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INHIBITION OF ADENYLATE CYCLASE AND ATPASE ACTIVITIES FROM RAT LIVER PLASMA MEMBRANE BY HEXACHLOROPHENE*

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Abstract—Adenylate cyclase activity from a rat liver plasma membrane preparation purified according to Neville's procedure was inhibited *in vitro* by low concentrations (1 μM to 0·1 mM) of hexachlorophene. Complete inhibition was obtained with 1 mM hexachlorophene. Similar effects were observed whether the activity was assayed in the presence of 10 μM GTP, 0·1 μM glucagon, 10 mM NaF or without any addition. The effect of hexachlorophene was not due to inhibition of the regenerating system present in the incubation medium, since the effect of the drug was preserved in its absence. The inhibition brought about by hexachlorophene was not reversed by increasing the concentration of the substrate ATP–Mg. The inhibition was immediate and irreversible spontaneously; it was not affected by the simultaneous addition of 2-mercaptoethanol. Hexachlorophene inhibited also the "total" Mg^{2+}-ATPase, as well as its Na^+, K^+-dependent fraction. These results suggest that the interaction of hexachlorophene with the plasma membrane, as reflected in the inhibition of two major enzymes activities, might play a key role in the toxicity of the drug.

Symptoms of poisoning by hexachlorophene† are mainly neurological. However, even in lethal cases, the morphological changes in the brain are limited to a spongy degeneration of the white matter [1] and have yet to be related to precise biochemical alterations. Miller and Buhler [2] recently proposed that hexachlorophene could directly alter the permeability properties of the cellular membrane. We report here that hexachlorophene exerts a potent inhibitory effect upon two plasma membrane enzymes, the ATPase and adenylate cyclase systems. The data suggest that the interaction between hexachlorophene and membranes, and particularly in plasma membranes, might play a pivotal role in its toxicity.

EXPERIMENTAL PROCEDURE

Materials

Hexachlorophene was purchased from Sigma; porcine, crystalline glucagon was purchased from Novo Laboratories; cyclic AMP and creatine phosphate were from Calbiochem. Nucleotides (disodium salts), 2-mercaptoethanol, and bovine serum albumin (fraction V) were obtained from Sigma. Creatine kinase was purchased from Boehringer. NaF and all other chemicals were from Merck. [α-^{32}P] ATP (1·5–1·9 Ci/m-mole) and cyclic [8-^{3}H] AMP (13 Ci/m-mole) were obtained from the CEA (Saclay, France).

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† Abbreviations: cyclic AMP, cyclic adenosine 3′, 5′ monophosphate; hexachlorophene 2,2′-methylene bis (3,4,6-trichlorophenol). Enzymes: creatine kinase, ATP: creatine phosphotransferase (EC 2.7.3.2); ATPase, ATP phosphohydrolase (EC 3.6.1.3); adenylate cyclase, ATP: pyrophosphate-lyase (cyclizing) (EC 4.6.1.1.).

Methods

Plasma membrane preparation. Female, albino, Wistar rats (about 100 g body weight) were used. Liver plasma membranes were prepared according to the procedure devised by Neville [3] up to step 11. The purified membranes were suspended in 1 mM $NaHCO_3$ and stored up to six weeks in liquid nitrogen without any loss of activity of ATPase or of adenylate cyclase. Several batches of liver membranes were used in the experiments reported here and gave similar results. The brain particulate fraction was obtained by centrifugation at 1,500 *g* of a homogenate of the whole rat brain in 3 volumes (w/v) of 1 mM $NaHCO_3$.

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Krishna, Weiss and Brodie [4] as modified by Pohl *et al.* [5] and as previously reported [6, 7]. The assay medium contained 0·5 mM [α-^{32}P] ATP (10^6 counts/min), 2 mM $MgCl_2$ (except when otherwise indicated), 1 mM cyclic AMP, 25 mM Tris–HCl, pH 7·6, an ATP regenerating system consisting of 25 mM phosphocreatine and 1 mg/ml of creatine phosphokinase, and 20–30 μg of membrane proteins in a final volume of 60 μl. Variations from this composition are indicated in the legends to figures. The reaction was initiated by addition of the membranes and was performed for 5 or 10 min in a shaking water bath at 30°. Reactions were terminated by the addition of 200 μl of 20 mM unlabeled ATP, pH 7·6 and rapid cooling at 0°. Under these conditions the degradation of ATP was found to be negligible over a 20 min period. When the effect of glucagon was tested, 0·1 per cent bovine serum albumin was added to the assay mixture. Labeled cyclic AMP was isolated according to Ramachandran [8] and the yield calculated by previous addition of cyclic [8-^{3}H] AMP. Samples were counted after addition of 10 ml Unisolve (Koch–Light), in an Intertechnique SL 30 liquid scintillation counter. Protein was

