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CYCLIC AMP AND PERMEABILITY COEFFICIENT OF ALBUMIN OF THE ISOLATED RAT MESENTERY

EFFECTS OF *ESCHERICHIA COLI* ENDOTOXIN

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Summary

We have investigated the mechanisms whereby *Escherichia coli* endotoxin exerts its exudative effects, by using an isolated rat mesentery placed as a separation membrane between the two compartments of a diffusion cell. The permeability coefficient of albumin (P_A) can be easily computed from the equilibration rate of ^{125}I-labeled albumin added to one compartment. *E. coli* endotoxin increased P_A in a concentration-related manner. Direct measurements revealed an early and transient increase in cyclic AMP and prostaglandin E-immunoreactive material. These effects of endotoxin could be inhibited by indomethacin. Calcium-depleted tissues have a low P_A, even though cyclic AMP levels could still be increased by endotoxin. If incubations were prolonged beyond 90 min, P_A remained elevated, but prostaglandin E and cyclic AMP levels fell to control values. Similar results were observed with trypsin-treated tissues. These results suggest that transmesenteric passage of albumin is increased in the presence of endotoxin. During the earlier part of the incubation (up to 90 min), the effects could be related to a local synthesis of prostaglandin E, and are controlled by cyclic AMP and intracellular calcium levels. During longer incubations (90—280 min) mesothelial exfoliation could occur, allowing free diffusion of albumin through the remaining interstitial tissue.

Introduction

The course of sepsis and shock due to Gram-negative bacteria is often marked by the development of protein-rich edemas. This generalized exudative

state probably reflects a widespread increase in vascular permeability to plasma albumin, as suggested by both clinical observations [1,2] and experiments with animal models [3—5]. It is still unclear, however, whether this increase in capillary permeability is a direct effect of endotoxin liberated by the lysis of the bacteria, or whether it merely reflects high circulatory levels of agents like histamine, kinins or prostaglandins [6—8]; they are known to increase vascular permeability [9], and they reproduce many of the histological modifications observed during endotoxin shock [10,11]. Little is known about how these mediators act, at the cellular level, to increase endothelial permeability [6,9]. In a recent report [12], we provided information on how they modify the permeability of a related tissues, the mesothelium. Using an experimental setup in which the permeability coefficient of albumin of the isolated mesentery could be easily measured, we showed that histamine, bradykinin, serotonin and various prostaglandins, added to the incubation medium, significantly increase this parameter. We also showed that the transmesenteric passage of albumin is at least partly controlled by cyclic AMP and intracellular calcium levels [12].

Our aim in the present study was to gain similar insights concerning the exudative effects of endotoxin. We, therefore, used experimental conditions closely similar to those described earlier to determine the permeability coefficient and cyclic AMP levels under the influence of endotoxin, alone or in combination with other agents. We also probed the system for possible calcium requirements, and finally, we were led to consider the possibility that endogenous prostaglandins might be involved in the early permeability effects of endotoxin on the mesothelium.

Material and Methods

A detailed account of the technique can be found elsewhere [12], and can be summarized as follows:

1. General aspects. Male albino rats (200—250 g) were used in all experiments. Usual chemicals and reagents were purchased from Merck (Darmstadt, F.R.G.). ^{125}I-Labeled albumin, tritiated cyclic AMP and cyclic AMP-binding protein came from the Radiochemical Centre (Amersham, U.K.). Prostaglandin E was a generous gift from Upjohn Co (Belgium). *E. coli* endotoxin (serotype 055 B5) bovine serum albumin and trypsin came from Sigma (St. Louis, USA); indomethacin from Merck, Sharp and Dohme (Belgium), and anti-prostaglandin E serum from Calbiochem (San Diego, U.S.A.).

^{125}I was determined in a nuclear gamma-spectrometer, and ^{3}H in a Packard Tri-Carb liquid scintillation spectrometer.

