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AGGREGATION OF RAT NEUTROPHILS BY NUCLEOTIDE TRIPHOSPHATES

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1 Adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) at concentrations of 3×10^{-7} M and greater cause a rapid partially reversible aggregation of rat polymorphonuclear leucocytes.

2 Other neucleotide phosphates are much less active at producing aggregation responses; the agonist potencies being UTP>ATP➤guanosine 5′-triphosphate, cytidine 5′-triphosphate, thymidine 5′-triphosphate; ATP>adenosine 5′-diphosphate (ADP)>adenosine 5′-monophosphate (AMP); and ADP>uridine 5′-diphosphate, thymidine 5′-diphosphate, guanosine 5′-diphosphate, cytidine 5′-diphosphate. Adenosine is inactive.

3 The hydrolysis resistant analogues of ATP, α-β-methylene ATP and β-γ-methylene ATP, do not cause neutrophil aggregation suggesting that hydrolysis of ATP and UTP may be required to initiate the aggregation response.

4 It is postulated that ATP and UTP may be important stimulants of neutrophil function and may be involved in the adherence of these cells to the vascular endothelium.

Introduction

The aggregation of polymorphonuclear leucocytes (PMNs) can be induced by a number of substances. Rapid reversible aggregation responses are induced by chemotactic factors such as the complement derived peptide C5a, the synthetic peptide formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Pne) and leukotriene B_4 (O'Flaherty, Kreutzer & Ward, 1977; Craddock, Hammerschmidt, White, Dalmasso & Jacob, 1977; Ford-Hutchinson, Bray, Doig, Shipley & Smith, 1980) as well as 1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphoryl-choline (platelet activating factor, PAF) (O'Flaherty, Showell, Becker & Ward, 1978; O'Flaherty, Cousart, Lineberger, Bond, Bass, De Chatelet, Leake & McCall, 1980; O'Flaherty, Wykle, Miller, Lewis, Waite, Bass, McCall & de Chatelet, 1981b). Nucleotide 5′-triphosphates have not been tested as aggregating agents for PMNs and there are conflicting reports on the ability of adenosine 5′-diphosphate (ADP) to induce neutrophil aggregation. O'Flaherty, Showell, Becker & Ward (1979) observed that ADP does not induce aggregation of human neutrophils over the concentration range $5 \times 10^{-6} - 3 \times 10^{-4}$ M, whereas Camussi, Tetta, Bussolino, Cappio, Coda, Masera & Segolini (1980) found that ADP is active as an aggregating agent at 10^{-4} M. The present paper describes the effects of a number of nucleotide 5′-phosphates on the aggregation of rat PMNs.

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Methods

Neutrophil aggregation assay

Neutrophil cell suspensions (> 85% PMNs) were prepared from peritoneal exudates obtained 24 h after the injection of 12% (w/v) sodium caseinate into 250–400 g male Wistar rats (Cunningham, Smith, Ford-Hutchinson & Walker, 1979). The cells were washed and resuspended at a concentration of 1×10^7 cells/ml in Eagle's minimum essential medium (MEM) buffered to pH 7.4 with 30 mM N-2-hydroxy-ethypiperazine-*N*′-2-ethane sulphonic acid (HEPES). Neutrophil aggregation was assessed by nephelometry in a Payton aggregometer (Cunningham, Shipley & Smith, 1980). All experiments were carried out within 3 h of isolation of the cells. After this time the responses to adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) were diminished. The results are expressed as the increase in light transmission measured in mm on the recorder. One mV output on the recorder is equivalent to 12 mm. The settings on the aggregometer were as follows: range, 5; level, 0.3; zero, 40; output, 80; temperature, 37°C and stir speed, 800 rev/min. The recorder amplifier was set at 20 mV. For comparison a standard concentration of leukotriene B_4 (1 ng/ml) causes an aggregation response of 41.2 ± 0.9 mm ($n = 30$) and a standard concentration of platelet activating factor (1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphoryl-choline; 10 ng/ml) a response of 64.5 ± 1.2 mm ($n = 25$), results being expressed as

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means ± s.e.mean. Stock solutions of nucleotide 5'-phosphates were made up in Eagle's MEM buffered to pH 7.4 with 30 mM HEPES, the pH adjusted, where appropriate, to pH 7.4 with 0.1 M NaOH, and 10 μl aliquots added to 500 μl aliquots of stirred cell suspensions. Stock solutions of indomethacin and nordihydroguaiaretic acid were made up in dimethylsulphoxide (DMSO). The final DMSO concentration in the cell suspensions was always $< 0.1\%$ and all suspensions were preincubated with drugs for 4 min before addition of ATP, UTP or ADP.

