Requirement for Guanosine Triphosphate for Cholera-Toxin-Catalysed Incorporation of Adenosine Diphosphate Ribose into Rat Liver Plasma Membranes and for Activation of Adenylate Cyclase

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(Received 2 August 1979)

1. Cholera toxin was shown to require the presence of GTP to activate rat liver plasma-membrane adenylate cyclase. ATP did not affect the activation process. 2. Cholera toxin catalysed the incorporation of ^{32}P from NAD labelled in the α -phosphate group of the ADP moiety into a rat liver plasma-membrane protein with a subunit mol.wt. of 42 500. This is taken to demonstrate ADP-ribosylation. The ADP-ribosylation of this protein also required GTP and was unaffected by ATP. 3. Nicotinamide inhibited both the activation of adenylate cyclase by cholera toxin and the ADP-ribosylation of the protein of 42 500 subunit mol.wt. Neither the activation nor the ADP-ribosylation could be reversed by treatment with nicotinamide in the presence of cholera toxin.

In recent years there has been considerable progress towards an understanding of the mechanism by which cholera toxin activates adenylate cyclase. It has been clearly established that the activation has an absolute requirement for NAD^+ (Gill, 1975; Martin *et al.*, 1977), and the presence of GTP is required for the effect to be expressed as increased adenylate cyclase activity (Cassel & Selinger, 1977). There is convincing evidence that in avian erythrocyte plasma membranes the toxin catalyses the ADP-ribosylation of a GTP-binding protein of the adenylate cyclase (Cassel & Pfeuffer, 1978; Gill & Meren, 1978). Cassel & Selinger (1976, 1977) have proposed that adenylate cyclase is activated by the binding of GTP and that the activation is reversed by GTP hydrolysis. They suggest that cholera toxin modifies the GTP-binding protein by inhibiting the hydrolysis of GTP, which leads to a potentiation of the GTP-activation process.

The aim of the present study was to establish whether the model outlined above can provide an adequate explanation for the effects of cholera toxin on a mammalian adenylate cyclase and to characterize further the nature of the interaction between cholera toxin and GTP in the activation process.

Experimental

Materials

ATP, 3':5'-cyclic AMP, GTP, p[NH]ppG (guanosine 5'-[β -imidol]triphosphate) and creatine kinase were obtained from Boehringer (London), Lewes, East Sussex BN7 1LG, U.K. ATP was purified

by chromatography on DEAE-cellulose as described by Kimura *et al.* (1976). Phosphocreatine was obtained from Sigma (London) Chemical Co., Kingston on Thames, Surrey KT2 7BH, U.K. [^{32}P]- P_i and 3':5'-cyclic [8- ^3H]AMP was obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. [α - ^{32}P]ATP was prepared as described by Martin & Voorheis (1977). [^{32}P]NAD labelled in the α -phosphate of the ADP moiety was prepared as described by Cassell & Pfeuffer (1978). Cholera toxin was a gift from Dr. C. E. Miller, National Institutes of Health, Bethesda, MD 20014, U.S.A.

Methods

Adenylate cyclase assay. Adenylate cyclase activity was determined by the method of Salomon *et al.* (1974).

Rat liver plasma membranes. These were prepared from the livers of female Wistar rats (140–170 g) by the method of Pilgis *et al.* (1974).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate was carried out as described by Neville & Glossman (1971), except that a slab-gel system was used. After electrophoresis the gels were fixed for 1 h in 7% (v/v) acetic acid and then cut into 2 mm slices. Each slice was incubated in 0.5 ml of 10M- H_2O_2 at 70°C until it dissolved. The resulting solution was counted for radioactivity in 4 ml of scintillant [67% (v/v) xylene, 33% (v/v) Triton X-114, containing 3 g of diphenyloxazole/litre].

Protein. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Cytosol extract. Rat liver (10g) was homogenized in 10ml of ice-cold buffer containing 20mM-Tris/HCl, pH 7.4, and 250mM-sucrose. The homogenate was centrifuged at 100000g for 1h at 4°C. The supernatant was dialysed overnight against 20mM-Tris/HCl, pH 7.4.

