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EFFECTS OF THE SCHISTOSOMICIDE 1,7-bis(*p*-AMINOPHENOXY)HEPTANE (153C51) ON LYSOSOMES AND MEMBRANE STABILITY

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Abstract—The schistosomicide 1,7-bis(*p*-aminophenoxy)heptane (153C51) stabilised mouse erythrocytes and rat liver lysosomes against osmotic lysis. Chloroquine was less potent in either system in terms of the maximum stabilisation achieved or the concentration needed to produce the maximum effect. Two lysosomal enzymes, acid phosphatase and β-acetylglucosaminidase, were partially inhibited by 153C51. Binding of [^{3}H]-153C51 by rat liver lysosomes was directly proportional to the drug concentration. The results are discussed in relation to the amphiphilic, cationic properties of the drug molecule and the effect of 153C51 on lysosomes in the tegument of the blood fluke *Schistosoma mansoni*.

The experimental schistosomicide 1,7-bis(*p*-aminophenoxy)heptane (153C51) caused the accumulation of large numbers of residual lysosomal bodies in the tegument of male *Schistosoma mansoni* soon after treatment of the murine host [1]. Although not previously reported in a helminth parasite, this cellular reaction to drug treatment is not uncommon elsewhere. For example, chloroquine caused the formation of similar bodies in malaria parasites [2] as well as in mouse [3] and rat [4] livers, in various tissues of pigs [5] and other mammals [6] and in macrophages cultured *in vitro* [7].

Lysosomal residual bodies might be expected to accumulate if secondary lysosomes are deprived or depleted of hydrolytic activity as a result of changes either in membrane stability or in their internal composition [8, 9]. The accumulation of such bodies after chloroquine treatment has been explained on these biochemical grounds by experiments *in vitro* concerning the effects of chloroquine on the stability of biological membranes [10, 11, 12] and on the activity of certain lysosomal enzymes [12]. The results of these experiments also help to explain the retinotoxic side effects of chloroquine [13] because phagocytosis, one of the intracellular digestive functions of lysosomes, is of great importance within the retina in the turnover of membranes associated with the visual process [14]. It may be significant that in addition to exhibiting a similar ultrastructural effect on schistosomes as chloroquine on malaria parasites, 153C51 also had retinotoxic effects on the mammalian host [15].

In view of the similarities between the biological effects of 153C51 and chloroquine, it was decided to examine the biochemical effects of 153C51, *in vitro*, for comparison with the results previously reported for chloroquine.

MATERIALS AND METHODS

(a) *Effects on mouse erythrocyte stability.* The effects of drugs on the stability of mouse red blood cells subjected to hypotonic shock was determined essentially as described by Seeman and Weinstein [16]. Blood was collected by cardiac puncture from male CD1 mice weighing approximately 40 g and pooled in a heparinised tube. The erythrocytes were packed by centrifugation, the plasma removed and the cells washed three times in equal volumes of 154 mM-NaCl in 10 mM-potassium phosphate buffer. A stock suspension of these erythrocytes was prepared in the same solution to give a density of 120–400 × 10^6 cells per 200 μl or an optical density of approximately 0.5 O.D. units when totally haemolysed in NaCl-free buffer. The degree of haemolysis was assayed by adding 200 μl of erythrocyte stock suspension to 3 ml 10 mM-potassium phosphate buffer containing various hypotonic concentrations of NaCl. The concentration of NaCl was adjusted so that in control preparations there was a 50 per cent haemolysis of added erythrocytes. After mixing the preparation was allowed to stand for 5 min before centrifugation at 1500 *g* for 5 min. The optical density of the supernatant was measured on a Pye Unicam SP500 spectrophotometer at 543 nm. Blanks (zero haemolysis) were prepared from erythrocytes added to 154 mM-NaCl in phosphate buffer. Drugs were added to the incubation mixture in 30 μl DMSO (153C51) or water (chloroquine) to the desired concentration before addition of the erythrocyte stock suspension. DMSO in these proportions did not affect the lysis of mouse erythrocytes. At concentrations above 10^{-3} M, 153C51 was only partially soluble in the incubation mixture. At 10^{-3} M-153C51, the pH of the mixture was lowered by 0.1 pH unit and at 10^{-2} × 0.5 M by 0.5 pH unit.