Leucocyte chemokinesis assay

Leucocyte suspensions were prepared by dextran sedimentation of fresh heparinized human venous blood. Chemokinetic activity was assayed in an agarose microdroplet assay as previously described (Bray, Ford-Hutchinson, Shipley & Smith, 1980; Smith & Walker, 1980). The cells were allowed to migrate for 2.25 h and the result were recorded as the area of migration in the presence of UTP, ATP or ADP when compared to that of cells in the presence of medium alone. Leukotriene B_4 at a final concentration of 0.3 and 1 ng/ml was used as a positive control.

Materials

Nucleotide mono-, di- and tri-5'-phosphates were used as the sodium salts (Sigma). Other compounds used were indomethacin (Merck, Sharpe and Dohme), nordihydroguaiaretic acid (Sigma), theophylline (Sigma) and leukotriene B_4, 5S,-12R-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (a gift from Dr J. Rokach, Merck Frosst Laboratories, Kirkland, Quebec, Canada).

Results

Figure 1 shows the effects of various nucleotide 5'-triphosphates on the aggregation of rat PMNs. All these compounds produced rapid, partially reversible, aggregation responses. The shape of the aggregation curves was similar to that previously observed following the addition of zymosan activated serum or F-Met-Leu-Phe to rat PMNs (Cunningham *et al.*, 1980). UTP and ATP were active at concentrations as low as 3×10^{-7} M and were considerably more potent than guanosine 5'-triphosphate (GTP), deoxyadenosine 5'-triphosphate (deoxy ATP), thymidine 5'-triphosphate (TTP) and cytidine 5'-triphosphate (CTP). UTP showed maximal activity at 10^{-4} M and the concentrations of ATP and UTP required to produce a half maximal UTP response in dose-response curves from three separate experiments for ATP and two for UTP were 0.9, 1.8 and 2.1×10^{-5} M (mean 1.6×10^{-5} M) and 2.2 and 2.3×10^{-6} M respectively.

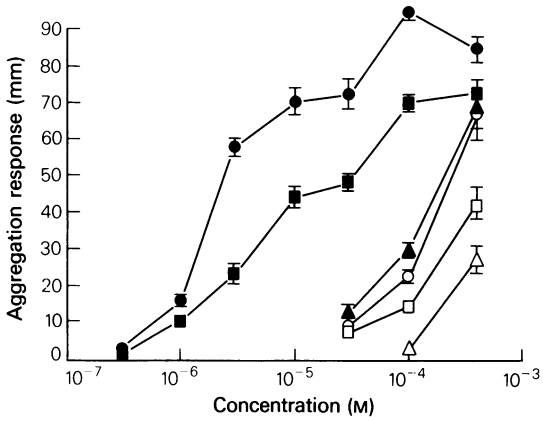

Figure 1 The effects of UTP (●), ATP (■), GTP (▲), deoxy ATP (○), TTP (□) and CTP (△) on the aggregation of rat neutrophils. Bars represent the s.e.mean. $n = 5-35$. These results are the combined results from up to 12 separate experiments. Each concentration of agonist was tested in at least 2 separate experiments.