2. Determination of the permeability coefficient of albumin (P_A). Placed vertically between the two halves of a diffusion cell a sheet of mesentery covers a 1 cm diameter circular communicating window. Both compartments contained 2 ml Krebs-Ringer bicarbonate buffer (pH 7.4), 1 mg/ml glucose and 1 mg/ml bovine serum albumin. The medium was continuously gassed with an O_2/CO_2 (95 : 5) mixture and was warmed at 38°C by a water bath. The agent under study was present at a known concentration in both chambers and was absent from control cells. At time $t = 0$, a tracer amount of ^{125}I-labeled albumin was added to one of the compartments. After 10 min, required for thermal

equilibration, 50 μl were taken from both compartments and the protein precipitated with trichloracetic acid. After centrifugation, the precipitates were counted and the difference in radioactivity between each pair of samples computed. As no filtration occurs in the system, the difference is a decreasing exponential function of time. The permeability coefficient of albumin, P_A, is easily computed from consecutive values of the difference in radioactivity, using an exponential fit program and expressed in cm/s.

3. Determination of the cyclic nucleotides and endogenous prostaglandins. The incubation methods have been described elsewhere [12]; the protein-binding assay for cyclic AMP was derived from the method of Gilman [13], and the radioimmunoassay for cyclic GMP was derived from the method of Steiner et al. [14].

For the determination of prostaglandin E, disks of mesentary (diameter 1 cm) were incubated in 15-ml tubes with ground-glass stoppers. Incubations were stopped with 1.5 ml ethyl acetate/0.1 M HCl (3 : 1, v/v). After standing overnight at 4°C, 2 ml ethyl acetate were added; the organic phase was isolated and dried in nitrogen stream. The prostaglandins were assayed according to Orczyk and Behrman [15]. The prostaglandin E antiserum used cross-reacted slightly with prostaglandin F (about 10%). As no separation of the two families of prostaglandins was performed, the results are expressed as 'prostaglandin E-reactive material', referred to as 'prostaglandin E' hereafter.

4. Statistical method. Probability, P, values were calculated using Student's t-test.

Results

Table I shows the effects of three concentrations of endotoxin upon P_A. During both the 0—100- and 180—280-min incubations, the increases in P_A are related to the concentrations of endotoxin. The effect is manifested early (at 10-min incubations) and lasts as long as 280 min. No investigations can be done before 10 min as this delay is required for thermal equilibration.

TABLE I

EFFECTS OF ESCHERICHIA COLI ENDOTOXIN ON P_A

Incubations up to 100 min and up to 280 min in the presence of various concentrations of endotoxin. Comparison of effects upon P_A, with controls taken from the same animals; means ±S.E. The numbers in brackets represent the number of incubations performed. The percentage increase is that compared to control values.

Endotoxin (μg/ml)	P_A (cm/s)($\times 10^{-5}$)			
	0—100 min incubation	Increase (%)	180—280 min incubation	Increase (%)
0	6.54 ± 0.75 (21)		6.21 ± 0.77 (18)	
2.5	6.97 ± 0.61 (21)	7 *	6.80 ± 0.51 (20)	10 *
0	5.14 ± 0.33 (16)		5.16 ± 0.68 (15)	
50	7.04 ± 0.58 (19)	37 **	6.92 ± 0.71 (14)	34 **
0	5.37 ± 0.42 (13)		—	
150	8.00 ± 0.53 (21)	50 ***	—	

* Not significant; ** $P < 0.05$; *** $P < 0.025$.

The effect observed with 150 μg/ml endotoxin is of the same magnitude as that induced by 5 μg/ml prostaglandin E_1 or E_2 added to the medium [12]. A limited number of experiments with 300 μg/ml did not give higher values; for reasons discussed previously, it is likely that the +50% value represents the hindrance of unstirred layers or of the extracellular matrix, rather than of actual saturation kinetics [12].

If the tissue is washed after 1 min contact with endotoxin, the effect of the compound is nearly suppressed: P_A reached $(7.20 \pm 0.57) \cdot 10^{-5}$ cm/s ($N = 17$), instead of 9.22 ± 1.18 as in unwashed tissues ($n = 18$). The former value is not statistically different from that observed in controls not treated with endotoxin ($(6.22 \pm 0.70) \cdot 10^{-5}$ cm/s, $n = 16, P > 0.05$). It, thus, seems that a 1 min period is nto enough for the full effect of the compound to develop. Comparable observations were obtained with 5 μg/ml prostaglandin E_1 under similar experimental conditions. The effect of endotoxin, thus, differs from that of cholera toxin, which is unaffected by subsequent washing [16].