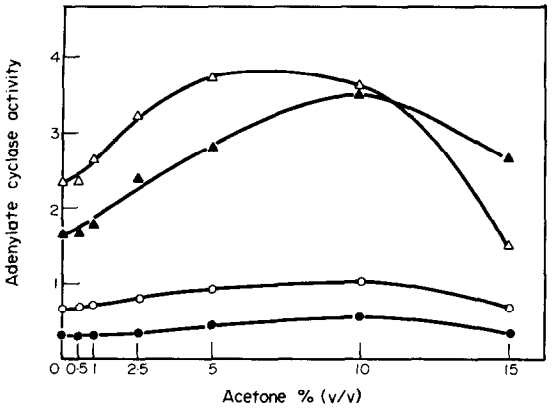

Fig. 1. Effect of acetone on liver adenylate cyclase. Purified membranes from rat liver (20 μg protein per assay) were incubated for 10 min in the presence of increasing concentrations (v/v) of acetone with no other addition (●), or with 10 μM GTP (○), 0·1 μM glucagon (▲), or 10 mM NaF (△). The enzyme activity is expressed as nmoles of cyclic AMP formed in 10 min per mg protein at 30°.

estimated by Lowry's procedure [9], using bovine serum albumin as standard. Results are expressed in nmoles of cyclic AMP formed in 5 or 10 min per mg protein at 30°. The results, obtained from triplicate determinations, agreed within ±5%.

Hexachlorophene was dissolved in acetone, diluted and buffered with NaOH at pH 7·6. The final concentration of acetone in the incubation medium was 5% (v/v) for the highest concentration of hexachlorophene (1 mM) and was lower, in proportion, for the other concentrations tested. Appropriate controls were performed to assess a possible inhibitory effect of the solvent alone upon the cyclase system. As shown in Fig. 1, acetone added to the assay medium up to 10% final concentration, never inhibited but actually increased significantly all the cyclase activities (basal activity and activity assayed in the presence of NaF, GTP or glucagon). The mechanism of this effect of acetone upon adenylate cyclase is unknown.

ATPase assay. "Total" ATPase was assayed using the conditions described by Emmelot and Bos [10] and as previously reported [11]; the medium contained 66 mM NaCl, 33 mM KCl, 5 mM $MgCl_2$, 25 mM Tris pH 7·4, 5 mM ATP pH 7·2 (sodium or Tris salt) and 40 μg protein in a total volume of 1 ml. The Na^+, K^+-independent Mg^{2+} ATPase was tested in the complete absence of sodium and in the presence of 100 mM KCl. The Na^+, K^+-stimulated activity was estimated by difference between these two activities. The reaction, initiated by the addition of the membranes, was performed at 37° for 10 minutes, and was stopped by adding ice-cold trichloroacetic acid (5% final concentration). Protein was removed by centrifugation and inorganic phosphate in the supernatant was estimated by the method of Fiske and Subbarow [12]. Results are expressed in μmoles of P_i liberated in 10 min per mg protein. Each enzyme assay was done in duplicate and found to agree within ± 3%. Unlike its action upon adenylate cyclase, acetone had no activating effect upon ATPase activity. On the contrary, it was slightly inhibitory. The effect of hexachlorophene was corrected with appropriate controls using acetone alone.

RESULTS

Figs. 2a and 2b show that adenylate cyclase from rat brain and liver plasma membranes is extremely sensitive to *in vitro* addition of hexachlorophene. Hexachlorophene, added at the beginning of the incubation, inhibited all the cyclase activities tested (namely the basal activity and the activities stimulated by 10 μM GTP, 0·1 μM glucagon or 10 mM NaF for the liver system; the basal and the NaF stimulated activities for the brain system). The liver basal activity was already slightly inhibited by 0·1 μM hexachlorophene whereas concentrations higher than 10 μM were necessary to inhibit the fluoride, the GTP and the glucagon stimulated activities (Fig. 2a). The apparent I_{50} was 60 μM for the liver enzyme (20 μg