Figure 2 shows the effects of various adenosine derivatives upon the aggregation of rat neutrophils. The agonist potencies were ATP>adenosine 5'-diphosphate (ADP)>adenosine 5'-monophosphate (AMP). Adenosine itself produced no aggregation response. The concentrations of ADP required to

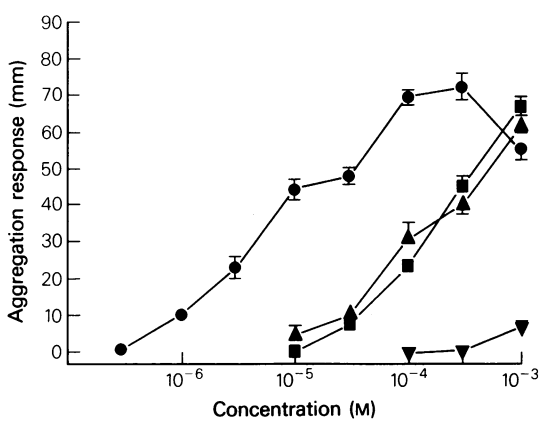

Figure 2 The effects of ATP (●), ADP (▲) and AMP (■) on the aggregation of rat neutrophils. Bars represent ± s.e.mean. $n = 5-35$. These results are the combined results from up to 12 separate experiments. Each concentration of agonist was tested in at least 2 separate experiments.

Table 1 The effects of nucleotide 5′-diphosphates on the aggregation of rat neutrophils

	Aggregation response (mm)			
Agonist	10^{-5} M	3×10^{-5} M	10^{-4} M	3×10^{-4} M
ADP	5.0 ± 1.9 (12)	10.5 ± 0.7 (16)	31.2 ± 3.5 (18)	40.2 ± 2.4 (45)
UDP	–	9.2 ± 1.7 (5)	16.5 ± 2.6 (5)	33.4 ± 3.9 (5)
TDP	–	–	9.2 ± 2.6 (5)	5.6 ± 1.1 (5)
GDP	–	–		5.7 ± 1.0 (5)
CDP	–	–	–	1.6 ± 0.4 (5)

Results are expressed as the height of the aggregation response measured in mm on the chart recorder and shown ± s.e.mean with the number of determinations in parentheses.

produce a half maximal UTP response in three dose-response curves in separate experiments were 1.0, 3.2 and 4.4×10^{-4} M (mean 3.1×10^{-4} M). The effects of various nucleotide 5′-diphosphates are shown in Table 1. All these compounds are considerably less active than the corresponding 5′-triphosphates. The agonist potencies were ADP>uridine 5′-diphosphate (UDP)>thymidine 5′-diphosphate (TDP), guanosine 5′-diphosphate (GDP) and cytidine 5′-diphosphate (CDP). The effects of the stable analogues of ATP, β-γ-methylene ATP and α-β-methylene ATP on the aggregation of rat neutrophils were also investigated. Neither of these compounds produced any evidence of an aggregation response over the concentration range 3×10^{-6} to 3×10^{-4} M.

UTP, ATP and ADP were also tested for their ability to induce the chemokinesis of human PMNs in an agarose microdroplet assay. In two separate experiments, using different donors, these compounds had no stimulant or inhibitory effect on the random migration of human PMNs over the concentration range 10^{-7}–10^{-4} M. Leukotriene B_4 at a concentration of 0.3 and 1 ng/ml caused a significant increase in migration in the same experiments. The percentage increases over control at 0.3 ng/ml were 366 ± 60 and 378 ± 41 and at 1 ng/ml were 426 ± 63 and 419 ± 34 in the two experiments (means ± s.e.mean, $n = 6$).

Table 2 shows the effects of 4 min preincubations with indomethacin or nordihydroguaiaretic acid, on the aggregation response induced by 5×10^{-5} M ATP and UTP and 3×10^{-4} M ADP. Nordihydroguaiaretic acid (10^{-5} M) significantly reduced the response to 5×10^{-5} M UTP and ATP by 45% and 14% respectively. The response to 5×10^{-5} M UTP was also significantly reduced by 10^{-6} M nordihydroguaiaretic acid (36% reduction) and 10^{-5} M indomethacin (35% reduction).