Fig. 1 shows the increase in cyclic AMP observed with various concentrations of endotoxin, after incubations of 2, 20 and 40 min. The increases in cyclic AMP are related to the concentrations of endotoxin and can be observed as early as the second minute of incubation. The increases in cyclic AMP levels are short-lived, as by 90 min they return to control values.

After 220-min incubation, cyclic AMP levels were 0.54 ± 0.10 pmol/cm^2, as compared to 0.48 ± 0.05 pmol/cm^2 in controls incubated during the same period of time without endotoxin ($n = 10$ in both series, $P > 0.05$).

Increases in capillary permeability have been related to the concentrations of Ca^{2+} in the endothelial cells [17]. We have also presented results compatible with a similar role for calcium in the mesentery, where tissues depleted of calcium by pretreatment with EDTA became insensitive to prostaglandin E_1 [12]. It was of interest to test whether endotoxin increased P_A through a calcium-dependent mechanism, thereby resembling mediators of inflammation, or whether it acted in a different way. The results of these experiments are shown in Tables II and III. In the absence of any preincubation, P_A is significantly increased by 50 μg/ml endotoxin (group I). If the tissue is preincubated with 10^{-4} M EDTA, washed twice and incubated with the standard buffer con-

TABLE II

EFFECTS OF ENDOTOXIN AND Ca^{2+} ON P_A

P_A measured after 100-min incubation with or without 50 μg/ml endotoxin, means ±S.E. Group I: preincubation with 2.5 mM Ca^{2+}, and incubation with Ca^{2+}; Group II: preincubation without Ca^{2+}, but with 10^{-4} M EDTA, followed by an incubation as in Group I; group III: preincubation as in group II; incubation without Ca^{2+}, but with 1.2 mM Mg^{2+}. The numbers in brackets represent numbers of mesenteries isolated. * $P < 0.05$; ** not significant.

Group	P_A (cm/s)(×10^{-5})		Increase (%)
	No endotoxin	50 μg/ml endotoxin	
I	6.13 ± 0.50 (12)	8.30 ± 0.65 (10)	35 *
II	5.82 ± 0.24 (9)	8.10 ± 0.98 (12)	39 *
III	4.15 ± 0.95 (9)	4.57 ± 0.45 (15)	10 **

TABLE III

EFFECTS OF ENDOTOXIN AND Ca^{2+} ON CYCLIC AMP

Values represent concentrations of cyclic AMP (pmol/cm^2 membrane) after 20-min incubations with or without endotoxin. Group I: incubation in buffer containing 2.5 mM Ca^{2+}; group II: incubation without Ca^{2+}, but with 10^{-4} M EDTA. Each mean ±S.E. is calculated on ten incubations. * $P < 0.05$; ** $P < 0.025$.

Group	No endotoxin	50 μg/ml endotoxin	Increase [a] (%)
I	1.70 ± 0.33	3.02 ± 0.95	78 *
II	3.97 ± 1.11	6.85 ± 1.51	73 *
Increase [b]	134 **	124 **	

[a] Percentage increase compared to control without endotoxin.
[b] Percentage increase of group II compared to group I.

taining Ca^{2+} (group II), the effect is similar to that observed in group I. If the final incubation is performed in a medium that contains no Ca^{2+}, but does contain 1.2 mM Mg^{2+}, there is a significant decrease in P_A, and the effect of endotoxin is virtually abolished (group III). Similar procedures lead to increases in cyclic AMP in control tissues not treated with endotoxin, probably as a consequence of phosphodiesterase inhibition by EDTA; but a further increase was observed in the presence of endotoxin.

Although intracellular calcium levels were not measured and calcium fluxes were not monitored, these results support our view that calcium is indeed required for endotoxin to augment P_A. They also show that high cyclic AMP levels are not necessarily followed by a rise in permeability, and it may be tentatively concluded that the step(s) controlled by calcium presumably lie distal to those regulated by cyclic AMP.

