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Evidence for a mechanism for the initiation of acid hydrolase secretion by macrophages that is functionally independent of alternative pathway complement activation

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A mechanism for the initiation of selective acid hydrolase secretion from macrophages by weak bases that is functionally independent of complement activation is proposed on the basis of the following findings. (1) The release of β-galactosidase from macrophages exposed to methylamine and chloroquine was found to be highly dependent on the pH of the incubation medium; the degree of lysosomal secretion correlated closely with the amount of free base in solution at each pH investigated. (2) The secretion of β-galactosidase induced by methylamine was additively enhanced by a fixed dose of zymosan; likewise, chloroquine additively enhanced the secretion of β-galactosidase during exposure to zymosan. By contrast, chloroquine did not additively enhance the release of lysosomal enzyme effected by exposure to methylamine. (3) Two new secretagogues, imidazole and benzamidine, like chloroquine, failed to initiate any activation of the alternative complement pathway. The possible relationship between secretagogue induced vacuolization and lysosomal secretion is discussed.

Previous studies have shown that many of the macromolecular and particulate stimuli that are capable of inducing the selective extrusion of lysosomal acid hydrolases from mouse macrophages also possess a capacity to initiate activation of the alternative pathway of complement (Schorlemmer *et al.*, 1977; Riches & Stanworth, 1981). Indeed, in view of the observation of Schorlemmer *et al.* (1976), that complement component C3b is also capable of eliciting such a secretory response by macrophages, it was suggested by Schorlemmer *et al.* (1977) that the macrophage could comprise a self-activating unit upon confrontation with potential activators of the alternative pathway, thereby generating component C3b via a factor-B-dependent C3-convertase formed from complement proteins that are derived from the macrophages themselves.

However, whilst this series of events may be evoked in the initiation of lysosomal enzyme release following exposure of macrophages to stimuli such as zymosan particles and streptococcal cell walls, we now propose, on the basis of observations that several weak-base amines can also induce this response (Riches & Stanworth, 1980*a*; Riches *et al.* 1981), another means by which macrophage acid hydrolase secretion can be initiated and which is clearly independent of complement activation.

Materials and methods

Animals

Adult male BALB/C mice, weighing approx. 15–25g, were obtained from Banting and Kingman Ltd., Kingston-upon-Hull, U.K.

Tissue culture materials

Tissue culture medium 199 was obtained from Wellcome Research Labs, Beckenham, Kent, U.K.; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), streptomycin, penicillin and heat inactivated foetal calf serum were obtained from Gibco Bio-Cult Ltd, Paisley, Scotland, U.K. Tissue culture grade plastic petri-dishes (30mm diameter) were obtained from Sterilin, Teddington, Middlx., U.K.

Biochemical reagents

Lactic acid, NAD^+ grade AA-1, NADH grade III, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-*N*-acetylβ-D-glucosaminide, 4-methylumbelli-

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ferone, zymosan A, methylamine hydrochloride, chloroquine phosphate, benzamidine, imidazole, and Triton WR-1339 were obtained from Sigma. Triton X-100 was obtained from BDH.

Complement test reagents

Complement fixation test diluent tablets were obtained from Oxoid, Basingstoke, Hants., U.K. Rabbit erythrocytes were obtained from normal adult half-lop rabbits by venepuncture of the marginal ear vein.

Macrophage collection, purification and culture

Mouse peritoneal macrophages were isolated, and purified as previously described (Riches & Stanworth, 1980*a*). After being cultured overnight in medium 199 buffered with 20mM-Hepes and supplemented with 100μg of streptomycin/ml, 100i.u. of penicillin/ml and 10% (v/v) heat-inactivated foetal calf serum, the purified macrophage monolayers were rinsed with serum-free medium 199 before being challenged with various stimulating agents also contained in serum-free medium 199. At the end of the incubation period, the supernatants were removed, and the cells were lysed in 0.1% Triton X-100 in 0.9% NaCl.