Results and Discussion

Requirement for ATP and GTP

Table 1 shows the effects of ATP and GTP on the activation of adenylate cyclase by cholera toxin. Pretreatment of the membranes with cholera toxin alone and NAD⁺ followed by extensive washing did not result in activation of the adenylate cyclase subsequently assayed in the presence of GTP. If GTP, cholera toxin and NAD⁺ were present in the pretreatment medium, there was a marked increase in adenylate cyclase activity with no further added cholera toxin in the assay medium. It appears therefore that there is no activation unless both GTP and cholera toxin as well as NAD⁺ are present in the same incubation. The activation of adenylate cyclase by cholera toxin and NAD⁺ was maximal at 10μM-GTP. Approx. 50% of this stimulation was ob-

served at 1μM-GTP. The activation of adenylate cyclase under these conditions persists in the subsequent assay in the absence of NAD⁺ and cholera toxin provided that GTP is present.

There was no increase in adenylate cyclase activity when the membranes were pretreated with ATP, cholera toxin and NAD in the absence of GTP; indeed there was a slight decrease in the activity. If both ATP and GTP as well as cholera toxin were included in the preincubation, there was no significant increase in the activation observed compared with the activation with GTP and cholera toxin (Table 1a). It appears therefore that ATP was unable to support the activation of adenylate cyclase by cholera toxin and was not required for activation to take place in the presence of GTP.

A number of workers have reported that ATP is essential for the activation by cholera toxin of adenylate cyclase in avian erythrocyte plasma membranes (Gill & Meren, 1978; Cassel & Pfeuffer, 1978), and in an earlier study (Martin *et al.*, 1977) we found, in common with Van Heyningen (1976), that ATP was necessary for the activation of liver plasma-membrane adenylate cyclase. However, Kimura *et al.* (1976) have demonstrated that all commercial preparations of ATP are significantly contaminated with GTP, and no attempt was made to purify the ATP used in these earlier studies. In the

Table 1. *Effect of ATP, GTP and cytosol on the activation of rat liver plasma-membrane adenylate cyclase by cholera toxin and NAD⁺*

Rat liver plasma membranes (0.2mg of protein) were preincubated at 30°C for 20min in 0.4ml of medium containing 25mM-Tris/HCl, pH 7.4, 10mM-MgCl₂, 1mM-dithiothreitol and 1mM-NAD⁺. The membranes were then centrifuged (12000g for 1min) and washed three times in 50mM-Tris/HCl (pH 7.4)/1mM-dithiothreitol. They were then assayed for adenylate cyclase at 30°C for 20min in 0.1ml of incubation medium containing 25mM-Tris/HCl, pH 7.4, 10mM-MgCl₂, 0.5mM-ATP, [α -³²P]ATP (10μCi/ml), 0.1mM-3':5'-cyclic AMP, 1mM-dithiothreitol, 5mM-phosphocreatine, creatine kinase (50 units/ml) and 0.01mM-GTP. Results are means $\pm$ S.E.M. of three observations.

Additions to preincubation	Adenylate cyclase activity (nmol/20min per mg of protein)
(a)	
NAD ⁺ (1 mM)	0.582 $\pm$ 0.040
NAD ⁺ (1 mM), cholera toxin (10μg/ml)	0.624 $\pm$ 0.008
NAD ⁺ (1 mM), ATP (0.5 mM)	0.471 $\pm$ 0.019
NAD ⁺ (1 mM), cholera toxin (10μg/ml), ATP (0.5 mM)	0.394 $\pm$ 0.011
NAD ⁺ (1 mM), GTP (0.01 mM)	0.505 $\pm$ 0.020
NAD ⁺ (1 mM), cholera toxin (10μg/ml), GTP (0.01 mM)	1.268 $\pm$ 0.032
NAD ⁺ (1 mM), ATP (0.5 mM), GTP (0.01 mM)	0.692 $\pm$ 0.022
NAD ⁺ (1 mM), cholera toxin (10μg/ml), ATP (0.5 mM), GTP (0.01 mM)	1.374 $\pm$ 0.051
(b)	
NAD ⁺ (1 mM)	0.815 $\pm$ 0.033
NAD ⁺ (1 mM), cholera toxin (10μg/ml), GTP (0.01 mM)	2.924 $\pm$ 0.136
NAD ⁺ (1 mM), cholera toxin (10μg/ml), ATP (0.5 mM), GTP (0.01 mM)	2.985 $\pm$ 0.256
NAD ⁺ (1 mM), cholera toxin (10μg/ml), GTP (0.01 mM), cytosol (2.5 mg of protein/ml)	3.181 $\pm$ 0.084
NAD ⁺ (1 mM), cholera toxin (10μg/ml), GTP (0.01 mM), ATP (0.5 mM), cytosol (2.5 mg of protein/ml)	3.218 $\pm$ 0.230