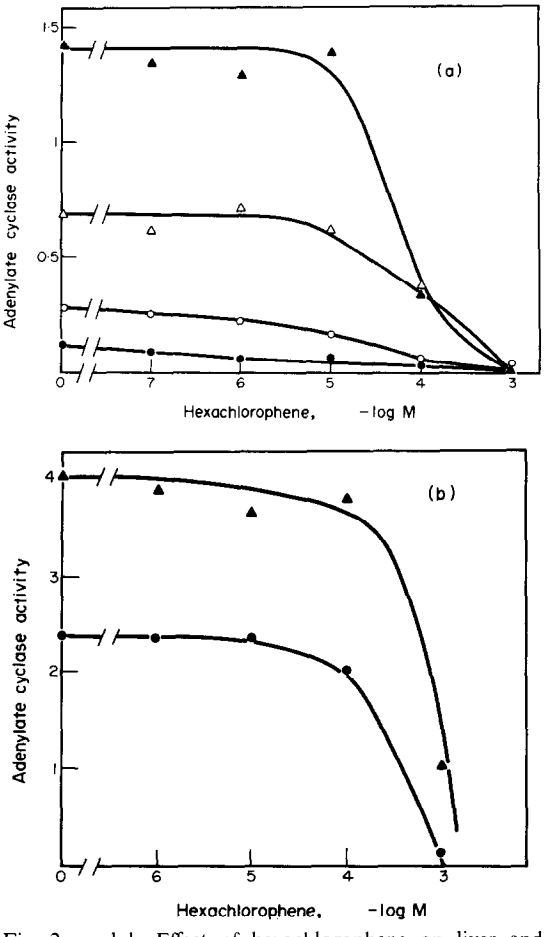

Fig. 2a and b. Effect of hexachlorophene on liver and brain adenylate cyclase. a) Purified membranes from rat liver (20 μg protein per assay) were incubated for 10 min in the presence of increasing concentrations of hexachlorophene with no other addition (●), or with 10 μM GTP (○), 0·1 μM glucagon (△) or 10 mM NaF (▲). Assay conditions were as described in the text. Hexachlorophene was dissolved in acetone, buffered, and diluted to the indicated final concentration so that the final concentration of acetone never exceeded 5% (v/v). Appropriate controls were performed with acetone alone. The enzyme activity is expressed as nmoles of cyclic AMP formed in 10 min per mg protein at 30°. b) A particulate fraction from rat brain (200 μg protein per assay) was incubated as described in the text except that ATP was 3 mM, $MgCl_2$ was 6 mM and that 1 mM EDTA was added. (●) basal activity; (▲) activity assayed in the presence of 10 mM NaF.

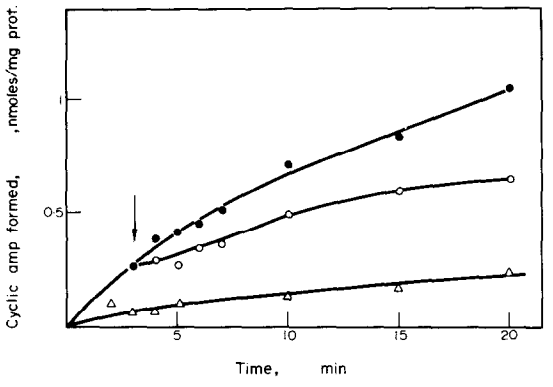

Fig. 3. Time course of the effect of hexachlorophene on rat liver adenylate cyclase. The assay was performed, as described in the text, in the presence of 10 mM NaF. Hexachlorophene (0·1 mM final concentration) was introduced either at zero time (△) or after 3 min of incubation (○). The incubation was continued for varying time periods, as indicated. The control (●) contained no hexachlorophene.

of membrane protein per assay). The inhibition of the brain enzyme required a higher hexachlorophene concentration (Fig. 2b), in accordance with the tenfold higher amount of protein per assay. It should be noted that, unlike acetone, even low concentration of hexachlorophene did not activate the cyclase. At high concentration (1 mM), all the enzyme activities tested were completely inhibited. From these results, it would appear that hexachlorophene exerted its effect directly on the cyclase system and not through any specific hormone receptor or "transducer".