Table 2 The effects of indomethacin and nordihydroguaiaretic acid on the aggregation of rat neutrophils induced by ATP, UTP and ADP

	Aggregatory agent		
	5×10^{-5} M ATP	5×10^{-5} M UTP	3×10^{-4} M ADP
No drug	33.9 ± 1.1	55.7 ± 1.8	32.5 ± 5.5
Indomethacin 10^{-6} M	nd	52.2 ± 3.0	nd
Indomethacin 10^{-5} M	30.9 ± 0.4	36.1 ± 0.7*	34.2 ± 6.0
Nordihydroguaiaretic acid 10^{-7} M	nd	46.6 ± 4.0	nd
Nordihydroguaiaretic acid 10^{-6} M	35.0 ± 0.7	35.8 ± 0.4*	41.7 ± 5.5
Nordihydroguaiaretic acid 10^{-5} M	29.0 ± 0.7*	30.7 ± 1.4*	34.7 ± 2.5

Results are expressed as the height of the aggregation response in mm as measured on the chart recorder and are shown ± s.e.mean.

* $P < 0.001$ when compared to the control response. $n = 6$ for control response and 5 for drug-treated groups. nd = not determined.

Discussion

ATP has been reported to have a number of effects on leucocyte function. Hsu & Becker (1974) have demonstrated rapid Ca^{2+} and Mg^{2+}-dependent contraction and volume changes in rabbit peritoneal PMNs which are probably related to the aggregation response described in the present work. In contrast ADP and ATP inhibit chemotaxis and random migration in the same cell type (Rivkin & Becker, 1976) and ATP enhances enzyme release from guinea-pig peritoneal PMNs treated with cytochalasin B (Tou & Maier, 1976). ATP is known to induce Ca^{2+}-dependent histamine release from mast cells and has also been reported to induce the aggregation of rat platelets in the presence of plasma. This effect is due to the high level of creatine kinase in rat plasma and in the absence of this enzyme, ATP is an inhibitor of both rat and human platelet aggregation (Agarwal, Haskel & Parks, 1980).

The present work demonstrates that ATP and UTP, and to a lesser extent other nucleotide phosphates, cause the aggregation of rat PMNs. One possible explanation for this is that aggregation by nucleotide phosphates is linked to a P_2-purinoceptor site (Burnstock, 1976; 1978). In support of this are the observed agonist ratios (ATP>GTP,TTP and CTP and ADP>UDP>TDP,GDP and CDP). However, if there is a P_2-receptor site on the rat neutrophil cell surfaces it differs from other P_2-receptor sites. First UTP is more active than ATP as an aggregating agent and secondly the stable analogues of ATP, α-β-methylene ATP and β-γ-methylene ATP, which are resistant to hydrolysis by enzymes such as ATPases, are inactive. In other systems such as the rat urinary bladder, these compounds are more active than ATP because of the extracellular breakdrown of ATP (Brown, Burnstock & Cocks, 1979). The lack of activity of the hydrolysis-resistant analogues of ATP suggests that metabolism of ATP and UTP may be required for aggregation to occur.

In certain systems ATP acts indirectly by stimulating arachidonic acid metabolism (Needleman, Minkes & Douglas, 1974; Burnstock, Cocks, Paddle & Staszewska-Barczak, 1975). Arachidonic acid induces neutrophil aggregation through its metabolism to leukotriene B_4 (Ford-Hutchinson, Bray & Smith, 1979; Ford-Hutchinson *et al.*, 1980). Two pieces of evidence argue against ATP or UTP acting exclusively via leukotriene B_4. First the heights of the maximal aggregation responses induced by either ATP or UTP are over twice that produced by maximal doses of leukotriene B_4. Secondly nordihydroguaiaretic acid, which in this cell population inhibits leukotriene B_4 synthesis completely at concentrations of 10^{-6} M and 10^{-5} M (Bray *et al.*, 1980; Ford-Hutchinson *et al.*, 1980), has no effect on the response to ADP and only partially affects responses to ATP and UTP (Table 2). Indomethacin (10^{-6} and 10^{-5} M) completely suppresses cyclo-oxygenase activity in this cell system (Ford-Hutchinson *et al.*, 1979) and has no effect on ADP and ATP responses and only a small effect on UTP responses. These results suggest that stimulation of arachidonic acid metabolism to produce a potent aggregating agent such as leukotriene B_4 may potentiate the responses to UTP and ATP but not to ADP. In this context it has been proposed that other aggregating agents may work in part through the release of leukotriene B_4 (O'Flaherty, Hammett, Shewmake, Wykle, Love, McCall & Thomas, 1981a).