Enzyme assays

Both supernatant and cell fractions were analysed for the following enzyme activities: cytoplasmic lactate dehydrogenase and lysosomal β-galactosidase and *N*-acetylglucosaminidase, by using an automated continuous flow method that has been described in detail elsewhere (Riches & Stanworth, 1981). All enzyme assays were conducted under optimal conditions giving a linear release of reaction product with time. The general range of absolute enzyme activities recorded for β-galactosidase and *N*-acetylglucosaminidase was 100–200nmol of 4-methylumbelliferone liberated/min per culture, whilst that of lactate dehydrogenase varied between 6 and 12μmol of NADH oxidized/min per culture.

Complement assay

Lysosomal secretagogues were tested for a capacity to activate the alternative pathway of complement using the method of Riches & Stanworth (1980*b*).

Calculation of free base concentrations

Weak bases exist in solution in the free base form $R\text{-}NH_2$ and as its conjugate acid $R\text{-}NH_3^+$. The proportion of each component in a given solution is dependent on both the pH of the solution, and the p*K*a of the base. The molar ratio of free base and conjugate acid can be calculated at a given pH from the Henderson–Hasselbalch equation:

$$pH = pKa + \log \frac{(R\text{-}NH_2)}{(R\text{-}NH_3^+)}$$

Thus:

$$\frac{(R\text{-}NH_2)}{(R\text{-}NH_3^+)} = \text{antilog}\,(pH - pKa)$$

Statistical analyses

Comparisons of means were made using the Student's *t*-test. Correlation coefficients were calculated by linear regression of data using the method of least squares. All statistical analyses were computed on a Texas TI59 programmable calculator.

Results

pH-dependence of weak-base-induced lysosomal secretion

The pH-dependence of weak-base induced lysosomal enzyme secretion was studied by exposing mouse macrophage monolayers to either methylamine (25mM) or chloroquine (100μM) for 60min at 37°C in serum-free medium 199 whose pH had been adjusted to give range of pH values between 6.0 and 8.5 by the addition of either 0.5M-HCl or 0.5M-NaOH. pH measurements were made with a Philips PW 9410 digital pH meter equipped with a calibrated Russell glass electrode.

As will be seen from Figs. 1 and 2, the ability of both methylamine and chloroquine to initiate lysosomal enzyme release was found to be highly dependent on the pH of the incubation medium.

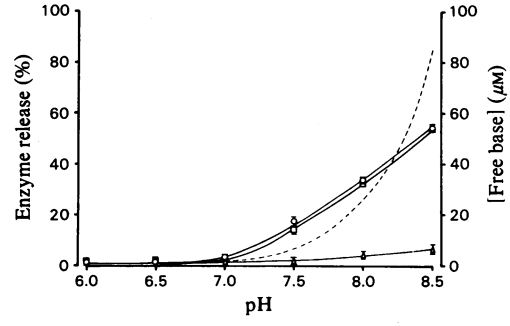

Fig. 1. *Effect of pH on methylamine (25mM, 60min exposure)-induced lysosomal enzyme release from macrophages*

O, β-galactosidase; □, *N*-acetylglucosaminidase; △, lactate dehydrogenase; – – – –, concentration of free methylamine base. Each point represents the mean ± S.D. for four experiments.

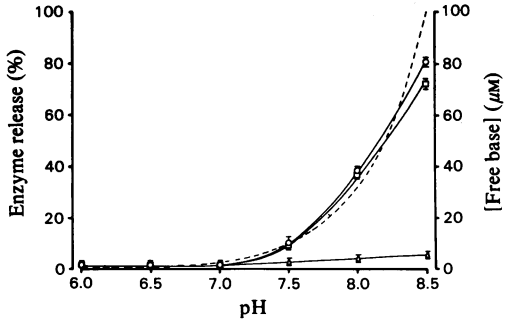

Fig. 2. *Effect of pH on chloroquine (100 μM, 60 min exposure)-induced lysosomal enzyme release from macrophages*

O, β-galactosidase; □, *N*-acetylglucosaminidase; △, lactate dehydrogenase; ——, concentration of free chloroquine base. Each point represents the mean ± S.D. for four experiments.