The effect of hexachlorophene was extremely rapid since no lag phase was observed. Preincubation of the membranes with various concentrations of inhibitor were performed for different time periods. Data (not shown) indicated that hexachlorophene reached its inhibiting sites within one min after its addition and that its action was not enhanced by increasing the preincubation period up to 10 min. This was further demonstrated by a time-course study. Fig. 3 depicts such an experiment with the rat liver membranes, where hexachlorophene was added either at the beginning of the incubation or three minutes after. The enzyme was assayed in the presence of 10 mM NaF. The activity assayed in the presence of hexachlorophene was decreased compared with the control at the first time point studied (after 1 min). Furthermore, the same figure shows that the inhibitory action of hexachlorophene was sustained for the whole time period studied (20 min), thus having no tendency to spontaneous reversibility.

The inhibition of adenylate cyclase by hexachlorophene was not due to an effect on the enzyme creatine-kinase which is used together with creatine phosphate as an ATP-regenerating system in the cyclase assay. The enzyme assay was performed, with and without regenerating system, for a 5 min incubation period (Fig. 4). It was verified, that under these conditions, the enzyme activity, tested in the presence of 0·1 μM glucagon or 10 mM NaF, was still linear with time. Similar inhibiting effects of hexachlorophene were obtained on both enzyme activities, whether the regenerating system was present, or not. This indicates that hexachlorophene acts directly on the membrane enzyme and not via the regenerating system.

The inhibitory effect was not altered by addition of 10 mM 2-mercaptoethanol to the assay mixture, suggesting that no reactive-SH groups were involved in the effect of hexachlorophene.

The effect of hexachlorophene was further tested as a function of the substrate concentration. This was performed under the optimal conditions for the enzyme activity, i.e. in the presence of a constant excess (2 mM) of Mg over that of the concentration of ATP, at all the ATP concentrations tested. This procedure keeps all the ATP in the form of an equimolar ATP–Mg complex with a concentration of Mg low enough not to be inhibitory. This would not be the case if the ratio ATP vs Mg was kept constant. This procedure is reported in detail in a previous publication from this laboratory [7]. Under these conditions and in the presence of 10 mM NaF, hexachlorophene appeared to lower the maximal velocity of the reaction while the affinity of the catalytic site for the substrate was even more altered: the apparent K_m for ATP–Mg was 1400 μM in the presence of 0·1 mM hexachlorophene and 110 μM in the presence of 50 μM hexachlorophene, vs 59 μM in its absence (Fig. 5). ATP–Mg exerted no protection against the effect of the inhibitor.

The addition of a large excess of bovine serum albumin has been described to reverse the inhibition brought about by hexachlorophene upon the mitochondrial oxidative phosphorylation [13], indicating that the bound hexachlorophene could be displaced by serum albumin. No such data could be obtained in the rat liver system with up to 5% (w/v) final concentration of bovine serum albumin (Table 1). This could be ascribed to a higher affinity of the binding of the drug to the plasma membrane.

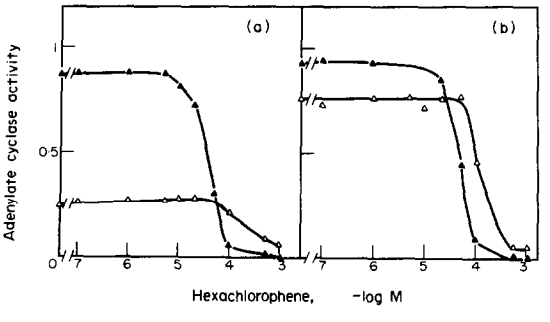

Fig. 4. Effect of hexachlorophene on adenylate cyclase activity assayed in the presence or in the absence of a regenerating system. Liver plasma membranes (20 μg protein per assay) were incubated for 5 min in the presence of 10 mM NaF (▲), or 0·1 μM glucagon (△), and in the absence (Fig. 4a) or in the presence (Fig. 4b) of the regenerating system (creatine kinase–creatine phosphate). The enzyme activity is expressed in nmoles of cyclic AMP formed in 5 min. Basal activity was 0·03 nmoles of cyclic AMP formed in 5 min/mg protein with no regenerating system. The activities tested in the presence of 5% acetone, with no hexachlorophene added and in the presence of 10 mM NaF or 0·1 μM glucagon were respectively 1·8 and 0·35 with no regenerating system and 1·7 and 1·1 in the presence of a regenerating system.

