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DIFFERENTIAL EFFECTS OF CHLORAMPHENICOL AND ITS NITROSOANALOGUE ON PROTEIN SYNTHESIS AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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Abstract—Protein synthesis and oxidative phosphorylation were chosen as measures to study the differences between the effects of chloramphenicol (CAP) and its derivative nitroso-chloramphenicol (NO-CAP) on rat liver mitochondria. [^{14}C]Leucine incorporation into mitochondrial protein was inhibited 83 per cent by 30 μM CAP and was equally inhibited by a similar concentration of thiamphenicol; 30 μM NO-CAP, however, inhibited [^{14}C]leucine incorporation only 34 per cent and 30 μM nitrosobenzene had no effect. A millimolar concentration of CAP was required to inhibit oxidative phosphorylation, whereas 75 μM NO-CAP was inhibitory. Unlike CAP, NO-CAP at 100 μM slightly inhibited state 4 respiration with glutamate as substrate, but slightly activated it with succinate. Respiratory state 3 with glutamate was completely inhibited by 75 μM NO-CAP, whereas the same concentration of CAP was only 10 per cent inhibitory. With succinate, 250 μM NO-CAP was required to inhibit state 3, whereas 600 μM CAP had no effect. The uncoupled state triggered by 2,4-dinitrophenol in the presence of either glutamate or succinate was inhibited totally by NO-CAP, but not by CAP. The inhibition by NO-CAP was mitochondrial protein dependent, for more NO-CAP was required for inhibition with a larger amount of protein. NO-CAP effects could be prevented or released by cysteine, but not by washing. Oxidative phosphorylation was also inhibited by another nitroso compound, nitrosobenzene, which, however, did not affect mitochondrial protein synthesis. The results indicate that, unlike CAP, NO-CAP is a potent inhibitor of the energy conserving mechanism.

The clinical use of chloramphenicol (CAP)‡ is commonly associated with a dose-dependent, reversible bone marrow suppression and rarely with irreversible bone marrow aplasia. Reversible marrow suppression from CAP is a consequence of mitochondrial injury [see Ref. 1 for review]. At the subcellular level the effect of CAP is a function of its concentration in the reaction medium. At therapeutic levels it inhibits mitochondrial protein synthesis by blocking peptidyl transferase [2, 3] whereas at millimolar levels (10- to 15-fold therapeutic levels) other metabolic processes such as site 1 of the respiratory chain [4, 5] and DNA synthesis [6–8] are inhibited. Furthermore, CAP has been reported to inhibit other activities in mitochondria [9, 10] and chloroplasts [11, 12].

The pathogenesis of CAP-induced bone marrow aplasia remains uncertain. The extensive clinical use of the CAP analogue, thiamphenicol, in Europe and the Far East without any associated cases of aplastic anemia has reopened the question of the structure–toxicity relationship in the CAP molecule. Furthermore, recent comparative studies on the two analogues suggest that the *p*-NO_2 group may somehow be related to the development of aplastic anemia from CAP [7, 13].

Bioreduction of CAP and other nitrocompounds in various tissues has been clearly demonstrated [14–16]. Nitroso and hydroxylamine groups are presumed to be among the reduction intermediates and could, therefore, play an important role in CAP toxicity *in vivo*. The recent success in the preparation of nitroso-chloramphenicol (NO-CAP) by Corbett and Chippo [17] has made possible a direct examination of the metabolic effects of these intermediates compared to CAP, the parent molecule. We compared the effects of CAP and NO-CAP on mitochondrial protein synthesis and oxidative phosphorylation. The results indicate that NO-CAP is less effective than CAP as an inhibitor of protein synthesis but is much more potent as an inhibitor of the energy conserving mechanism.