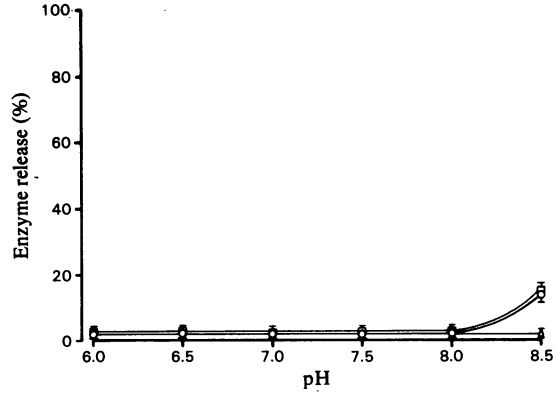

Fig. 3. *Effect of pH alone (60 min exposure) on lysosomal secretion from macrophages*

O, β-galactosidase; □, *N*-acetylglucosaminidase; △, lactate dehydrogenase. Each point represents the mean ± S.D. for four experiments.

Between pH 6.0 and 7.0 neither methylamine or chloroquine were found to trigger a significant release of lysosomal β-galactosidase or *N*-acetylglucosaminidase activities compared with control cultures that had not been challenged with weak-bases ($P > 0.1$). However, as the pH of the incubation medium was further increased, there was a progressive release of lysosomal enzymes from the cells into the culture supernatants; a response that was significant for methylamine above pH 7.0 ($P < 0.001$) and for chloroquine at pH 7.5 ($P < 0.01$).

Interestingly, the degree of lysosomal secretion was found to parallel closely the amount of free base (i.e. the non-protonated component) present in the incubation medium, as calculated using the Henderson–Hasselbalch equation (see Methods). Indeed, when the β-galactosidase data were analysed by linear regression, a highly significant association was found between free base concentration and the degree of lysosomal secretion for both methylamine ($r = 0.94$; $P < 0.001$) and chloroquine ($r = 0.98$; $P < 0.001$). The pH-dependent release of lysosomal β-galactosidase and *N*-acetylglucosaminidase from macrophages was found to be due to the presence of the weak base in the incubation medium, since, as shown in Fig. 3, incubation of macrophages in media of between 6.0 and 8.5, in the absence of weak bases, only triggered the secretion of a significant level of lysosomal enzymes at pH 8.5 ($P < 0.01$).

The selectivity of the lysosomal secretory response, which occurred in the absence of cell death, is indicated by the failure to detect significant levels of cytoplasmic lactate dehydrogenase in culture supernatants following challenge with either methylamine of chloroquine. Furthermore, the total culture activities of lactate dehydrogenase (as well as the lysosomal enzymes) remained constant during the challenge period, indicating that none of the enzymes were newly synthesized.

Additive effects of weak-bases and zymosan

Similarities between zymosan, methylamine and chloroquine induced lysosomal acid hydrolase release were sought by challenging macrophages as follows:
(*a*) with increasing concentrations of methylamine in the presence of a fixed dose of chloroquine (*b*) with increasing concentrations of methylamine in the presence of a fixed dose of zymosan (a commonly used activator of the alternative pathway of complement); and (*c*) with increasing concentrations of zymosan in the presence of a fixed dose of chloroquine.

All incubations were carried out at pH 7.4 and at 37°C.

(*a*) *Methylamine and chloroquine.* The effect of challenging macrophage monolayers for 2 h with increasing doses of methylamine (0–25 mM) in the presence of a fixed dose of chloroquine (25 μM) is illustrated in Fig. 4. It will be seen that chloroquine (the partial agonist) potentiates the response to methylamine (the full agonist) at low concentrations of methylamine. However, as the concentration of methylamine is increased, the additive effect of chloroquine on methylamine induced β-galactosidase secretion is progressively diminished until at high concentrations of methylamine, it becomes statistically indistinguishable ($P > 0.1$) from that of methylamine alone. This type of non-additive response is typical of partial agonist and full agonist interactions, and may be quantitatively analysed to obtain an estimate of the dissociation constant of the partial agonist for its 'receptor'. Thus, by plotting the

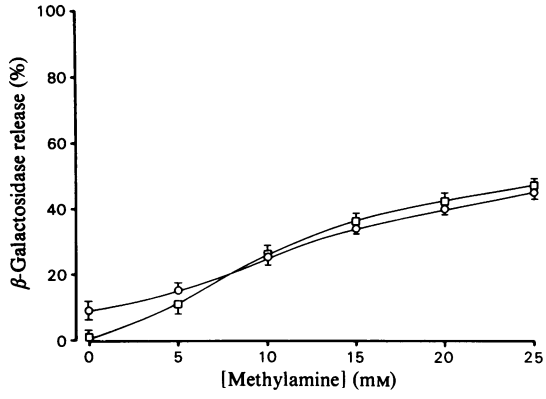

Fig. 4. *Effect of chloroquine (25 μM, ○) on the dose-dependent release of lysosomal β-galactosidase induced by exposure of mouse macrophages for 2 h to methylamine (0–25 mM, □)*

Each point represents the mean ± S.D. for four experiments.