(b) *Effects on lysosomes.* The effects of 153C51 on the release and activity of enzymes from a rat liver lysosome preparation were determined, after minor modifications, by the method of Smith *et al.* [12]. Fresh liver from male Cobs Wistar rats weighing approximately 40 *g* was cut into small pieces and a 10% (w/v) homogenate made at 40° in 0.25 M-sucrose–0.02 M-Tris acetate buffer, pH 7.4, by use of a hand-held

Teflon/glass homogeniser. The homogenate was centrifuged at 4° and 600 *g* for 5 min and the pellet reserved for experimentation. The supernatant was diluted with an equal volume of the same buffer and re-centrifuged at 4° and 3500 *g* for 15 min. The 3500 *g* pellet was resuspended in a volume of 0.45 M-sucrose–0.04%-glycogen–0.02 M-Tris acetate, pH 7.4, one tenth that of the buffer used for the original homogenisation. This stock lysosome suspension was kept at 4° and used within 2 hr of preparation. Ultrastructural examination of the 3500 *g* pellet fixed in phosphate buffered glutaraldehyde revealed that it consisted of a mixed preparation of lysosomes, mitochondria and other membranous cellular constituents.

The effects of 153C51 on lysosomal membrane stability were measured by assaying the enzyme activity released from 400 μl of the stock suspension after incubation for 1 hr at 37° in a metabolic shaker with 4.0 ml 0.18 M-sucrose–0.1 M-Tris acetate buffer, pH 7.4, with or without added drug. Under these conditions the buffering capacity of the incubation medium was not exceeded. Drug was added in 40 μl of the appropriate solvent. After incubation, intact lysosomes were pelleted by centrifugation at 27,000 *g* for 15 min at 4° and samples of the supernatant were assayed for the activity of lysosomal enzymes released during incubation. The enzymes assayed were acid phosphatase (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase), aryl sulphatase (EC 3.1.6.1; aryl sulphate sulphohydratase) and β-acetylglucosaminidase (EC 3.2.1.30; β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase). Aryl sulphatase activity was measured by the method of Smith *et al.* [12] except that 250 mM-acetate buffer, pH 3.8, was used in an incubation of 30 min duration. Acid phosphatase activity was determined in a final volume of 400 μl containing 100 μl 4 mM-*p*-nitrophenylphosphate, 80 μl 250 mM-sodium acetate buffer, pH 3.8, plus 100 μl 27,000 *g* supernatant and 120 μl H_2O. The reaction was started by the addition of enzyme and stopped by the addition of 1600 μl 0.05 M-NaOH. The optical density at 420 nm was compared with a blank to which had been added 100 μl 0.45 M-sucrose–0.04%-glycogen–0.02 M-Tris acetate, pH 7.4, and the amount of *p*-nitrophenol (PNP) produced was calculated by reference to standards incubated as above but without substrate. Acetylglucosaminidase activity was determined by incubating 200 μl 20 mM-*p*-nitrophenol-*N*-acetyl-β-D-glucosaminide in identical fashion to the acid phosphatase substrate. The final pH of the acid phosphatase and acetylglucosaminidase media was 4.5, that of the aryl sulphatase medium 4.0.

The effects of 153C51 upon lysosomal enzyme activity were determined by the addition of drug to the media used for assay of enzymes released from lysosomes incubated in hypotonic sucrose without drug. Addition of 153C51 did not change the pH of the assay medium.

The total enzyme activity in the lysosome preparations was determined by incubating 50 μl lysosome suspension in 2 ml 0.2%-Triton X-100–0.04 M-Tris acetate, pH 7.4, for 15 min at 37° and assaying the enzymes released.