The mechanism by which these compounds induce neutrophil aggregation is unclear. Unlike other reversible aggregating agents which are chemotactic factors, UTP, ATP and ADP do not promote the chemokinesis of human PMNs and inhibit the chemotaxis and random migration of rabbit PMNs (Rivkin & Becker, 1976). This may either reflect a species difference, although human PMNs will aggregate in response to ADP (Camussi *et al.*, 1980), or may reflect rapid hydrolysis of the nucleotide 5′-phosphates by exoenzymes released by PMNs. One possible mode of action is through the stimulation of a neutrophil Ca^{2+}-dependent ATPase, an enzyme that is thought to play an important role in cellular activation (Schneider, Mottola & Romea, 1979).

The results suggest that the receptor site which interacts with UTP and ATP on the neutrophil may represent a fifth aggregating receptor site, in addition to those that already have been proposed for C5a, F-Met-Leu-Phe, leukotriene B_4 and PAF (Ford-Hutchinson, 1981; O'Flaherty, *et al.*, 1981a). The neutrophil aggregation phenomena is thought to be related to the ability of PMNs to adhere to the vascular endothelium (O'Flaherty *et al.*, 1978). ATP and UTP may play a role in the adherence of PMNs to the vascular endothelium and in the pathogenesis of shock lung. In this context it is of considerable interest that cultured vascular endothelial cells have been shown to release ATP, apparently by an active secretory process (Carleton, Gordon, Hutchings & Pearson, 1979).

Reprint requests to Canadian address, please.

References

AGARWAL, K.C., HASKEL, E.J. & PARKS, R.E. (1980). Effects of adenosine analogs and adenine nucleotides on adenosine 5-diphosphate induced rat platelet aggregation. *Biochem. Pharmac.*, **29,** 1799–1805.

BRAY, M.A., FORD-HUTCHINSON, A.W., SHIPLEY, M.E. & SMITH, M.J.H. (1980). Calcium ionophore A23187 induces release of chemotactic and aggregating factors from polymorphonuclear leucocytes. *Br. J. Pharmac.*, **71,** 507–512.

BROWN, C., BURNSTOCK, G. & COCKS, T. (1979). Effects of adenosine 5′-triphosphate (ATP) and β-γ-methylene ATP on the rat urinary bladder. *Br. J. Pharmac.*, **65,** 97–102.

BURNSTOCK, G. (1976). Purinergic receptors. *J. Theor. Biol.*, **62,** 491–503.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G., COCKS, T., PADDLE, B. & STASZEWSKA-BARCZAK, J. (1975). Evidence that prostaglandin is responsible for the rebound contraction following stimulation of non-adrenergic, non-cholinergic ('purinergic') inhibitory nerves. *Eur. J. Pharmac.*, **31,** 360–362.

CAMUSSI, G., TETTA, C., BUSSOLINO, F., CAPPIO, F.C., CODA, R., MASERA, C. & SEGOLINI, G. (1980). Mediators of immune-complex-induced aggregation of polymorphonuclear leucocytes. II. Platelet-activating factor as the effector substance of immune-induced aggregation. *Int. Archs Allergy appl. Immun.*, **64,** 1–15.

CARLETON, J.S., GORDON, J.L., HUTCHINGS, A. & PEARSON, J.D. (1979). Secretion and extracellular metabolism of adenine nucleotides by endothelial cells in culture. *J. Physiol.*, **291,** 40P.

CRADDOCK, P.R., HAMMERSCHMIDT, D.E., WHITE, J.G., DALMASSO, A.P. & JACOB, H.S. (1977). Complement (C5a) induced granulocyte aggregation *in vitro*. A possible mechanism of complement-mediated leukostasis and leukopenia. *J. clin. Invest.*, **60,** 260–264.

CUNNINGHAM, F.M., SHIPLEY, M.E. & SMITH, M.J.H. (1980). Aggregation of rat polymorphonuclear leucocytes *in vitro*. *J. Pharm. Pharmac.*, **32,** 377–380.

CUNNINGHAM, F.M., SMITH, M.J.H., FORD-HUTCHINSON, A.W. & WALKER, J.R. (1979). Migration of peritoneal polymorphonuclear leucocytes in the rat. *J. Path.*, **128,** 15–20.