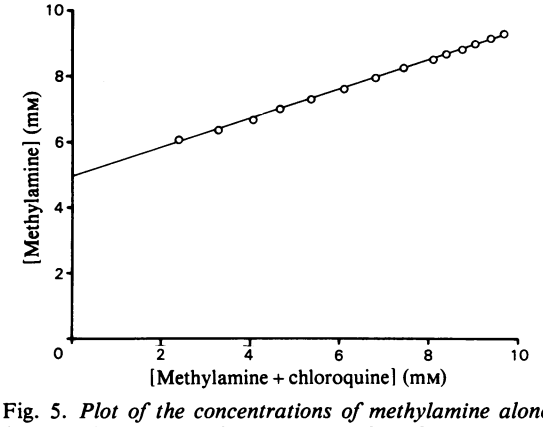

Fig. 5. *Plot of the concentrations of methylamine alone (0–10 mM), and in the presence of a fixed dose of chloroquine (25 μM) required to produce an equal degree of β-galactosidase release*

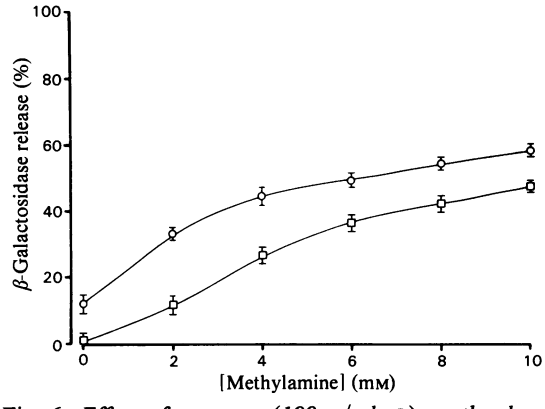

Fig. 6. *Effect of zymosan (100 μg/ml, ○) on the dose-dependent release of β-galactosidase induced by exposure of mouse macrophages for 2 h to methylamine (0–25 mM, □)*

Each point represents the mean ± S.D. for four experiments.

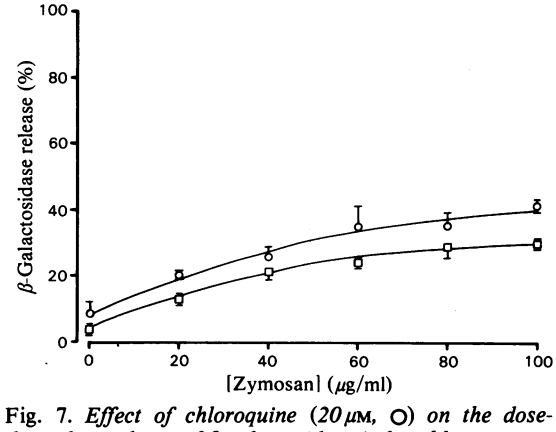

Fig. 7. *Effect of chloroquine (20 μM, ○) on the dose-dependent release of β-galactosidase induced by exposure of mouse macrophages for 4 h to zymosan (0–100 μg/ml, □)*

Each point represents the mean ± S.D. for four experiments.

concentrations producing equal responses of the full agonist alone versus the full agonist in the presence of a fixed dose of the partial agonist, the dissociation constant for the partial agonist (K_D), can be calculated from the equation (Foreman & Lichtenstein, 1980):

$$K_d = \frac{[\text{partial agonist}]}{\frac{1}{\text{slope}} - 1}$$

Fig. 5 illustrates such a plot for chloroquine and methylamine, from which the K_D for chloroquine was calculated to be 2.01×10^{-5} mol · l^{-1}.

