

Alternatively, PMA under these conditions may simply have provoked the release of all of the mobilizable azurophil granules. The data in Table 3 indicated that it was necessary to inhibit microfilament assembly (with cytochalasin D) for stimulation by GTP[S] to be fully expressed. This also suggests that the guanine nucleotide is acting at a distal site in this capacity.

Inhibition of degranulation

Our most unusual finding was that under certain circumstances GTP[S] profoundly inhibited degranulation. This inhibition was most pronounced when secretion of specific granules was elicited by low concentrations of Ca^{2+} (Fig. 3). Inhibition required millimolar concentrations of Mg^{2+} (Fig. 2) and was independent of the presence or absence of adenine nucleotides (Figs. 1 and 2, Table 1). Inhibition by GTP[S] took place at relatively low concentrations (30 μM) and was not mimicked by any other nucleotide (Table 1). The inhibitory effect of GTP[S] was abolished merely by incubating these cells at 37 °C, precluding a determination of whether or not this effect was inhibitable by PT or cholera toxin (results not shown).

Such inhibitory actions of GTP[S] are very rare in the literature. An inhibitory G-protein has been postulated for phospholipase C activation in permeabilized pituitary cells (Limor *et al.*, 1989). Also, guanine nucleotides have been shown to inhibit arachidonic acid release in saponin-permeabilized platelets (Rehm *et al.*, 1988). The only previous report of GTP[S] inhibiting degranulation was found in permeabilized AtT-20 cells; in these experiments, a cell-surface agonist, somatostatin, could inhibit Ca^{2+}-induced release in a GTP-dependent manner (Luini & De Matteis, 1990). These authors suggested that a G-protein coupled to somatostatin receptors inhibited secretion at some step distal to second messengers, perhaps at an exocytotic site. Our data also suggested that GTP[S] acted at a distal site. As shown in Fig. 4 and Table 2, the guanine nucleotide could inhibit secretion in the presence of PMA. Thus inhibition by GTP[S] took place either at some step after PKC activation or along some independent parallel pathway. The ability of GTP[S] to inhibit activation by a wide variety of nucleotides was consistent with this distal-site hypothesis (Fig. 1, Table 1); previously published data indicate that these other nucleotides share a common mode of cell stimulation, namely interaction with a purine receptor ultimately leading to PKC activation (Smolen & Sandborg, 1990).

One possible site of action is suggested by studies on endocytosis, rather than exocytosis, and vesicular traffic in the Golgi stack. Transport through the Golgi stack is mediated by the budding off of membrane vesicles, followed by fusion of these vehicles with other lamellae (reviewed by Rothman & Orci, 1990). The budding process is apparently initiated by GTP[S]-dependent proteins, which must be removed from the vesicle for fusion to proceed. Consequently, GTP[S] inhibits fusion in this system (Rothman & Orci, 1990). Also, GTP[S]-inhibitable fusion events have been measured in macrophage fractions (Mayorga *et al.*, 1989); sub-micromolar concentrations of GTP[S] inhibited endosome–endosome fusion, plasma-membrane–endosome fusion and fusion-dependent endosomal proteolysis. Thus, GTP[S]-inhibitable proteins involved in the fusion process have been observed in other systems, and it is possible that inhibition mediated by this guanine nucleotide in electroporated neutrophils is analogous to one or more of these. An additional aspect of the Golgi system is that, among the various factors required for fusion, there is a protein that is sensitive to NEM (designated 'NEM-sensitive fusion protein' or NSF) (Rothman & Orci, 1990; Weidman *et al.*, 1989). We did find that degranulation in our system was sensitive to NEM, so it is possible that neutrophils possess analogous mechanisms.