METHODS

Isolation of mitochondria. Rat liver mitochondria were isolated according to the method of Weinbach [18], in a homogenizing medium consisting of 250 mM sucrose, 10 mM Tris–HCl, and 1 mM EDTA, pH 7.4

Mitochondrial protein synthesis. Protein synthesis was carried out in a metabolic shaker at 30° in the following medium: 50 mM bicine, 15 mM P_i, 10 mM succinate, 1 mM EDTA, 50 mM KCl, 5 mM ATP, 5 mM nicotinamide, 6 mM $MgCl_2$, 50 units penicillin G/ml, adjusted to pH 7.4 with KOH, 50 μg of a

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‡ Abbreviations: CAP, chloramphenicol; M, mitochondria; NO-CAP, nitroso-chloramphenicol; NO-Benz, nitrosobenzene; PABA, *p*-aminobenzoic acid; TAP, thiamphenicol; and DNP, 2,4-dinitrophenol.

complete amino acid mixture minus leucine as described by Roodyn *et al.* [19], and 2.5–3.5 mg of mitochondrial protein. All materials used to measure mitochondrial protein synthesis were sterilized either by autoclaving or by filtration through Millipore filters with a pore size of 0.45 μm. Other procedures were as described [20]. Synthesis of mitochondrial protein was unaffected by 300 μg cycloheximide/ml.

Respiration and oxidative phosphorylation. Determinations were by oxypolarography using a Clark electrode and oxygen monitor (YSI, Yellow Springs, OH), and a standard incubation medium consisting of 24 mM glycylglycine, 87 mM sucrose, 60 mM KCl, and 9 mM $MgCl_2$, pH 7.4. State 4 and state 3 are the metabolic states defined by Chance [21]. Uncoupled state is the state of mitochondria in the presence of an uncoupler such as 2,4-DNP.

Protein. Determination was by the biuret method [22]; bovine serum albumin (fraction V) was used as a standard.

RESULTS

Effects of CAP and NO-CAP on mitochondrial protein synthesis. Table 1 shows that both CAP and thiamphenicol (TAP) in a concentration of 30 μM caused over 80 per cent inhibition of [^{14}C]leucine incorporation into mitochondrial protein. However, the same concentration of NO-CAP produced only 34 per cent inhibition, and NO-Benz, which is part of the NO-CAP molecule (cf. Fig. 1), did not inhibit incorporation at all.

Effects of CAP and NO-CAP on oxidative phosphorylation. Figure 1 shows oxypolarographic traces of rat liver mitochondria preincubated for 2.5 min with 100 μM CAP, NO-CAP and other related compounds. In accordance with previous results [20], therapeutic concentrations of CAP did not affect either state 4/state 3 transition or uncoupling by DNP (Fig. 1B). When NO-CAP was used under the same conditions, however, it inhibited completely both transition and uncoupling (Fig. 1C). NO-Benz, which has a nitroso radical in common with NO-CAP, also inhibited completely state 3 and the uncoupling state (Fig. 1D). On the other hand, parallel assays showed that TAP, which differs from chloramphenicol by substitution of the *p*-nitro group with a methylsulfonyl moiety, and *p*-aminobenzoic acid, which has an aminobenzene moiety, did not inhibit oxidative phosphorylation [Fig. 1 (E and F)]; neither did the *p*-amino analogue of CAP tested at 100 μM (not shown).

Table 1. Inhibition of mitochondrial protein synthesis by CAP and NO-CAP compared to inhibition by thiamphenicol and nitrosobenzene*

	cpm/mg protein	% Inhibition
Control	5809 ± 143 (6)	
CAP	985 ± 71 (5)	83
TAP	952 ± 26 (3)	84
NO-CAP	3866 ± 166 (5)	34
NO-Benz	5842 ± 255 (5)	0

* Inhibitors were added at a concentration of 30 μM, equivalent to almost 10 μg/ml in the case of CAP or NO-CAP. After 60 min of incubation with 1 μCi of [^{14}C]-L-leucine (sp. act. 339 mCi/mmole), samples of 0.5–0.6 mg of mitochondrial protein were applied per paper disc, washed, and counted (see Methods). Numbers in parentheses = number of assays ± S.E.