(c) *The binding of* 153C51 *to lysosomes.* The binding of [G-^{3}H]-153C51 (sp. act. 12.37 mCi/mg) to the constituents of the lysosome preparation was measured by use of a Millipore 1225 sampling manifold loaded with 2.5 cm diameter Whatman GF/C glass fibre discs. Incubation vessels were prepared to contain 3.86 ml 0.35 M-sucrose–0.1 M-tris acetate, pH 7.4, 40 μl DMSO or 40 μl DMSO plus 153C51. This mixture was pre-incubated at 37° in a shaking water bath for 5 min before addition of either 100 μl of lysosome preparation or of buffer. Lysosomes were prepared as in (b) except that a 0.25 M-sucrose–glycogen–buffer mixture was used to suspend the 3500 *g* pellet. The lysosome–drug mixture was incubated for 5 min and then filtered under vacuum through the Millipore apparatus. The filters were washed with four 4.0 ml volumes of incubation buffer at 4° which had first been used to rinse out the incubation vessel. The glass fibre discs were placed in a scintillation phial with 1.0 ml Nuclear Chicago Solubiliser and stored overnight at 37°. Next day, 40 μl glacial acetic acid was added before counting the radioactivity in the samples as described previously [17] at an efficiency of 37–39 per cent. The entire incubation and filtration process was repeated in triplicate with and without added lysosomes and 153C51.

The enzyme substrates were obtained from Sigma Chemical Company and all other reagents were either obtained from Sigma or British Drug Houses. The [G-^{3}H]-153C51 was custom-synthesised at the Radiochemical Centre, Amersham and was chromatographically pure.

Snedecor's Variance Ratio test was used in the statistical analyses of results.

Protein concentrations were determined by the method of Lowry *et al.* [18] with bovine serum albumin as the standard.

RESULTS

(a) *Effects on mouse erythrocyte stability.* As shown in Fig. 1, 153C51 both labilised and stabilised mouse erythrocytes subjected to osmotic shock depending on the drug concentration employed. Greatest stabilisation was evident at neutral pH at 10^{-4} M. At higher concentrations than this there was a sudden decrease in stabilisation until, around 0.5×10^{-3} M, labilisation was first observed. Changing the pH of the buffer to 6.0 or 8.0 did not dramatically affect the profile of activity. As described by Seeman [23] mouse erythrocytes were more stable at alkaline pH and more fragile at acid pH. The following molarities of NaCl produced 50% haemolysis of control samples in the present work: pH 6.0, 99–103 mM; pH 7.0, 77 mM; pH 8.0, 57–62 mM.

(b) *Effects on lysosomes.* Throughout the experiments on lysosomes there was some variability in results from sample to sample. The variability was evident in the protein content of samples, the total enzyme activity released after lysis and in the drug binding studies. However, there was relatively little variation in the described effects of 153C51 and chloroquine. Appropriate reference of results to the total enzyme activity and percentage lysis of control samples was made wherever possible. Control samples not treated with drug were always incubated simultaneously with drug-treated samples.

A comparison of acid phosphatase activity released from three fractions of rat liver is shown in Table 1 together with the effects of 10^{-5} M 153C51. There was considerable latency of acid phosphatase in all fractions