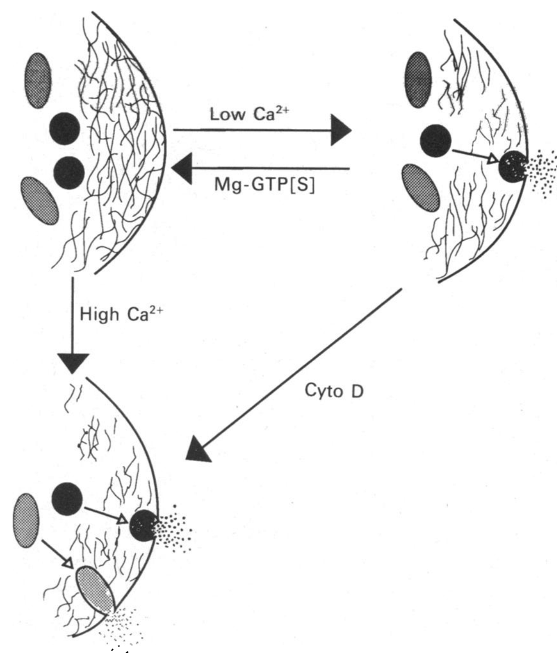

Fig. 6. Model for regulation of secretion by GTP[S] and microfilaments

The freshly permeabilized neutrophil is shown in the upper left, in which a microfilament matrix, stabilized by the absence of Ca^{2+}, restrains granules from contact with the plasma membrane. In the presence of high concentrations of Ca^{2+}, the microfilament network is severed by the bivalent cation, which also drives secretion of both granule types. Under these conditions, high Ca^{2+} concentrations inhibit GTP[S]-induced microfilament assembly such that direct stimulatory effects of the nucleotide on azurophil-granule release can be observed. However, under low Ca^{2+} concentrations, the microfilament network may be partially destabilized and only specific granules are released (upper right). GTP[S], in the presence of Mg^{2+} and low Ca^{2+}, can promote microfilament assembly and stabilization, and thus inhibit secretion. The presence of cytochalasin D (Cyto D) can inhibit microfilament assembly, thus reversing the effects of GTP[S] as an inhibitor and more clearly revealing its capacity to stimulate azurophil-granule release.

The data in Fig. 5 indicate that a plausible site for the action of GTP[S] would be actin polymerization. Other investigators have shown that, in permeabilized neutrophils, GTP[S] and other guanine nucleotides can cause polymerization of actin (Therrien & Naccache, 1989; Downey *et al.*, 1989; Bengtsson *et al.*, 1990). Furthermore, these effects of GTP[S] are insensitive to pertussis toxin. One group of investigators (Burgoyne *et al.*, 1989) has reported contrary results, but did note high specificity for GTP[S]. It is conceivable that the creation and stabilization of microfilaments by GTP[S] could be serving physically to restrain granules from interaction with fusion sites on the plasma membrane, thereby inhibiting degranulation. Indeed, our data (Fig. 5) confirmed that GTP[S] enhanced F-actin polymerization, a process which was inhibited by high Ca^{2+}. One potential model that is in accord with our data is shown in Fig. 6. The freshly permeabilized neutrophil is shown in the upper left, in which a microfilament matrix, stabilized by the absence of Ca^{2+}, restrains granules from contact with the plasma membrane. In the presence of high concentrations of Ca^{2+}, the microfilament network is severed by the bivalent cation (Burgoyne *et al.*, 1989; Downey *et al.*, 1990), which also drives secretion of both granule types. Under these conditions, high Ca^{2+} inhibits GTP[S]-induced microfilament assembly such that direct stimulatory effects of the nucleotide on azurophil-granule release can be observed. However, under low Ca^{2+} concentrations, the microfilament network

may be partially destabilized, and only specific granules are released (upper right); this may be due to the size of the pores in the resulting matrix, the relative Ca^{2+}-sensitivities for granule release, peculiarities of the biochemistry for secretion for each granule type, or some combination of these factors. Whatever is involved, GTP[S] in the presence of Mg^{2+} and low Ca^{2+} can promote microfilament assembly and stabilization, and thus inhibit secretion. The presence of cytochalasin D can inhibit microfilament assembly, thus reversing the effects of GTP[S] as an inhibitor and more clearly revealing its capacity to stimulate azurophil-granule release. That the microfilament system might regulate secretion is a conclusion also reached by others (Narasimhan *et al.*, 1990). Although this model is certainly not proved by our data, our findings are nonetheless in accord with it, and it has the advantage of simplicity.

The profound qualitative differences between activation and inhibition by GTP[S] implied that two distinct mechanisms were operating. Indeed, the activation pathway appeared to be closely associated with high Ca^{2+} concentrations and with azurophil granules. Inhibition, on the other hand, required Mg^{2+} and low concentrations of Ca^{2+}, was associated with specific granules and may well have been closely allied with the microfilament system. These dual roles for a guanine nucleotide could imply highly flexible regulatory mechanisms for this agent.

This work was supported by N.I.H. grant DK32471 (J.E.S.).

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Received 11 March 1991/5 June 1991; accepted 12 June 1991