To provide further insight into the difference between the actions of CAP and NO-CAP on oxidative phosphorylation, we examined the effect of the drug concentration on state 4, state 3, and the uncoupled state by using two different substrates: glutamate and succinate, NAD- and FAD-linked substrates, respectively. Figure 2 indicates that, in

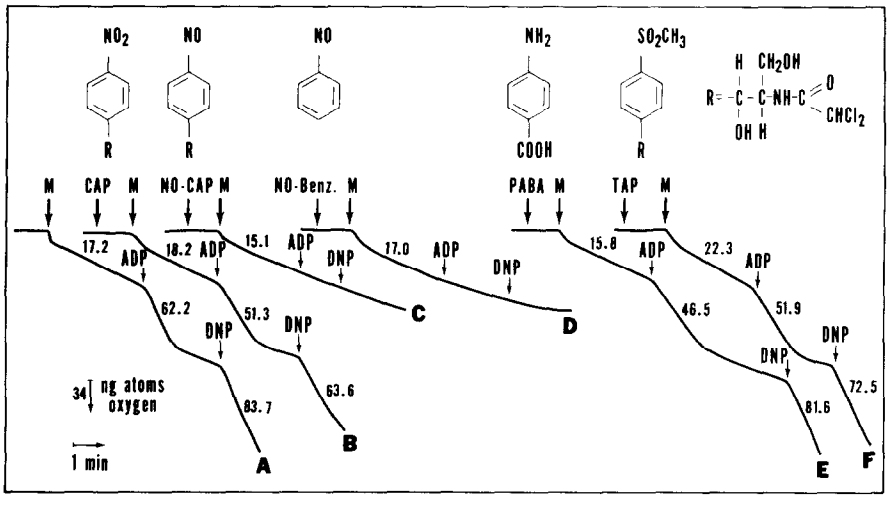

Fig. 1. Effects of chloramphenicol (CAP), nitroso-chloramphenicol (NO-CAP), nitrosobenzene (NO-Benz), *p*-aminobenzoic acid (PABA) and thiamphenicol (TAP) on the transition state 4/state 3 and uncoupling in mitochondria, determined by oxypolarography. Mitochondria (1 mg protein/2 ml) were incubated for 2.5 min at 30° in the standard reaction medium (see Methods) with 10 mM glutamate, 5 mM phosphate, and, when present, 100 μM of each reagent. ADP (200 nmoles) and DNP (0.18 mM) were added as indicated. The numbers along the traces give the initial rates of respiration in ng-atoms $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$. Plot A is a control. The formula of each added reagent is shown.

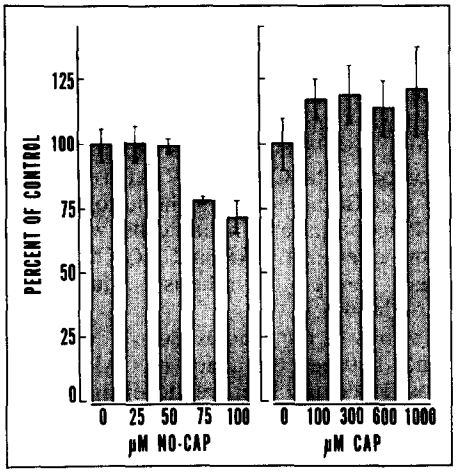

Fig. 2. Effect of CAP and NO-CAP concentrations on mitochondrial respiration in the presence of glutamate. Mitochondria (0.75–1 mg protein) were incubated as in Fig. 1, with various concentrations of drugs. The results are expressed as percentages of control.

the presence of 0.75–1 mg mitochondrial protein, 75 μM NO-CAP (which totally inhibited phosphorylation under similar conditions) slightly inhibited respiration with glutamate. In contrast, 1 mM CAP did not inhibit respiration, rather, a slight respiratory activation was observed; this is presumably due to the uncoupling action of CAP as has been suggested previously [9]. When succinate was the oxidizable substrate (Fig. 3), CAP slightly increased repiration as it did with glutamate; but NO-CAP, in contrast to its effect on glutamate oxidation, caused an activation of state 4 that did not seem to be concentration dependent.

Figure 4 shows the inhibition of the transition state 4/state 3 by CAP and NO-CAP in the presence of either glutamate or succinate. Respiratory state 3 with glutamate was completely inhibited by 75 μM NO-CAP, whereas the same concentration of CAP was only 10–15 per cent inhibitory. To have complete inhibition of state 3 with glutamate, a millimolar concentration of CAP was needed (Fig. 4). With succinate as substrate, 250 μM NO-CAP was

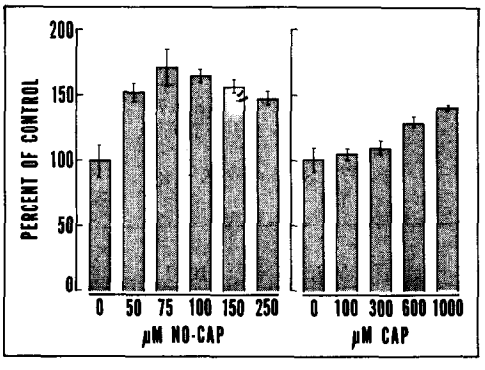

Fig. 3. Effect of CAP and NO-CAP concentrations on mitochondrial respiration in the presence of succinate. Conditions were as in Fig. 2, but with 10 mM succinate instead of glutamate.