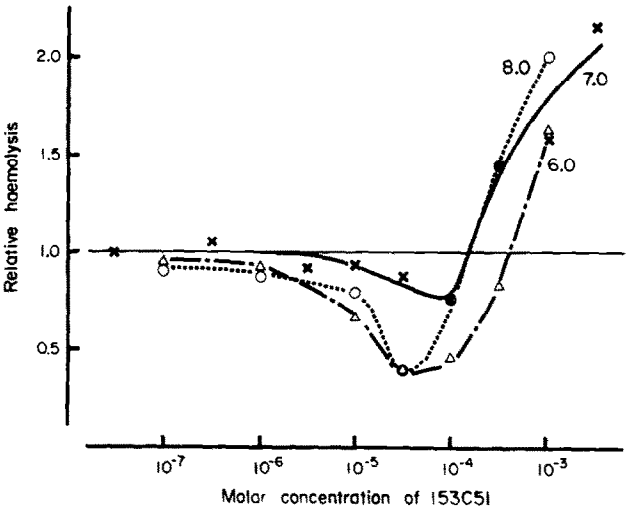


Fig. 1. The effect of 153C51 on mouse erythrocyte lysis after osmotic shock (see Methods for techniques). The results are expressed as Relative Haemolysis or the

$$\frac{\text{O.D.}_{543\text{nm}} \text{ drug treated sample}}{\text{O.D.}_{543\text{nm}} \text{ control sample}}$$

Experiments were performed with various drug concentrations at pH 6.0, 7.0 and 8.0. At least three separate preparations of erythrocytes were used at each drug concentration for each pH value. The coefficient of variation about the mean at each concentration and pH was, routinely, 12%.

examined when compared with the total activity of a detergent-treated fraction. The latency apparent in the resuspended material from the 3500 *g* pellet was in good agreement with that reported by Smith *et al.* [12]. A concentration of 10^{-5} M 153C51 was without effect on enzyme activity in all fractions although it did appear to stabilise the rat liver lysosome preparation so that only 51 per cent of the control activity was released after incubation with drug. The effect of 153C51 on the release and/or activity of several lysosomal enzymes at various drug concentrations was examined in detail. The results are recorded in Table 2. No attempt was made to distinguish between the effects of 153C51 on the release of the enzymes and the effects on enzyme activity in this series of experiments. With all these enzymes there was a similar dependence of the enzyme activity detected after incubation on the concentration of drug used. The highest reduction in activity occurred at 10^{-5} M 153C51 and the effects were reduced at higher and lower concentrations until at 10^{-7} M they were undetected. In Table 3 the effects of 153C51 on drug release from lysosomes are recorded separately from the effects on enzyme activity for acid phosphatase and *β-N*-acetylglucosaminidase. The drug prevented the release of both enzymes from osmotically shocked lysosomes at all three concentrations examined. Similarly, at all three concentrations the drug inhibited *β-N*-acetylglucosaminidase activity although the per cent inhibition did not increase with the concentration of drug used. At 10^{-3} M, 153C51 inhibited acid phosphatase activity by 25 per cent.

The quantity of 153C51 bound by the lysosome preparation was directly proportional to the concentration of drug in the medium (Fig. 2). No attempt was made to establish equilibrium conditions for the drug binding.

Table 1. The release of acid phosphatase activity (μmoles *p*-nitrophenol released/mg protein/30 min) from washed * fractions of rat liver homogenate after osmotic shock and the effects of 10^{-5} M 153C51

	600 *g* pellet	3500 *g* pellet	3500 *g* supernatant
No drug	0.24 ± 0.09	0.23 ± 0.15	0.79 ± 0.20
Incubated + drug	0.20 ± 0.09	0.12 ± 0.05†	0.75 ± 0.24
Assayed + drug	0.25 ± 0.09	0.20 ± 0.11	0.75 ± 0.18
Total activity after Triton X-100 treatment	1.50 ± 0.35	0.98 ± 0.30	1.45 ± 0.24
μg Protein/sample analysed (100 μl)	541 ± 102	1797 ± 511	450 ± 78

* The 600 *g* and 3500 *g* pellets were dispersed and washed in a volume of buffer equal to that in which they were originally suspended and re-centrifuged.

† Significantly lower than control value ($P < 0.05$). Values were derived from three experiments.