experiment presented here, contaminating GTP was removed from ATP by DEAE-cellulose chromatography.

It has been found by a number of groups (Gill, 1975; Gill & Meren, 1978; Cassel & Pfeuffer, 1978) that avian erythrocyte plasma membranes require the presence of a cytosolic extract for full activation of adenylate cyclase by cholera toxin. Gill (1975) found a 6-fold increase in the response in the presence of cytosol. We have found no increase in activation on addition of cytosol in the presence of cholera toxin, NAD⁺ and GTP in either the presence or the absence of ATP (Table 1b). Indeed it would be surprising to find any substantial effect, since the adenylate cyclase activity observed with cholera toxin under optimum conditions is about 80% of the maximum activity that can be achieved when glucagon is used together with GTP or p[NH]ppG (Table 2; Martin *et al.*, 1977).

It has been proposed that the activation by cholera toxin results from the inhibition of hydrolysis of GTP by the GTP-binding component of adenylate cyclase. This prevents the reversal of the activation resulting from the binding of GTP. In Table 2, the effects of GTP on the adenylate cyclase activity of membranes that had been pretreated with cholera toxin are shown. When the membranes had been pretreated with cholera toxin together with GTP, the activity in the absence of GTP was elevated, but the addition of GTP caused a further marked increase in activity. This suggests that the effect of the toxin is to decrease but not to abolish the rate of hydrolysis of GTP and the resulting reversal of activation. If the hydrolysis of GTP was completely inhibited, an irreversible activation analogous to that seen with the GTP analogue p[NH]ppG (Rodbell *et al.*, 1975) would be expected, with no activation on the subsequent addition of GTP. This is consistent with the observations of Cassel & Selinger (1977), who showed that the hormone-sensitive GTPase activity of turkey erythrocyte plasma membranes is decreased but not abolished by cholera toxin. We have been unable to confirm this observation in rat liver plasma membranes, as the endogenous non-specific GTPase activity is too high to allow direct measurement of the hormone-sensitive GTPase, a problem also encountered by Cassel & Selinger (1976) in liver plasma membranes.

Fig. 1 shows the incorporation of radioactivity from [³²P]NAD⁺ labelled in the α -phosphate of the ADP moiety into rat liver plasma-membrane proteins. Membranes were incubated with [³²P]NAD⁺ and additions as described in the legend. At the end of the incubation the membrane was sedimented by centrifugation and washed three times as described in the legend to Table 1. The pellet was dissolved in a buffer containing 2% sodium dodecyl sulphate and

Table 2. *Effect of glucagon, GTP and p[NH]ppG on the activity of adenylate cyclase pretreated with cholera toxin*
Rat liver plasma membranes (0.1 mg) were preincubated and washed as described in the legend to Table 1. The membranes were then assayed for adenylate cyclase with the additions shown in the Table. Results are means $\pm$ s.e.m. of three observations.