A similar release profile was observed for *N*-acetylglucosaminidase; at all concentrations of chloroquine and methylamine, viability was maintained at greater than 98% as indicated by lactate dehydrogenase release from the cells.

(*b*) *Methylamine and zymosan.* A totally different picture was seen when macrophages were challenged for 2 h with methylamine (0–25 mM) in the presence of a fixed dose of zymosan (100 μg/ml). As will be seen from Fig. 6, zymosan was found to enhance additively the methylamine stimulated release of

lysosomal β-galactosidase at both low and high concentrations of methylamine. Thus, since clearly there is no competition between the two stimuli for an activation site involved in triggering lysosomal secretion, one must conclude that methylamine and zymosan stimulate this response via different triggering mechanisms.

(*c*) *Zymosan and chloroquine.* The effect of exposing mouse macrophages to increasing concentrations of zymosan (0–100 μg/ml) in the presence of a fixed dose of chloroquine (20 μM) for 4 h is illustrated in Fig. 7. Here, as with the case of methylamine and zymosan, chloroquine potentiated the release of β-galactosidase from the cells at all concentrations of zymosan, i.e. the capacities of these two secretagogues are additive to one another and must therefore act on independent cell activation systems.

Effects of other lysosomotropic agents

Imidazole and benzamidine. The effects of exposing macrophage monolayers to the weak-base amines imidazole (10–50 mM) and benzamidine (5–25 mM) for 5 h at pH 7.4 and at 37°C are illustrated in Figs. 8 and 9 respectively. It will be seen that, during exposure to imidazole, 60% of the total culture activities of lysosomal acid hydrolases were released by 50 mM-weak base, while with benzamidine, 85% of the activity was released by exposure to 25 mM-weak base. Concomitant with the secretory process, both weak bases also initiated a pronounced development of large vacuoles throughout the cytoplasm, as is seen with other weak bases such as methylamine and chloroquine.

Sucrose and Triton WR-1339. Previous investigations have shown that both sucrose and the non-haemolytic surfactant Triton WR-1339 are taken up by mouse macrophages by a process of fluid phase pinocytosis, and, due to the absence of a suitable lysosomal degrading enzyme, are stored intracellularly resulting in the formation of large vacuoles/vesicles (Cohn & Ehrenreich, 1969).

However, whilst exposure of macrophages to sucrose at concentrations between 5 and 50 mM for 6 h resulted in the formation of many phase-lucent cytoplasmic vacuoles, it did not result in the release of either cytoplasmic or lysosomal enzymes. Likewise, macrophages exposed to Triton WR-1339 (0.001–0.1%) for 4.5 h also developed many cytoplasmic vacuoles, though lysosomal and cytoplasmic enzymes were not released.

Complement activating properties of secretagogues

The capacity of zymosan and methylamine to activate the alternative pathway of complement has been demonstrated previously (Riches & Stanworth, 1981) by application of a simple haemolytic procedure developed in our laboratory (Riches & Stanworth, 1980*b*). In the present study, chloroquine, benzamidine, imidazole, sucrose and Triton WR-1339 were similarly examined for an ability to activate the alternative pathway. The concentrations of substance studied were identical with those used in the lysosomal secretion experiments

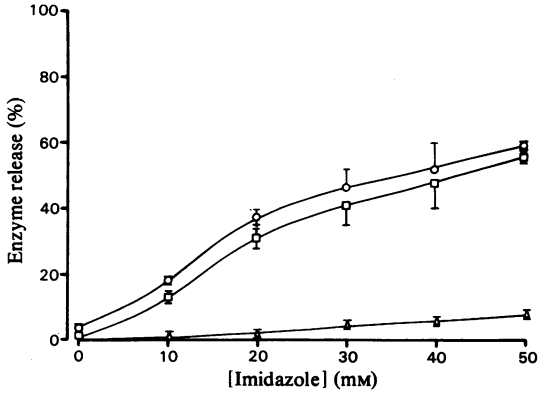

Fig. 8. *Dose-dependent release of lysosomal enzymes from macrophages exposed to imidazole (0–50 mM) for 5 h at 37°C*

O, β-galactosidase; □, *N*-acetylglucosaminidase; △, lactate dehydrogenase. Each point represents the mean ± S.D. for four experiments.