Table 2. The release of lysosomal enzymes from rat liver after osmotic shock: The effects of various concentrations of 153C51

Drug Concn	Per cent of control (untreated) activity released		
	Acid phosphatase	β-*N*-Acetyl-glucosaminidase	Aryl-sulphatase
10^{-3} M	69	57	61
10^{-4} M	50	43	29
10^{-5} M	45	35	19
10^{-6} M	50	48	47
10^{-7} M	98	100	100
10^{-8} M	105	100	102
Control value:	0.42 ± 0.18 μmoles PNP/mg protein/ 30 min	0.23 ± 0.16 μmoles PNP/mg protein/ 30 min	27.3 ± 15.0 μg *p*-nitrocatechol/ mg protein/30 min

(All drug values are derived from the mean of at least 3 separate experiments).

Comparison of the effects of 153C51 and chloroquine on the release and/or activity of aryl sulphatase from rat liver lysosomes revealed that 153C51 was a more potent drug in this respect (Table 4). A concentration of 10^{-5} M chloroquine was without effect whilst at 10^{-3} M it resulted in a yield of 59 per cent of control activity. At 10^{-5} M 153C51 yielded only 35 per cent of control activity. Combinations of chloroquine and 153C51 were no more effective than 153C51 alone. The results obtained with chloroquine alone were in good agreement with those reported by Smith *et al.* [12].

DISCUSSION

The schistosomicide 153C51 had a very similar stabilising effect to chloroquine on the mammalian membrane systems examined in this study and had a direct inhibitory effect on two lysosomal enzymes. These biochemical observations help to explain the accumulation of residual lysosomes in the tegument of schistosomes treated with 153C51 assuming that the lysosomes of that tissue react in the same way as rat liver lysosomes. In practice, it would be difficult to examine the effects of 153C51 on isolated, intact, tegument lysosomes since the available techniques for stripping the tegument clear of the underlying parenchyma involve the chemical or physical destruction of membrane integrity. A study of lysosomes prepared from homogenates of whole schistosomes would be likely to yield misleading results since dissimilar effects of a drug on lysosomes from different tissues of the same organism are known to occur [19] and only the lysosomes of the tegument are visibly affected by 153C51 [1].

The effects of both 153C51 and chloroquine on lysosomes are probably related to certain molecular properties which they share with many drugs which are known to cause the abnormal storage of polar lipids in lysosomes [20]. All the compounds in question are amphiphilic and have basic nitrogen groups with the capacity to form cations under appropriate pH conditions, depending on their individual pK_a's. Therefore, the lipophilicity and membrane permeability coefficients of the ionised and un-ionised forms of these compounds are vital factors in determining the effects on membranes in general and on lysosome structure and function in particular [21, 22]. Thus 153C51,

Table 3. The effect of 153C51 on the release and activity of rat liver lysosomal enzymes. Values are μmoles PNP released/mg protein/30 min

	Drug concn 10^{-5} M	10^{-4} M	10^{-3} M
Acid phosphatase			
No drug (control)	0.36 ± 0.02	0.33 ± 0.2	0.71 ± 0.02
Incubated + drug	0.16 ± 0.01*	0.19 ± 0.06*	0.36 ± 0.07*
Assayed + drug	0.36 ± 0.03	0.32 ± 0.04	0.53 ± 0.11‡
β-Acetylglucosaminidase			
No drug (control)	0.17 ± 0.01	0.12 ± 0.03	0.31 ± 0.02
Incubated + drug	0.04 ± 0.02*	0.07 ± 0.02*	0.14 ± 0.05*
Assayed + drug	0.12 ± 0.01*	0.09 ± 0.01‡	0.23 ± 0.03†

Protein/100 μl lysosome preparation analyzed = 1694 ± 339 μg [7]. Enzyme activity values were routinely derived from the means of three experiments.

* Significantly different from control, $P < 0.001$.

† Significantly different from control, $P < 0.01$.

‡ Significantly different from control, $P < 0.05$.