Additions to preincubation	Adenylate cyclase (nmol/20 min per mg of protein)			
	None	GTP (0.01 mM)	Glucagon (1 μ M) + GTP (0.01 mM)	Glucagon (1 μ M) + p[NH]ppG (0.1 mM)
NAD ⁺ (1 mM)	0.280 $\pm$ 0.010	0.683 $\pm$ 0.045	1.131 $\pm$ 0.020	3.092 $\pm$ 0.024
Cholera toxin (10 μ g/ml), NAD ⁺ (1 mM)	0.337 $\pm$ 0.009	1.183 $\pm$ 0.087	1.830 $\pm$ 0.100	2.064 $\pm$ 0.009
GTP (0.01 mM), NAD ⁺ (1 mM)	0.359 $\pm$ 0.014	1.017 $\pm$ 0.042	1.626 $\pm$ 0.028	4.143 $\pm$ 0.140
Cholera toxin (10 μ g/ml), GTP (0.01 mM), NAD ⁺ (1 mM)	1.200 $\pm$ 0.055	3.475 $\pm$ 0.150	4.291 $\pm$ 0.177	4.124 $\pm$ 0.100

Fig. 1. Effect of cholera toxin, ATP and GTP on the incorporation of label from $[^{32}\text{P}]\text{NAD}^+$ into rat liver plasma membranes

Rat liver plasma membranes were preincubated and washed as described in the legend to Table 1, except that $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ ($50\mu\text{Ci/ml}$) was added. Further additions were: (a) no additions; (b) cholera toxin ($10\mu\text{g/ml}$); (c) ATP (0.5mM); (d) cholera toxin ($10\mu\text{g/ml}$) and ATP (0.5mM); (e) GTP (0.01mM); (f) cholera toxin ($10\mu\text{g/ml}$) and GTP (0.01mM). The membranes were then solubilized as described under 'Methods' before being subjected to electrophoresis on 9.5% polyacrylamide gels in the presence of sodium dodecyl sulphate.

10% (v/v) 2-mercaptoethanol at 37°C for 16 h and electrophoresed as described under 'Methods' on a 9.5% polyacrylamide gel. Where the membranes were treated with labelled NAD^+ in the presence of GTP and cholera toxin, a prominent peak of labelling appeared (Fig. 1), which was not present under any of the other conditions used. There were also a number of other peaks of much higher molecular weight. These were not consistently present (see Fig. 3), and we have not investigated them any further. The pattern of labelling was very similar to that found under similar conditions in pigeon erythrocyte plasma membranes by Cassel & Pfeuffer (1978). The incubations were carried out under

Fig. 2. Effect of nicotinamide on the activation of rat liver plasma-membrane adenylate cyclase by cholera toxin in the presence of NAD^+ and GTP

Rat liver plasma membranes (0.1mg of protein) were preincubated in 0.4ml of preincubation medium as described in the legend to Table 1 in the presence of nicotinamide ($0\text{--}100\text{mM}$): ●, no additions; O, cholera toxin ($10\mu\text{g/ml}$). The membranes were then washed and assayed for adenylate cyclase in the presence of GTP (0.01mM). The results are means $\pm$ S.E.M. of the three observations.

exactly the same conditions as the preincubations in the experiment described in Table 1, except that $[^{32}\text{P}]\text{NAD}^+$ was used. Thus the conditions required for activation of adenylate cyclase by cholera toxin are also essential for the labelling of the major component. A second sample of the same labelled material was subjected to electrophoresis on a more highly cross-linked polyacrylamide gel (13%) together with cytochrome *c* (12000), lactate dehydrogenase (35000), bovine serum albumin (67000) and bovine liver catalase (57500) as molecular-weight markers. In this case the peak appeared in the centre of the gel and its mol.wt. was determined as 42500. This is very close to the mol.wt. of 42000 determined by Cassel & Pfeuffer (1978) and Gill & Meren (1978) for a protein labelled under similar conditions in pigeon erythrocyte plasma membranes. The extent of the incorporation was 93.6 ± 8.3 (mean $\pm$ S.E.M. of six observations) pmol/mg of membrane protein.

Effects of nicotinamide

Fig. 2 shows the effect of increasing concentrations of nicotinamide on the activation of rat liver membrane adenylate cyclase by cholera toxin. Nicotinamide has little effect on the adenylate cyclase activity in the absence of cholera toxin, causing only a slight decrease of about 13%. However, increasing concentrations of nicotinamide decrease the activation of the enzyme by cholera toxin, until at 100mM -nicotinamide the activation is abolished.