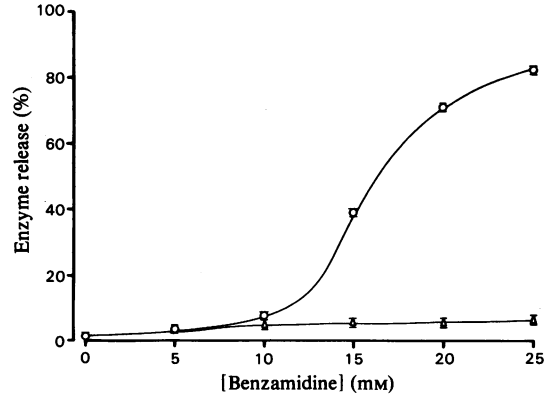

Fig. 9. *Dose dependent release of lysosomal enzymes from macrophages exposed to benzamidine (0–25 mM) for 5 h at 37°C*

O, β-galactosidase; △, lactate dehydrogenase. Each point represents the mean ± S.D. for four experiments.

Table 1. *Capacity of various lysosomotropic agents to initiate activation of complement C3 via the alternative pathway*
Each point represents the mean ± S.D. for three experiments.

Agent	Activation of C3 (%)					
Benzamidine (mM) ...	0	5	10	15	20	25
	3.0 (0.5)	0.2 (0.6)	1.8 (2.7)	2.2 (1.9)	2.8 (1.0)	4.3 (0.5)
Chloroquine (μM) ...	0	25	50	100	300	500
	0.4 (0.6)	4.1 (2.5)	2.3 (2.8)	3.9 (2.1)	3.7 (0.6)	3.1 (0.6)
Imidazole (mM) ...	0	10	20	30	40	50
	0.1 (0.5)	1.0 (0.8)	5.1 (4.3)	9.9 (1.5)	17.1 (5.1)	20.2 (5.7)
Sucrose (mM) ...	0	10	20	30	40	50
	5.3 (1.4)	7.2 (0.7)	3.9 (2.1)	3.5 (0.5)	1.0 (0.05)	2.0 (1.5)
Triton WR-1339 (%, v/v) ...	0	0.001	0.005	0.01	0.05	0.1
	3.0 (3.2)	1.9 (0.8)	5.0 (1.3)	1.1 (3.6)	2.8 (2.5)	0 (3.2)

referred to earlier, and are tabulated, together with their effects, in Table 1. It can be seen that, with the exception of higher concentrations of imidazole, none of the substances examined were found to activate the alternative pathway of complement.

Discussion

Previous investigations (Schorlemmer *et al.*, 1977; Riches & Stanworth, 1981) have tended to suggest that the secretion of lysosomal enzymes from macrophages during exposure to particulate secretagogues such as zymosan particles, and to soluble stimuli such as methylamine, may be mediated by endogenously generated components of the alternative complement pathway, particularly C3b. The results of the present study, however, clearly indicate that the release of lysosomal enzymes effected by exposure of macrophages to weak-base amines occurs via an activation mechanism that is functionally distinct from that of zymosan-induced lysosomal secretion, and which is also independent of complement activation. The evidence in support of this conclusion is threefold.

Firstly, the release of lysosomal enzymes from macrophages induced by exposure to methylamine and chloroquine was found to be highly dependent on the pH of the incubation medium. This finding could be attributed to a requirement for a cell surface ecto-enzyme, which is optimally active at or around pH 8.5, e.g. Ca^{2+}-ATPase (Cooper & Stanworth, 1976) or 5′-nucleotidase (Edelson & Cohn, 1976). However, an alternative, and in our opinion the most likely, explanation, is that since weak bases readily permeate biological membranes in un-ionized form, i.e. as $R\text{-}NH_2$ (de Duve *et al.*, 1974), the increase in pH merely elevates the amount of unprotonated weak base in solution according to the laws of simple acid–base equilibria, and that this is reflected by a concurrent increase in the degree of lysosomal secretion. This conclusion is supported by the highly significant association noted between β-galactosidase release from the macrophages and the absolute concentrations of methylamine and chloroquine free base calculated at the various pH values from the Henderson–Hasselbalch equation. The small, though statistically significant, level of lysosomal secretion that is seen at pH 8.5 in the absence of lysosomal secretagogue is possibly due to alkali-induced deamidation of glutamine and asparagine which are present in the culture medium, to yield free ammonia, which itself has been found to be a potent lysosomal secretagogue (Riches & Stanworth, 1980*a*).