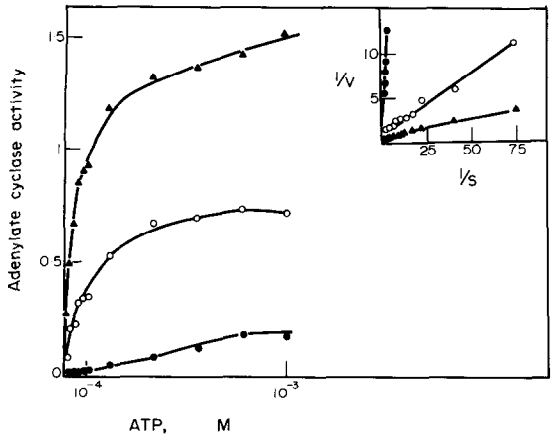

Fig. 5. Effect of varying concentrations of ATP on the effect of hexachlorophene. Liver plasma membranes (22 μg protein per assay) were incubated for 5 min in the presence of 10 mM NaF, with no hexachlorophene added (▲), or with 50 μM hexachlorophene (△), or 0·1 mM hexachlorophene (●). The final concentration of Mg was always higher than that of ATP by a fixed excess (2 mM). The enzyme activity refers to the nmoles of cyclic AMP formed in 5 min. Insert: Lineweaver Burk plot of the data.

A parallel study was performed upon the ATPase activity. As depicted in Fig. 6 hexachlorophene inhibited, in a dose dependent fashion, the two ATPase activities tested, namely the so called 'non specific' Mg^{2+}-ATPase and the Na^+, K^+-stimulated, Mg^{2+}-dependent ATPase. Inhibition was observed at 1 μM and was nearly complete at 0·1 mM hexachlorophene: from the values depicted, an apparent I_{50} of 8 μM was calculated for both activities. ATPase therefore appeared more sensitive to hexachlorophene than the adenylate cyclase system (I_{50} = 60 μM, Fig. 2a).

Since hexachlorophene can act as a detergent, we tried to assess its possible non-specific effect in our system by two procedures: i) Electron microscopic examination of the membranes was performed after incubation *in vitro* for 10 min in the absence or in the presence of hexachlorophene (from 10 μM to 1 mM final concentration). After fixation in 1·5% glutaraldehyde, the membranes were examined at low (× 12,000) and high (× 40,000) magnification. No morphological alteration of the structure of the membranes could be observed, even at high hexachlorophene concentration: ii) A fluorescence study was performed using 8-anilino-1-naphthalene (50 μM final concentration) as external probe. In the presence of the plasma membranes the usual increase in fluorescence of the dye (× 20), and the shift of the emission maximum from 520 nm to 470 nm, occurred; these phenomena were not altered by addition of increasing concentrations of hexachlorophene up to 0·16 mM (final concentration).

DISCUSSION

Although numerous studies [14] have dealt with the toxicity of hexachlorophene, little is known of the molecular mechanism through which hexachlorophene, and related compounds, are toxic. It was reported that hexachlorophene could inhibit the succino-oxidase activity of rat heart and liver [15], alter the permeability of membranes from muscle [16] and

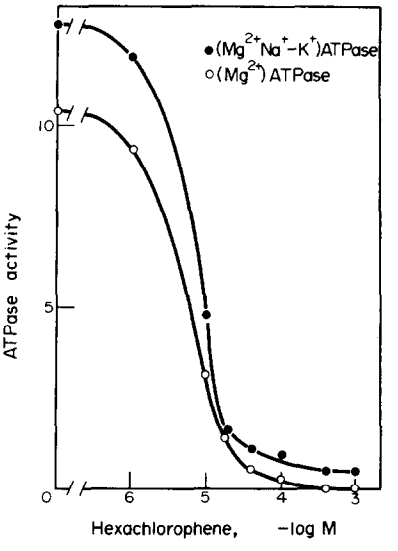

Fig. 6. Effect of hexachlorophene on "total" Mg^{2+}-ATPase (●) and Na^+, K^+-independent Mg^{2+}-ATPase (○). Liver plasma membranes (32 μg protein per assay) were incubated for 10 min as described in the experimental section. Enzyme activity is expressed as μmoles of inorganic phosphate released per mg protein in 10 min at 37°. In the presence of 5% acetone alone, the activities were 8·80 and 6·40 for "total" Mg^{2+}-ATPase and its Na^+, K^+-independent fraction respectively.

Table 1. Effect of increasing concentrations of bovine serum albumin on fluoride activated adenylate cyclase activity, in the presence and in the absence of 0·1 mM hexachlorophene. The enzyme activity is expressed in nmoles of cyclic AMP formed per mg protein in 10 min at 30°.