Fig. 3 shows the effect of 100 mM-nicotinamide on the incorporation of label from [32 P]NAD $^{+}$ into the protein of 42 500 mol.wt. Nicotinamide (100 mM) alone had no effect on the incorporation of label into the protein, but the incorporation of label in the presence of cholera toxin was completely suppressed by nicotinamide (100 mM). These observations are consistent with the proposal of Gill & Meren (1978) that the cholera-toxin-catalysed ADP-ribosylation of the GTP-binding protein may be described by the following equation:

It would be expected from this that nicotinamide used at a sufficiently high concentration should prevent the ADP-ribosylation and also the resulting activation of adenylate cyclase. It would also be expected that the addition of nicotinamide together with cholera toxin to preactivated membranes in the absence of NAD $^{+}$ should lead to the reversal of the ADP-ribosylation reaction and a deactivation of adenylate cyclase. Cassel & Pfeuffer (1978) showed that treatment of pigeon erythrocyte plasma membranes under these conditions resulted in a partial reversal of both the activation of adenylate cyclase and the incorporation of label from [32 P]NAD $^{+}$ into the 42 000-molecular-weight protein. We have been unable to demonstrate an analogous effect using rat liver plasma membranes. Treatment of preactivated rat liver plasma membranes with 100 mM-nicotinamide and cholera toxin did not result in any decrease of either the adenylate cyclase activity or the incorporation of label from NAD $^{+}$ (Table 3). The reason for this discrepancy is not clear. The reversal process is favoured by low pH, and Cassell & Pfeuffer (1978) used pH 6.1. We were unable to use

a pH of less than 6.5, since lower pH resulted in a substantial irreversible loss of adenylate cyclase activity. Thus our conditions were less favourable for the reversal process, but it seems unlikely that this difference is sufficient to explain the total lack of effect. Another possibility is that the environment of the GTP-binding protein in the rat liver plasma membrane may differ from that in avian erythrocyte plasma membranes. This would be consistent

Fig. 3. Effect of nicotinamide on the incorporation of label from [32 P]NAD $^{+}$ into rat liver plasma membranes

Rat liver plasma membranes were preincubated and washed as described in the legend to Table 1, except that [α - 32 P]NAD $^{+}$ (50 μ Ci/ml) was added. Further additions were: (a) no additions; (b) nicotinamide (100 mM); (c) cholera toxin (10 μ g/ml); (d) cholera toxin (10 μ g/ml) and nicotinamide (100 mM). The membranes were solubilized and subjected to electrophoresis on 9.5% polyacrylamide gels as described under 'Methods'.

Table 3. Effect of nicotinamide and cholera toxin on the adenylate cyclase activity and 32 P content of liver plasma membranes that had been pretreated with cholera toxin, [32 P]NAD $^{+}$ and GTP

Rat liver plasma membranes (0.2 mg) were preincubated and washed as described in the legend to Fig. 1 in the presence of cholera toxin (10 μ g/ml), GTP (0.01 mM), NAD $^{+}$ (1 mM) and [32 P]NAD $^{+}$ (50 μ Ci/ml). The membranes were then incubated for 20 min at 30°C in a medium containing 100 mM-Mes (4-morpholine-ethanesulphonic acid) buffer, pH 6.5, 10 mM-MgCl $_2$ and 1 mM-dithiothreitol with additions as shown in the Table. At the end of the incubation the membranes were centrifuged and either (i) resuspended in 50 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol and then assayed for adenylate cyclase in the presence of GTP (0.01 mM), or (ii) solubilized and subjected to electrophoresis on 13% polyacrylamide gels as described under 'Methods'. Results are means $\pm$ S.E.M. of three observations.