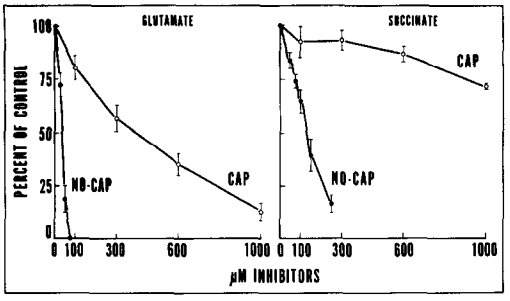

Fig. 4. Inhibition of transition state 4/state 3 by CAP and NO-CAP with 10 mM glutamate or succinate as substrate. Experimental conditions were as in Fig. 1.

required to produce 85 per cent inhibition of state 3, while 1 mM CAP only slightly affected that state. This result is in agreement with previous work [4] where it was shown that millimolar concentrations of CAP inhibit only site 1 of the electron transport chain.

The uncoupled state of mitochondria in the presence of 2,4-dinitrophenol with either glutamate or succinate as substrate was affected by CAP and NO-CAP in a pattern similar to their action on state 3. At low concentrations, NO-CAP completely inhibited the uncoupling effect of 2,4-DNP, whereas higher concentrations of CAP were required either to inhibit with glutamate or to activate slightly with succinate (Fig. 5).

Table 2 shows that the inhibition of state 3 and of the uncoupled state by NO-CAP was mitochondrial protein dependent. When 0.75 mg protein was used per assay, complete inhibition was observed at 75 μM NO-CAP, but when 2.25 mg protein was used, higher NO-CAP concentrations were required to produce a similar inhibition. This protein concentration dependence was less evident with CAP.

Influence of cysteine and of washing on the inhibition caused by NO-CAP. The addition of cysteine to the reaction medium after the mitochondria had been added did not influence their respiration, but it prevented the inhibition caused by NO-CAP. As shown in Fig. 6A, the addition of ADP and of DNP after that of cysteine and NO-CAP resulted in the release of the oxygen consumption as with the control. Furthermore, when 250 μM cysteine was added to the medium after preincubation of mitochondria

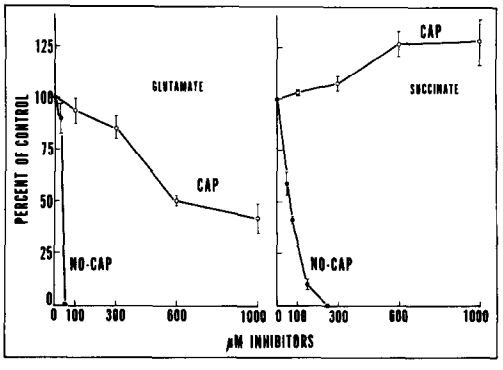

Fig. 5. CAP and NO-CAP inhibition of 2,4-dinitrophenol uncoupling. Conditions were as in Fig. 1.

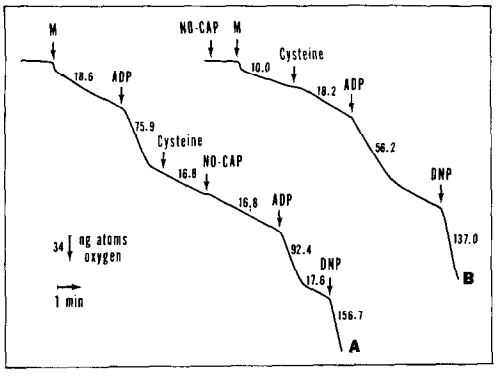

Fig. 6. Prevention and reversal of the inhibition caused by NO-CAP. Conditions were as in Fig. 1, with 1.2 mg of mitochondrial protein. NO-CAP was 125 μM and cysteine 250 μM. The numbers on the traces are the initial rates of respiration in ng-atoms $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.

with 125 μM NO-CAP, the inhibition of state 4, state 3, or the uncoupled state was reversed, as shown by addition of ADP and DNP (Fig. 6B).

