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MITOCHONDRIAL TOXICITY OF ULCEROGENIC CINCHOPHEN AND ITS DERIVATIVES *IN VITRO*

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Abstract—3-Hydroxycinchophen was found to be the most potent uncoupler of respiratory chain phosphorylation among the cinchophen derivatives studied: the following typical effects were observed (1) at low concentrations an increase in mitochondrial respiration in the absence of phosphate acceptor, (2) at high concentrations an inhibition of mitochondrial respiration, and (3) at intermediate concentrations a stage of respiratory decline in which ADP acts as an inhibitor. These effects on respiration were found, however, only when succinate was used as substrate. When substrates oxidized by NAD-linked dehydrogenases were used, the mitochondrial respiration was inhibited even at low concentrations of 3-hydroxycinchophen. A similar inhibition was also observed with ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylendiamine (TMPD) as substrate.

6-Methoxycinchophen had also a clear uncoupling effect. 8-Hydroxy-, 6-hydroxycinchophen and cinchophen itself had a slight effect on both respiration and ATPase activity, but 7-hydroxy-, 8-methoxy-, and 4′-hydroxycinchophen were without an effect.

Bovine serum albumin was partially effective in restoring oxidative phosphorylation after 3-hydroxycinchophen uncoupling.

CINCHOPHEN is known to cause gastric ulcerations in several animals.[1] The administration of the drug stops effectively the biosynthesis of mucus,[2] and this has been postulated to be the mechanism of cinchophen ulcerogenesis. We have shown previously that cinchophen inhibits protein biosynthesis *in vitro*[3] and amino acid synthesis by transamination reactions.[4] The maintenance of cellular integrity and the biosynthesis of polysaccharides and proteins *in vivo* is, however, a complicated ATP-requiring process. Cinchophen has previously been shown to interfere *in vitro* with the oxidative metabolism by inhibiting some dehydrogenases[5,6] and by lowering P/O-ratios in mitochondrial preparations.[7] The administration of this drug has been demonstrated to cause morphological damage in mitochondria of the gastric mucous membrane,[8] and to lower drastically lactate dehydrogenase isoenzyme LDH_1 in the small intestinal mucosa of the rat,[9] which isoenzyme has been postulated by several authors to be mitochondrial.[10]

The aim of the present study was to elucidate the ability of cinchophen and its derivatives to release respiratory control, to uncouple oxidative phosphorylation, to activate a latent ATPase and to cause swelling of rat liver mitochondria and to correlate their possible activity with the ulcerogenic potency.

METHODS

Rat liver mitochondria were prepared in a 0·22 M mannitol, 0·07 M sucrose and 0·2 mM ethylenediaminotetra-acetic acid (EDTA) medium as described by Chance and Hollunger.[11] Protein determinations were carried out by the biuret method.[12]

Measurements of respiratory and phosphorylative activity were determined polaro-

respiratory control was obtained with 3-hydroxycinchophen at about 0·5 mM concentration, which is approximately ten times higher than the concentration needed in case of 2,4-dinitrophenol (2,4-DNP) (Fig. 1). The maximal 2,4-DNP stimulated oxygen consumption was greater than that obtained in the presence of ADP or 3-hydroxycinchophen.

Oxygen electrode tracings in Fig. 2 show the effect of 3-hydroxycinchophen on the rate of oxygen consumption and on ADP:O ratio in mitochondrial preparations. It can be seen from the curve A that 220 μM 3-hydroxycinchophen increases the respiration 2·7-fold and decreases the ADP:O ratio from 1·9 to 1·2. So the stimulation in

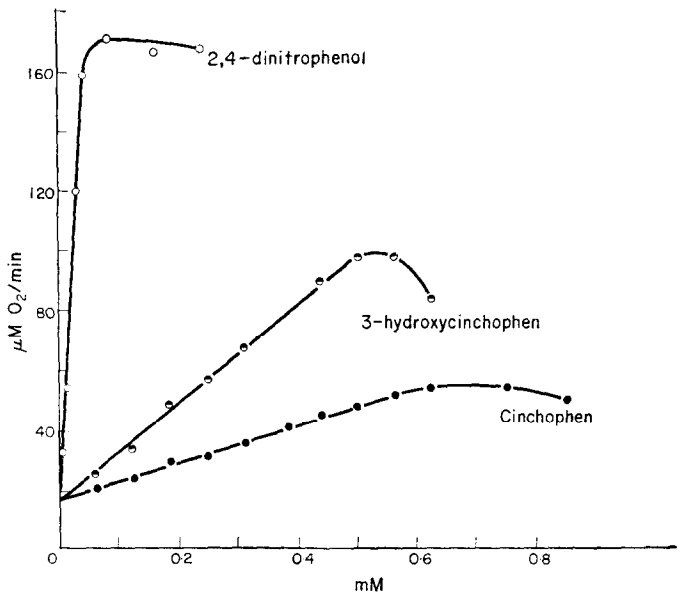

FIG. 1. The release of respiratory control in the presence of cinchophen, 3-hydroxycinchophen and 2,4-dinitrophenol in isolated rat liver mitochondria (2·7 mg of protein per ml) suspended in a MST-P_i-medium containing 8 mM succinate and 2 μM rotenone in the absence of phosphate acceptor.

mitochondrial respiration, i.e. loss of respiratory control, is paralleled by a decline in ADP:O ratio. Tracing B shows that the addition of bovine serum albumin partially restores oxidative phosphorylation. Tracing C indicates that even after the addition of 378 μM 3-hydroxycinchophen there still is some coupling between respiration and phosphorylation left, because by adding phosphate acceptor ADP the respiration is increased. When the 3-hydroxycinchophen concentration is increased to 0·5 mM, ADP addition does not further stimulate the oxygen consumption, and at still higher concentrations an inhibition of respiration by adding ADP, i.e. phosphate acceptor inhibition, becomes evident.