Table 4. The effects of chloroquine and 153C51 on the release of aryl sulphatase from a rat liver lysosome preparation

Drug treatment	Enzyme activity released (μg *p*-nitrocatechol formed/ mg protein/30 min)	% Control activity
Control (no drug)	10.15 ± 1.01*	—
10^{-5} M 153C51	3.53 ± 0.15	35
10^{-5} M Chloroquine	10.41 ± 0.59	103
10^{-5} M 153C51 + 10^{-5} M Chloroquine	3.85 ± 0.46	38
Control (no drug)	16.67 ± 2.51†	—
10^{-5} M 153C51	5.91 ± 2.85	35
10^{-3} M Chloroquine	9.80 ± 0.79	59
10^{-5} M 153C51 + 10^{-3} M Chloroquine	4.56 ± 1.36	27

* 29% of total activity (35.52 ± 5.34).
† 31% of total activity (53.54 ± 0.70).
All results are mean ± S.E. of 4 experiments.

which has a pK_a of 4.5, would be expected to permeate freely the lipid phase of biological membranes at neutral pH since over 99 per cent of the amine would be in the un-ionised form (Table 5). This explains the non-saturable association of [^{3}H]-153C51 with the rat liver lysosome preparation at pH 7.4 (Fig. 2) during a 5 min incubation. Under these conditions the solvent capacity of the lipid phase was obviously not exceeded even at 10^{-6} M. The ionisation data also indicate that it must have been the un-ionised form of 153C51 which was responsible for lysosome and erythrocyte stabilisation and explain why variation in pH between the limits 6.0–8.0 did not change the effects on erythrocytes (Fig. 1). Clearly, the compound would be almost completely in the un-ionised form throughout this range (Table 5). Certain other drugs do show pH-dependent effects on erythrocyte stability in this same range which may be explained by their dissociation characteristics [10, 23].

It is apparent from Fig. 2 that 153C51 is capable of association with lysosomal constituents of the rat liver cell *in vitro* and it may be assumed on the basis of the ultrastructural effects of this drug on *Schistosoma mansoni* [1] and the association of radioactive drug with schistosomes in the mouse [17] that the same may be true of the parasite's tegument lysosomes. Once within the lysosome, the drug would become protonated at the low intra-lysosomal pH—thought to be around 4.5 [21]—which is close to the pK_a of 153C51, and would potentially be able to complex with other suitably charged lysosomal constituents such as polar lipids [22]. So long as the intra-lysosomal pH could be maintained, it is possible to envisage a continual gradient encouraging the concentration of drug and drug complexes within the lysosome which would clearly

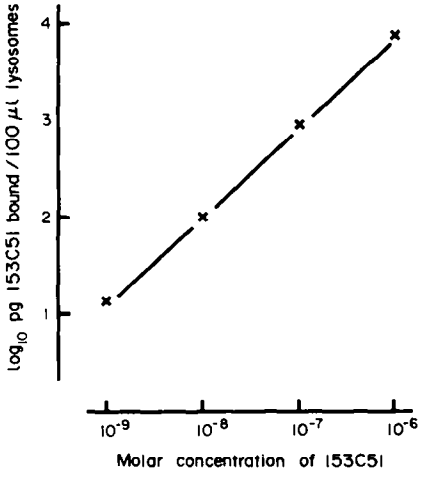
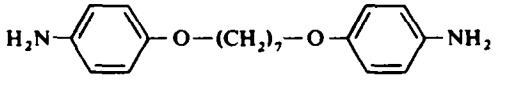


Fig. 2. Binding of [G-^{3}H] 153C51 to rat liver lysosomes (see Methods for technique). The protein concentration of the lysosome preparations was 2,008 ± 255 μg/100 μl sample. Four separate preparations were used and each examined in triplicate at all drug concentrations. The coefficient of variation about the mean at each concentration for the four experiments varied from 15 to 54%.