Additions to preincubation	Adenylate cyclase activity (nmol/20 min per mg of protein)	32 P incorporation (pmol/mg of membrane protein)
NAD $^{+}$ (1 mM)	2.013 $\pm$ 0.117	100
Nicotinamide (100 mM)	3.307 $\pm$ 0.039	129
Cholera toxin (10 μ g/ml)	2.078 $\pm$ 0.095	95.5
Nicotinamide (100 mM) + cholera toxin (10 μ g/ml)	2.522 $\pm$ 0.081	99.5

with the observation that GTP alone is a much more effective activator of rat liver plasma-membrane adenylate cyclase than of avian erythrocyte plasma-membrane adenylate cyclase. In the rat liver plasma membrane the activation observed was about 3-fold (Table 2), whereas in turkey erythrocyte plasma membranes the activation is only about 50% (B. R. Martin, unpublished work). It is possible therefore that in rat liver plasma membranes the equilibrium of the reaction is more in favour of ADP-ribosylation, such that reversal is difficult to achieve.

Cholera toxin has been shown to ADP-ribosylate itself (Trepel *et al.*, 1977), suggesting that there may be an ADP-ribosyl-toxin intermediate in the reaction with adenylate cyclase. If this is the case, the effect of nicotinamide in blocking the activation process might result from inhibition of the binding of NAD⁺ to the active site of the toxin or by removal of ADP-ribose from an ADP-ribosyl-toxin intermediate.

General discussion

The data that we have presented are consistent with the proposal that cholera toxin acts by ADP-ribosylating a GTP-binding component of adenylate cyclase, resulting in an increase in the response of the enzyme to GTP. Thus this model, first proposed by Cassel & Selinger (1977) for the adenylate cyclase of turkey erythrocyte plasma membranes, appears to describe adequately the action of cholera toxin in rat liver plasma membranes. We have also shown that the protein-GTP complex is a much more effective substrate for cholera toxin than the GTP-binding protein in the absence of GTP; indeed it is possible that the GTP-binding protein cannot be ADP-ribosylated at all in the absence of GTP. Cassel & Pfeuffer (1978) and Gill & Meren (1978) did not routinely include GTP in their cholera-toxin activation media and were still able to demonstrate activation and incorporation of label from [³²P]-NAD⁺. However, in all cases ATP was present, and Kimura *et al.* (1976) have pointed out that all commercial preparations of ATP are substantially contaminated with GTP. Using purified ATP we were able to demonstrate that ATP is neither required for the activation in the presence of GTP nor able to

support the cholera-toxin effect on rat liver plasma-membrane adenylate cyclase in the absence of GTP. The requirements for the activation of rat liver plasma-membrane adenylate cyclase by cholera toxin appeared to be much simpler than the requirements for activation of the avian erythrocyte plasma-membrane enzyme. The liver plasma-membrane adenylate cyclase requires a thiol reagent to release the active α -subunit of the toxin (Gill, 1976; Van Heyningen, 1976), Mg²⁺, NAD⁺ to act as an ADP-ribose donor, and GTP, presumably to induce the appropriate conformation of the acceptor protein. This relatively simple and well-defined system appears to offer considerable advantages for further studies of the mechanism of the activation process.

References

- Cassel, D. & Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2669–2673
- Cassel, D. & Selinger, Z. (1976) *Biochim. Biophys. Acta* **452**, 538–551
- Cassel, D. & Selinger, Z. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3307–3311
- Gill, D. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2064–2068
- Gill, D. M. (1976) *Biochemistry* **15**, 1242–1248
- Gill, D. M. & Meren, R. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3050–3054
- Kimura, N., Nakane, K. & Nagata, N. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1250–1256
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, B. R. & Voorheis, H. P. (1977) *Biochem. J.* **161**, 555–559
- Martin, B. R., Houslay, M. D. & Kennedy, E. L. (1977) *Biochem. J.* **161**, 639–642
- Neville, D. M. & Glossman, H. (1971) *J. Biol. Chem.* **246**, 6335–6338
- Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, C. R. (1974) *Biochim. Biophys. Acta* **343**, 250–267
- Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M. & Berman, M. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 3–29
- Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Trepel, J. B., Chuang, D. M. & Neff, N. H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5440–5442
- Van Heyningen, S. (1976) *Biochem. J.* **157**, 785–787