As discussed earlier, a general, non-specific effect of calcium depletion on cell-to-cell connections would have led to cell detachment, and hence to a rise

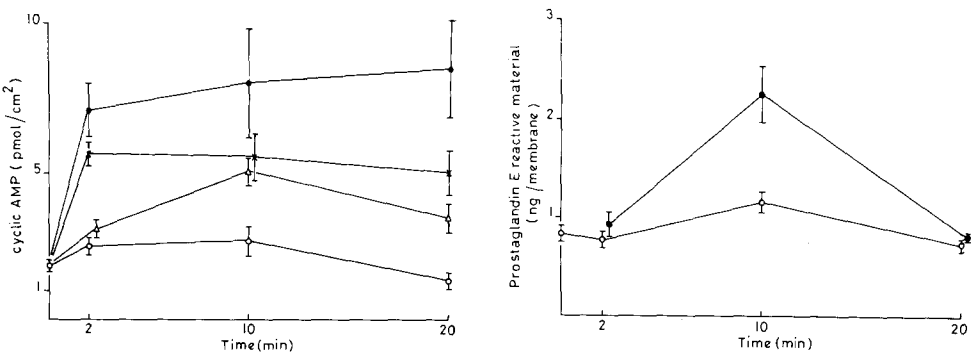

Fig. 1. Effects of endotoxin on cyclic AMP. Concentrations of cyclic AMP measured after 2-, 10- and 20-min incubation with various concentrations of endotoxin. ○, controls; △, 50 μg/ml endotoxin; X, 150 μg/ml endotoxin; ●, 250 μg/ml endotoxin. Each value represents the mean and S.E. of twelve incubations.

Fig. 2. Endotoxin and prostaglandin E-immunoreactive material. Concentrations of prostaglandin E-immunoreactive material after 2-, 10- and 20-min incubation. ●, with 50 μg/ml endotoxin; ○, no endotoxin. Each point corresponds to the mean ±S.E. of ten incubations.

TABLE IV

EFFECTS OF ENDOTOXIN AND INDOMETHACIN ON P_A AND ON CYCLIC AMP LEVELS

The permeability coefficient of albumin was measured during 100 min incubations and cyclic AMP was measured after 20 min incubation. The number of experiments are in parentheses. Values are means ±S.E. * Not significant; ** $P < 0.025$.

Agent	Concentration (μg/ml)	P_A (cm/s)($\times 10^{-5}$)	Increase (%)	Cyclic AMP (pmol/cm^2)	Increase (%)
Control	—	5.20 ± 0.61 (14)		1.83 ± 0.17 (12)	
Indomethacin	5	4.54 ± 0.71 (17)	−13 *	1.66 ± 0.24 (14)	−9 *
Endotoxin	50	7.83 ± 0.71 (13)	+50 **	3.44 ± 0.50 (14)	+89 **
Endotoxin + Indomethacin	50 5	4.54 ± 0.71 (14)	−13 *	1.82 ± 0.29 (12)	−6 *

in P_A rather than a decrease, similar to the one observed in mesenteries treated with trypsin [12].

Up to this point, the mechanism of action of endotoxin is quite reminiscent of those of histamine, kinins and exogenous prostaglandins described earlier [12]. Another similarity shared by endotoxin and the mediators of inflammation is that most changes in cyclic AMP levels take place in the mesothelial cells. In mesenteries preincubated with trypsin for 10 min, and thus reduced to sheets of loose connective tissue, only a minimal rise in cyclic AMP can be detected with 50 μg/ml endotoxin (0.86 ± 0.08 versus 6.21 ± 1.40 pmol/cm^2 tissue in non-trypsinized mesenteries).

TABLE V

CONCENTRATIONS OF PROSTAGLANDIN E-IMMUNOREACTIVE MATERIAL

Prostaglandin E-immunoreactive material measured after 10 min incubation: controls, various concentrations of endotoxin, indomethacin or simultaneous incubations with endotoxin and indomethacin. Means ±S.E. are calculated for 12 mesenteries.

Agent	Concentration (μg/ml)	Prostaglandin E-immunoactive material (ng/membrane)	Effect (%)
Control	—	1.06 ± 0.20	—
Endotoxin	10	1.83 ± 0.51	+73 **
Indomethacin	5	0.75 ± 0.07	−29 *
Endotoxin + Indomethacin	10 5	1.22 ± 0.30	+15 *
Control	—	1.19 ± 0.11	—
Endotoxin	50	2.29 ± 0.28	+92 ***
Indomethacin	5	1.04 ± 0.72	−14 *
Endotoxin + Indomethacin	50 5	1.13 ± 0.09	−5 *
Control	—	1.17 ± 0.37	—
Endotoxin	150	3.21 ± 0.14	+174 †
Indomethacin	5	1.24 ± 0.21	+6 *
Endotoxin + Indomethacin	150 5	1.33 ± 0.01	+14 *

* Not significant; ** $P < 0.05$; *** $P < 0.025$; † $P < 0.01$.