Table 5. The ionisation of 153C51 at various pH values. Figures are derived from the Henderson–Hasselbach equation

pH	% Ionised
3.0	97
4.0	76
4.5	50
5.0	24
5.5	9.1
6.0	3.0
6.5	0.9
7.0	0.3
8.0	<0.1
9.0	<0.1



The amino groups of 153C51 are protonated almost simultaneously. The pK_a at 40° is approximately 4.5 as determined spectroscopically (Personal communication, Dr. J. G. Vinter).

disturb the intra-lysosomal environment. This hypothesis might have particular relevance to the schistosome tegument which is known to be rich in phospholipid [24].

Amphiphilic drugs which are rapidly metabolised to more polar compounds e.g. by ring hydroxylation, are more or less devoid of the capacity for causing abnormalities in the lysosomal storage of biochemicals [20]. Goodwin *et al.* [15] found that diaminodiphenoxyalkanes such as 153C51 were without either retinotoxic or schistosomicidal activity if hydroxyl groups were substituted into the benzene rings. Therefore, in this chemical series, it would seem that the higher pK_a caused by the substitution (by analogy with aniline) and the expected enhancement of the intra-lysosomal effects must be outweighed by unfavourable changes in lipophilicity, membrane permeability or absorption from the gut of the host. On the basis of these observations, it may yet be possible to derive an effective orally-administered diaminodiphenoxyalkane which, although resulting in the death of schistosomes in the mesenteric blood plexus, would be metabolised in the liver into a non-toxic molecule before reaching the systemic circulation of the host animal.

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REFERENCES

1. S. D. M. Watts, A. Orpin and C. MacCormick. *Parasitology* **78**. 287 (1979).
2. D. Warhurst and D. J. Hockley, *Nature* **214**, 935 (1967).
3. R. Abraham, R. J. Hendy and P. Grasso, *Exp. Mol. Pathol.* **9**, 212 (1968).
4. Z. Hruban, A. Slesers and E. Hopkins, *Lab. Invest.* **27**, 62 (1972).
5. W. K. Read and W. W. Bay, *Lab. Invest.* **24**, 246 (1971).
6. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Critical Rev. Toxicol.* **4**, 185 (1975).
7. M. E. Fedorko, J. G. Hirsch and Z. A. Cohn, *J. Cell Biol.* **38**, 377 (1968).
8. G. Weissman, in *Lysosomes in Biology and Pathology* (Eds. J. T. Dingle and H. B. Fell) Vol. I, p. 276. North Holland, Amsterdam (1969).
9. D. Reijngoud and J. M. Tager. *Biochim. Biophys. Acta* **472**. 419 (1977).
10. A. D. Inglot and E. Wolna, *Biochem. Pharmac.* **17**, 269 (1968).
11. L. J. Ignarro, *Biochem. Pharmac.* **20**, 2847 (1971).
12. R. J. Smith, C. Sabin, H. Gilchrest and S. Williams, *Biochem. Pharmac.* **25**, 217 (1976).
13. R. Abraham and R. J. Hendy, *Exp. Mol. Pathol.* **12**, 185 (1970).
14. R. W. Young and D. Bok, *J. Cell Biol.* **42**, 392 (1969).
15. L. G. Goodwin, W. H. G. Richards and V. Udall, *Br. J. Pharmac.* **12**, 468 (1957).
16. P. Seeman and J. Weinstein, *Biochem. Pharmac.* **15**, 1737 (1966).
17. S. D. M. Watts, *Comp. Biochem. Physiol.* **60**, 109 (1978).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
19. L. J. Ignarro, *Biochem. Pharmac.* **20**, 2861 (1971).
20. H. Lüllmann and R. Lüllmann-Rauch, In *Drug Design and Adverse Reactions*, Alfred Benzon Symposium X, Munksgaard, P. 29. Academic Press, London.
21. C. De Duve, T. de Barsy, B. Poole, A. Trouet, P. Tulkens and F. Van Hoof, *Biochem. Pharmac.* **23**, 2495 (1974).
22. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).
23. P. Seeman, *Biochem. Pharmac.* **15**, 1753 (1966).
24. P. R. Wheater and R. A. Wilson, *Parasitology* **72**, 99 (1976).