When rat liver mitochondria were incubated with inhibitory concentrations of NO-CAP, then isolated from the reaction medium by rapid centrifugation and washed as indicated in Table 3, the inhibition of either state 3 or the uncoupled state was not reversed. This result shows that, unlike what is observed with CAP [23], inhibition by NO-CAP is not removed by washing.

DISCUSSION

This study was prompted by clinical observations with the CAP analogue TAP, which differs from CAP by substitution of the *p*-NO_2 group with a methylsulfonyl(—SO_2CH_3) moiety. TAP produces dose-dependent reversible bone marrow suppression as readily as CAP does, but in spite of its extensive use, there have been no documented cases of aplastic anemia in association with it. On the basis of these observations and our comparative studies with two drugs, we have postulated that some of the severe hematologic toxicity, such as aplastic anemia, that occurs in association with CAP may be related to an *in vivo* bioreduction of the *p*-NO_2 to toxic intermediates (nitroso, N=O; hydroxylamine, —NHOH). Our initial studies [13] demonstrated that, compared to CAP, NO-CAP is much more toxic *in vitro*. Because the mitochondrion is the target for CAP action in mammalian cells, we have compared the effects of CAP and NO-CAP on mitochondrial protein synthesis and oxidative phosphorylation.

The results of our studies clearly demonstrate a difference between the action of CAP and that of its nitroso analogue. Therapeutic levels of CAP (10–20 μg/ml) inhibited mitochondrial protein synthesis by over 80 per cent, whereas comparable levels of NO-CAP were considerably less inhibitory. The binding site of CAP with bacterial ribosomes has been shown to involve the propanediol portion of the molecule [24–26]. Because the propanediol moiety remains unaltered in NO-CAP, it is possible that the difference between the two compounds is in the permeability across the inner mitochondrial membrane limiting access of the NO-CAP to mitochondrial ribosomes.

In sharp contrast to their effects on mitochondrial protein synthesis, NO-CAP, unlike CAP, is a potent inhibitor of mitochondrial respiration and phosphorylation. At a concentration as low as 75 μM, NO-CAP completely inhibited ATP formation, as measured by oxypolarography. The inhibition was mitochondrial protein dependent, such that at lower concentrations of mitochondria less NO-CAP was required to cause complete inhibition.

The inhibition of mitochondrial respiration by NO-CAP was not reversed by washing but was prevented by the prior addition of cysteine. Furthermore, the addition of 250 μM cysteine after NO-CAP resulted in reversal of inhibition. Since nitroso groups have been recently reported to form conjugates with glutathione [27], our results suggest that NO-CAP may interact with some highly reactive –SH groups of the

Table 2. Mitochondrial protein dependence of the inhibition of oxidative phosphorylation by CAP and NO-CAP*

	0.75 mg Mitochondrial protein/assay				2.25 mg Mitochondrial protein/assay		
	NO-CAP (μM)						
	25	50	75	100	50	75	150
State 3	73 ± 6 (5)	19 ± 6 (3)	0 (5)	0 (3)	59 ± 18 (3)	48 ± 7 (4)	8 ± 0.5 (3)
Uncoupled state	90 ± 7 (3)	0 (3)	0 (5)	0 (3)	97 ± 8 (3)	63 ± 18 (3)	0 (3)
	CAP (μM)						
	100	300	600	1000	600	1000	
State 3	81 ± 5 (6)	57 ± 5 (5)	36 ± 4 (5)	13 ± 4 (5)	51 ± 6 (3)	24 ± 6 (4)	
Uncoupled state	94 ± 6 (5)	86 ± 5 (4)	51 ± 2 (3)	42 ± 7 (5)	61 ± 3 (3)	40 ± 5 (4)	

* Results are expressed as percentages of control values. Numbers in parentheses = number of assays ± S.E.