We investigated the possibility that endotoxin might stimulate a local synthesis of prostaglandins. Such an indirect mechanism of action has been suggested by Collier et al. [18]. As shown in Table IV, indomethacin suppresses the effects of endotoxin, both on P_A and on cyclic AMP production. Moreover, indomethacin also tends to reduce the basal P_A and cyclic AMP levels. In view of the results obtained earlier [12], it seems unlikely that, at the concentration used, indomethacin could have had indirect or unspecific effects unrelated to the inhibition of prostaglandin synthesis. Indeed, we have shown elsewhere [12] that the effects of histamine and bradykinin on both P_A and on cyclic AMP levels were not in the least modified by 5 μg/ml indomethacin. With this point of comparison in mind, we did not think necessary to investigate other concentrations of that compound.

The above reported observations lend support to the possible intervention of endogenous prostaglandins that could mediate the action of endotoxin on albumin permeation. To confirm this hypothesis, direct measurements of prostaglandin E were performed during incubations with endotoxin (Table V). Endotoxin significantly increases the prostaglandin E concentration in the medium. This effect is concentration-dependent, and is completely suppressed by indomethacin. As shown in Fig. 2, the rise in prostaglandin E concentration is observed as early as the second minute of incubation; it reaches a maximum at 10 min and returns to control values by 20 min. As a point of comparison, 10^{-4} M histamine, 10^{-4} M serotonin and 10^{-7} M bradykinin did not lead to any significant increase in prostaglandin E levels, although these compounds have occasionally been shown to stimulate prostaglandin release in other systems [17,19].

Discussion

The precise role of endotoxin in the increase in vascular permeability during septic shock has never been clearly determined [2]. Many humoral changes occur in such critical situations, including increases in the plasma concentrations of humoral mediators of inflammation [6—8,21] and in circulating cyclic AMP [22].

The present study, using an in vitro model free of any plasma factors, indicates that endotoxin possesses permeability effects of its own, at concentrations generally reported in experimental or clinical conditions [23,24]. This study also shows that, at least in its earlier phase, endotoxin interferes with some biochemical events in the mesothelial cells, leading to an increase in tissue permeability to albumin.

Earlier phase (0—90 min)

We have already called attention in an earlier article [12] to the major role played by cyclic AMP in the control of the diffusional parameters, P_A. We have shown that numerous physiologic and pharmacologic compounds that increase cyclic AMP production in the mesentery also increase P_A, both variables being to some extent linearly correlated. We have also shown that 10^{-3} M theophylline had the same effect, and that 10^{-3} M dibutyryl cyclic AMP could mimic the major mediators of inflammation in this respect.

The present experiments point again to cyclic AMP as an important intracellular substance, acting this time as a link between a bacterial endotoxin and an increased tissue permeability to albumin. In the present case, however, the rise in cyclic AMP production is concomitant with an augmented synthesis of prostaglandin E. These events are proportionally related to the concentrations of the toxin used, and can be completely abolished by indomethacin. At a further stage, the presence of Ca^{2+} seems required for the increase in P_A.

Tissue permeability is increased, but out experiments do not define whether the molecules follow an inter- or transcellular pathway. Both a more active microvesicular transport and/or a widening of intercellular junctions could account for our observations. Both mechanisms would require some changes in the configuration of the cellular membranes and perhaps the intervention of biochemical intracellular modifications, similar to those described here, which somewhat resemble excitation-contraction coupling in smooth muscle cells.

It should be added that prostaglandin E, measured in these experiments, could be but one of the various metabolites of arachidonic acid liberated in the presence of endotoxin [26]. Any of these or prostaglandin E itself could act in the same way as the classical mediators of acute inflammation.

Prolonged endotoxin incubations (up to 280 min)

Beyond 90-min incubation, prostaglandin-immunoreactive material and cyclic AMP levels return to control values, while P_A is still increased by +50%. This observation does not seem to be explained by any metabolic effect, but could rather be related to the exfoliation of the mesothelial cells. The interstitial tissue would then be directly exposed to albumin diffusion. Preliminary ultrastructural studies (unpublished data) support this scheme of action. The extensive shedding of mesothelial cell would, thus, be similar to that which affects endothelium in vivo [20]; both might be due to as yet unidentified cytotoxic effects of endotoxin.