Interestingly, it was noted from morphological examination that weak-base-induced vacuolization likewise became more pronounced with increases in the pH of the incubation medium, an observation that is in accordance with the findings of Yang *et al.* (1965) who studied the effect of pH on vacuolization induced by exposure of chick embryo fibroblasts to NH_4Cl. It has been proposed by de Duve *et al.* (1974) and by Ohkuma & Poole (1978) that such weak-base-induced responses are a consequence of the trapping, by protonation, of the weak base within the lysosomal compartment. When the intralysosomal concentration of the weak base becomes sufficiently high [up to 50 mM for chloroquine (Wibo & Poole, 1974)], the lysosomes undergo osmotic expansion to form large vacuoles. Hence, as the pH of the incubation medium is elevated, the absolute concentration of free base $R\text{-}NH_2$ becomes elevated for the reasons discussed above, thereby resulting in effectively, a concentration-dependent increase in lysosomal vacuolization. The possible relationship between vacuolization and lysosomal secretion will be discussed in more depth later.

The second and perhaps most convincing line of experimental evidence in favour of a functional difference between zymosan- and weak-base-induced lysosomal secretion came from our investigations into the additivity of the response to these two types

of secretagogue. Initially, the effect of a fixed dose of one weak-base secretagogue, chloroquine, on the capacity of another weak base, methylamine, to initiate acid hydrolase secretion was investigated. The results obtained were typical of partial agonist/full agonist interactions in that, at low doses of methylamine, the capacity of chloroquine to trigger lysosomal secretion was additive to that of methylamine. However, at high concentrations of methylamine, chloroquine inhibited β-galactosidase release, presumably by competition for the same activation site as methylamine. Thus the activation mechanism for the initiation of lysosomal secretion by chloroquine and methylamine is likely to be identical. The dissociation constant for chloroquine for its receptor was calculated from this experiment as being 2.01×10^{-5} mol·l^{-1}, i.e. it is an interaction of low affinity.

In marked contrast to the weak base/weak base interactions discussed above, zymosan was found to enhance additively the release of lysosomal enzymes effected by exposure to methylamine at all concentrations of methylamine that were studied, and, likewise, chloroquine was found to enhance additively zymosan-stimulated lysosomal secretion also over a wide range of zymosan concentrations. Thus, since clearly there is no competition between weak base and zymosan for the macrophage activation 'receptor', one must conclude that the receptors involved in zymosan-induced lysosomal secretion are distinct, separate entities from those involved in the initiation of this response by weak bases.

It has long been recognized that the injection of hypertonic sucrose into rodents resulted in massive vacuolization in both kidney and liver (Brewer & Heath, 1964), and that this response could also be evoked *in vitro*, in cells and tissues such as macrophages (Cohn & Ehrenreich, 1969) and chick embryo limb-bone rudiments (Dingle *et al.*, 1969). We therefore felt that it was pertinent, in the context of the present study, to examine whether lysosomal vacuolization induced by the pinocytosis of small molecules such as sucrose and Triton WR-1339 was capable of inducing acid hydrolase secretion. However, whilst much vacuolization was noted, challenge with both types of pinocytotic stimuli failed to initiate selective lysosomal secretion from macrophages.

Finally, a variety of lysosomal secretagogues, both previously described ones and novel compounds, have been tested for a capacity to activate the alternative pathway of complement in order to establish whether they might initiate lysosomal secretion via C3b generation as outlined by Schorlemmer *et al.* (1977). In our previously reported studies in this field, we reported that methylamine was capable of 'consuming' components of the alternative pathway over a similar concentration range to that required to initiate hydrolase secretion from macrophages, and hence therefore could conceivably trigger this response by endogenous C3 conversion (Riches & Stanworth, 1981). However, the findings presented here show that other potent weak-base secretagogues, such as benzamidine, chloroquine and probably imidazole, do not likewise activate the alternative complement pathway; hence, their mode of initiating macrophage lysosomal secretion is independent of complement activation, as probably is that of methylamine.

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