Table 3. Influence of washing on the inhibition of state 3 and uncoupled state caused by NO-CAP*

Mitochondria preincubated with NO-CAP, then isolated and washed	No wash†	One wash	Two washes
	Total inhibition		
State 3	+	+	+
Uncoupled state	+	+	+

* Mitochondria (1.5 mg protein) were incubated in the standard reaction medium with 10 mM glutamate, 5 mM P_i and 125 μM NO-CAP. After 2.5 min, the mitochondria were isolated by rapid centrifugation using an Eppendorf centrifuge 5412 for 2 min. The pellet was either resuspended in the reaction medium and tested at the oxypolarograph (one wash), or resuspended in the homogenizing medium (see Methods), then isolated again by rapid centrifugation and tested as above (two washes). Controls without NO-CAP were performed under the same conditions of isolation and washing.

† Mitochondria were tested as mentioned above prior to isolation and washing.

inner mitochondrial membrane, thereby blocking the energy conserving mechanism.

Although the exact mechanism of NO-CAP toxicity at the cellular level remains uncertain, our data suggest that the toxic manifestations that occur in cell culture [7] are, at least in part, a consequence of inhibition of the energy transducing mechanism.

REFERENCES

1. A. A. Yunis, in *The Year in Hematology* (Eds. R. Silber, J. LaBue and A. S. Gordon), p. 143. Plenum, New York (1978).
2. H. K. Das, A. Goldstein and L. C. Kanner, *J. molec. Pharmac.* **2**, 158 (1966).
3. M. J. Weber and J. A. Demoss, *Proc. natn. Acad. Sci. U.S.A.* **55**, 1224 (1966).
4. K. B. Freeman and D. Halder, *Can. J. Biochem.* **46**, 1003 (1968).
5. F. C. Firkin and A. W. Linnane, *Biochem. biophys. Res. Commun.* **32**, 398 (1968).
6. A. A. Yunis, D. R. Manyan and G. K. Arimura, *J. Lab. clin. Med.* **81**, 713 (1973).
7. A. A. Yunis, A. M. Miller, Z. Salem, M. D. Corbett and G. K. Arimura, *J. Lab. clin. Med.*, in press.
8. K. B. Freeman and H. Patel, *Molec. Pharmac.* **13**, 504 (1977).
9. J. B. Hanson, C. D. Stoner and T. K. Hodges, *Nature, Lond.* **203**, 258 (1964).
10. L. Tarago-Litvak, C. Desgranges, A. Araya and S. Litvak, *Eur. J. Biochem.* **93**, 271 (1979).
11. R. J. Ellis, *Science* **163**, 477 (1969).
12. O. Wara-Aswapati and J. W. Bradbeer, *Plant Physiol.* **53**, 691 (1974).
13. A. A. Yunis, in *Aplastic Anemia* (Eds. S. Hibino, F. Takaku and N. T. Shahide), p. 321. University of Tokyo Press, Tokyo (1978).
14. G. N. Smith and C. S. Worrel, *Archs. Biochem.* **28**, 232 (1950).
15. J. R. Fouts and B. B. Brodie, *J. Pharmac. exp. Ther.* **119**, 197 (1957).
16. C. Y. Wang, C. W. Chin and G. T. Bryan, *Biochem. Pharmac.* **24**, 1563 (1975).
17. M. D. Corbett and B. R. Chipko, *Antimicrob. Agents Chemother.* **13**, 193 (1978).
18. E. C. Weinbach, *Analyt. Biochem.* **2**, 235 (1961).
19. D. B. Roodyn, P. J. Reis and T. S. Work, *Biochem. J.* **80**, 9 (1961).
20. S. Abou-Khalil, Z. Salem and A. A. Yunis, *Am. J. Hemat.* **8**, 71 (1980).
21. B. Chance, in *Ciba Foundation Symposium on Regulation of Cell Metabolism.*, p. 91. Churchill, London (1959).
22. G. Allan, A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
23. T. F. McLeod, D. R. Manyan and A. A. Yunis, *J. Lab. clin. Med.* **90**, 347 (1977).
24. K. B. Freeman. *Can. J. Biochem.* **48**, 469 (1970).
25. D. R. Manyan, G. K. Arimura and A. A. Yunis, *Molec. Pharmac.* **11**, 520 (1975).
26. T. R. Tritton, *Archs Biochem. Biophys.* **197**, 10 (1979).
27. P. Eyer, *Chem. Biol. Interact.* **24**, 227 (1979).