Bovine serum albumin (final concentration, %, w/v)	Adenylate cyclase activity	
	With 10 mM NaF	With 10 mM NaF + 0·1 mM hexachlorophene
0	0·970	0·140
0·01	0·835	0·170
0·1	0·880	0·170
0·5	0·810	0·200
1·0	0·880	0·160
5·0	0·870	0·180

human erythrocytes [2], and uncouple the phosphorylating respiration in rat liver mitochondria [13, 17, 18]. From these data, it has been suggested that direct interaction of the drug with various cellular membranes could be important in its action [2, 19]. We have demonstrated a strong inhibitory effect of hexachlorophene on two marker enzymes of the liver plasma membrane, namely the ATPase and adenylate cyclase systems. The inhibition was observed with 1 μM hexachlorophene and was complete at 0·1 mM for the ATPase activities, and 1 mM for the adenylate cyclase activities. It is difficult to correlate these effects with the *in vivo* action of the drug. The concentrations of hexachlorophene used to inhibit the membrane activities *in vitro*, in this study as well as in others [2, 13, 17–20], are relatively high, ranging from 1 μM to 100 μM. These concentrations are higher than these reported to be present *in vivo* [20]. It should be noted however that membranes from mitochondria [13] have been shown to bind 2,000 times the amount of hexachlorophene necessary for 50 per cent uncoupling. A similar, tight binding of hexachlorophene was demonstrated in rat liver endoplasmic reticulum [19]. In this system, equilibrium dialysis experiments showed that microsomes could bind a maximum of 300 nmoles of hexachlorophene per mg of microsomal protein [19]. Such a strong interaction of the drug with the membrane is undoubtedly due to binding to multiple sites; of these sites, only a small fraction may be directly related to the enzyme activities studied. Preferential binding of hexachlorophene to membrane constituents probably occurs also *in vivo*, and might therefore explain the apparent discrepancy between the circulating levels of the drug and the concentrations necessary for the effect *in vitro*. Since the plasma membrane is the first to be encountered by hexachlorophene *in vivo*, it is possible that modifications of the biochemical properties of this membrane, in particular altered permeability [2], are responsible for some of the clinical findings during the intoxication.

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REFERENCES

1. R. D. Kimbrouch, *J. Clin. Pharmac.* **13**, 439 (1973).
2. T. L. Miller and D. R. Buhler, *Biochim. biophys. Acta* **352** 86 (1974).
3. D. M. Neville, *Biochim. biophys. Acta* **154**, 540 (1968).
4. G. Krishna, B. Weiss and B. B. Brodie, *J. Pharmac. exp. Ther.* **163**, 379 (1968).
5. S. L. Pohl, L. Birnbaumer and M. Rodbell, *J. biol. Chem.* **246**, 1849 (1971).
6. F. Leray, A. M. Chambaut, M. L. Perrenoud and J. Hanoune, *J. Eur. Biochem.* **38**, 185 (1973).
7. J. Hanoune, M. L. Lacombe and F. Pecker, *J. biol. Chem.* **250**, 4569 (1975).
8. J. Ramachandran, *Analyt. Biochem.* **43**, 227 (1971).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. P. Emmelot and C. J. Bos, *Biochim. biophys. Acta* **120**, 369 (1966).
11. A. M. Chambaut, F. Leray-Pecker, G. Feldmann and J. Hanoune, *J. gen. Physiol.* **64**, 104 (1974).
12. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
13. R. S. Caldwell, H. S. Nakaue and D. R. Buhler, *Biochem. Pharmac.* **21**, 2425 (1972).
14. J. D. Lockhart, *Pediatrics* **50**, 229 (1972).
15. B. S. Gould, N. A. Frigerio and W. B. Lebowitz, *Archs Biochem. Biophys.* **56**, 476 (1955).
16. B. A. Kovacs and L. Gyenes, *Arch. int. Pharmacodyn. Ther.* **163**, 210 (1966).
17. H. S. Nakaue, R. S. Caldwell and D. R. Buhler, *Biochem. Pharmac.* **21**, 2273 (1972).
18. W. Cammer and C. L. Moore, *Biochem. biophys. Res. Commun.* **46**, 1887 (1972).
19. A. J. Gandolfi, H. S. Nakaue and D. R. Buhler, *Biochem. Pharmac.* **23**, 1997 (1974).
20. T. R. Corner, *Chem. Biol. Interactions* **8**, 107 (1974).