FORD-HUTCHINSON, A.W. (1981). Neutrophil aggregation induced by PAF-acether and leukotriene B_4. *Br. J. Pharmac.*, **74,** 925P.

FORD-HUTCHINSON, A.W., BRAY, M.A., DOIG, M.V., SHIPLEY, M.E. & SMITH, M.J.H. (1980). Leukotriene B: a potent chemokinetic and aggregating substance released from polymorphonuclear leucocytes. *Nature*, **286,** 264–265.

FORD-HUTCHINSON, A.W., BRAY, M.A. & SMITH, M.J.H. (1979). The aggregation of rat neutrophils by arachidonic acid: a possible bioassay for lipoxygenase activity. *J. Pharm. Pharmac.*, **31,** 868–869.

HSU, L.S. & BECKER, E.L. (1974). Contraction and volume changes of glycerol treated rabbit polymorphonuclear leucocytes induced by ATP and Ca^{2+}. *Proc. Soc. exp. Biol. Med.*, **146,** 453–457.

NEEDLEMAN, P., MINKES, N.S. & DOUGLAS, J.R. (1974). Stimulation of prostaglandin biosynthesis by adenine nucleotides. Profile of prostaglandin release by perfused organs. *Circulation Res.*, **34,** 455–460.

O'FLAHERTY, J.T., COUSART, S., LINEBERGER, A.S., BOND, E., BASS, D.A., DE CHATELET, L.R., LEAKE, E.S. & MCCALL, C.E. (1980). Phorbol myristate acetate: *in vivo* effects upon neutrophils, platelets and lungs. *Am. J. Pathol.*, **101,** 79–92.

O'FLAHERTY, J.T., HAMMETT, H.J., SHEWMAKE, T.B., WYKLE, R.I., LOVE, S.H., MCCALL, C.E. & THOMAS, H.J. (1981a). Evidence for 5,12-dihydroxy-6,8,10,14-eicosatetraenoate as a mediator of human neutrophil aggregation. *Biochem. Biophys. res. Commun.*, **103,** 552–558.

O'FLAHERTY, J.T., KREUTZER, D.L. & WARD, P.A. (1977). Neutrophil aggregation and swelling induced by chemotactic agents. *J. Immunol.*, **119,** 232–239.

O'FLAHERTY, J.T., SHOWELL, H.J., BECKER, E.L. & WARD, P.A. (1978). Substances which aggregate neutrophils. *Am. J. Pathol.*, **92,** 155–166.

O'FLAHERTY, J.T., SHOWELL, H.J., BECKER, E.L. & WARD, P.A. (1979). Arachidonic acid aggregates neutrophils. *Inflammation*, **3,** 431–436.

O'FLAHERTY, J.T., WYKLE, R.L., MILLER, C.H., LEWIS, J.C., WAITE, M., BASS, D.A., MCCALL, C.E. & DE CHATELET, L.R. (1981b). 1-O-alkyl-sn-glyceryl-3-phosphorylcholines. A novel class of neutrophil stimulant. *Am. J. Pathol.*, **103,** 70–78.

RIVKIN, I. & BECKER, E.L. (1976). Effect of exogenous cyclic AMP and other adenine nucleotides on neutrophil chemotaxis and motility. *Int. Arch. Allergy appl. Immunol.*, **50,** 95–102.

SCHNEIDER, C., MOTTOLA, C. & ROMEO, D. (1979). Calcium ion-dependent adenosine triphosphatase activity and plasma-membrane phosphorylation in the human neutrophil. *Biochem. J.*, **182,** 655, 660.

SMITH, M.J.H. & WALKER, J.R. (1980). The effects of some antirheumatic drugs on an *in vitro* model of human polymorphonuclear leucocyte chemokinesis. *Br. J. Pharmac.*, **69,** 473–478.

TOU, J. & MAIER, C. (1976). Phospholipid metabolism and lysosomal enzyme secretion by leukocytes. Effects of dibutyryl cyclic adenosine 3′:5′-monophosphate and ATP. *Biochem. biophys. Acta*, **451,** 353–362.

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