Conclusions

Endotoxin liberated during Gram-negative sepsis and shock could directly increase vascular permeability: its presence in vascular tissues has been confirmed [25] and increased plasma leakage has been observed without gross cellular disruption [4,5]. These events could possibly be related to some metabolic events in the endothelial cells similar to the ones described in the present study.

The local synthesis of prostaglandin-like material by the target vascular cells would induce and inflammatory-like reaction by the tissue. Eventually endothelial necrosis and exfoliation would lead to a further increase in permeability [20].

Superimposed upon these direct endotoxin effects, the various humoral and hemodynamic changes related to sepsis could all contribute to the observed plasma exudation.

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References

1 Gilbert, H.C. and Moss, G.S. (1975) Trauma, Clinical and Biological Aspects, pp. 171—190, Plenum Medical Book Company, New York
2 Urbaschek, B. and Urbaschek, R. (1979) Microcirculation in Inflammation, pp. 74—104, Bibl. Anat. Vol. 17, Karger, Basel
3 Chien, S., Sinclair, D.G., Dellenback, R.J., Chang, C., Peric, B., Usami, S. and Gregersen, M.I. (1964) Am. J. Physiol. 207, 518—522
4 Berdjis, C.C. and Vick, J.A. (1968) J. Am. Med. Assoc. 3, 191—194
5 Pietra, G.C., Szidon, J.P., Carpenter, H.A. and Fishman, A.P. (1974) Am. J. Pathol. 77, 387—402
6 Hinshaw, L.B., Jordan, M.M. and Vick, J.A. (1960) Am. J. Physiol. 200, 987—989
7 Kobold, E.E., Lucas, R. and Thal, A.P. (1963) Surg. Forum 14, 16—19
8 Fletcher, J.R., Ramwell, P.W. and Herman, C.M. (1976) J. Surg. Res. 20, 589—594
9 Movat, H.S. (1971) Inflammation, Immunity and Hypersensitivity, pp. 38—72, Harper and Row, New York
10 Majno, G., Palade, G.E. and Schoefl, G.I. (1961) J. Biophys. Biochem. Cytol. 11, 607—626
11 Simpson, J.G., Neville, A.J. and Stalker, A.L. (1975) 8th European Conference on Microcirculation, pp. 301—302, Bibl. Anat. Vol. 13, Karger, Basel
12 Kahn, A. and Brachet, E. (1979) Biochim. Biophys. Acta 588, 219—231
13 Gilman, A.C. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 305—312
14 Steiner, A.L., Pagliara, A.S., Chase, L.R. and Kionis, D.M. (1972) J. Biol. Chem. 247, 1114—1120
15 Orczyk, G.P. and Behrman, H.R. (1972) Prostaglandins 1, 3—20
16 Bennett, V. and Cuatrecasas, P. (1975) J. Membr. Biol. 22, 29—52
17 Northover, A.M. and Northover, B.J. (1969) J. Pathol. 98, 265—276
18 Collier, J.C., Herman, A.G. and Vane, J.R. (1972) Proc. Roy. Soc. London 3, 19—20
19 Famaey, J.P., Fontaine, J., Seaman, I. and Reuse, J. (1977) Prostaglandins 14, 119—124
20 Gaynor, E., Bouvier, C. and Spaet, T.H. (1970) Science 170, 986—988
21 Markelonis, G. and Garbus, J. (1975) Prostaglandins 10, 1087—1106
22 Sibbald, W.J., Sardesai, V.W., Short, A. and Wilson, R.F. (1977) Surg. Gynecol. Obstet. 144, 199—204
23 Herman, A.G. and Vane, J.R. (1975) Arch. Int. Pharmacodyn. Ther. 213, 328—329
24 Jones, R.J. and Roe, E.A. (1979) J. Hyg. 83, 151—156
25 Rubenstein, H.S., Fine, J. and Coons, A.H. (1972) Proc. Soc. Exp. Biol. Med. 111, 458—467
26 Herman, A.G. and Moncada, S. (1976) Eur. J. Pharmacol. 39, 479—490