After the addition of 3-hydroxycinchophen or 2,4-dinitrophenol ATP synthesis decreases indicating an uncoupling of oxidative phosphorylation (Fig. 3).

3-Hydroxycinchophen was able to release the oligomycin-inhibited respiration, as did also 2,4-DNP.

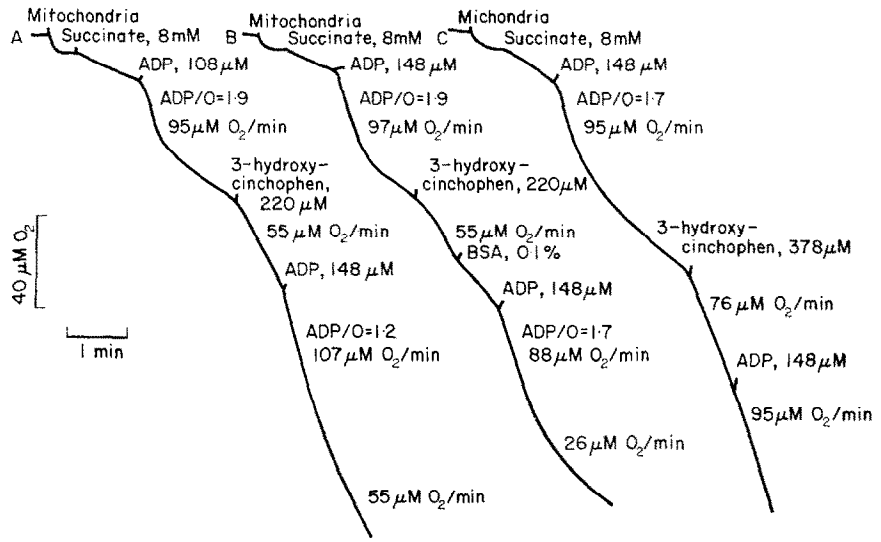

FIG. 2. The effect of 3-hydroxycinchophen on the respiration and oxidative phosphorylation in isolated rat liver mitochondria (2·2 mg protein/ml) suspended in KClST-P_i-medium. The restoring effect of bovine serum albumin (BSA) on oxidative phosphorylation is shown in tracing B. The final concentrations of reagents are indicated at the point of addition.

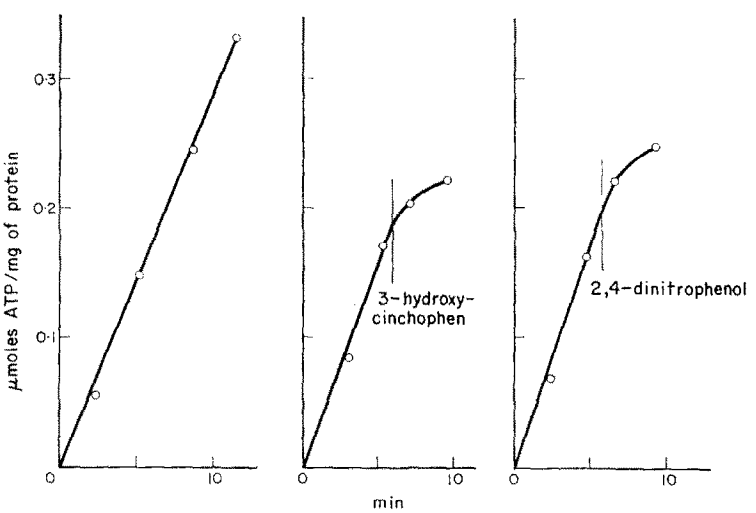

FIG. 3. The synthesis of ATP in isolated rat liver mitochondria suspended in a MST-P_i-medium containing rotenone (2 μM), succinate (8·25 mM), ADP (0·83 mM) and radioactive phosphate (11 μc) in the absence of any uncoupler and in the presence of 0·56 mM 3-hydroxycinchophen and 0·11 mM 2,4-dinitrophenol added into the reaction mixture during the incubation as indicated.

TABLE 1. EFFECT OF CINCHOPHEN, ITS DERIVATIVES AND 2,4-DINITROPHENOL (DNP) ON THE OXYGEN CONSUMPTION AND ATPase ACTIVITY IN THE PRESENCE AND ABSENCE OF OLIGOMYCIN

Compound	μM O_2/min	ATPase activity (μmoles P_i liberated/min.mg of protein) −Oligomycin	ATPase activity (μmoles P_i liberated/min.mg of protein) +Oligomycin (5μg/ml)
Control	18	2·33	1·84
Cinchophen	50	2·40	1·85
3-Hydroxycinchophen	98	5·95	1·71
6-Hydroxycinchophen	40	2·40	1·79
7-Hydroxycinchophen	20	2·07	1·69
8-Hydroxycinchophen	65	2·38	1·78
6-Methoxycinchophen	69	4·24	1·73
8-Methoxycinchophen	20	1·86	1·69
4′-Hydroxycinchophen	22	2·26	1·70
2,4-DNP	163	9·05	1·95

The concentrations of the compounds were in the oxygen consumption studies 470 μM, and 350 μM in case of the ATPase studies; the concentrations of 2,4-DNP were, however, 50 μM and 100 μM, respectively. Oxygen consumption was measured in the MST-P_i-medium containing 8 mM succinate as substrate, 2 μM rotenone and rat liver mitochondria (2·8 mg of protein per ml). ATPase activity was measured in the KClST-medium containing 2·5 mM ATP and rat liver mitochondria (0·43 mg of protein per ml).

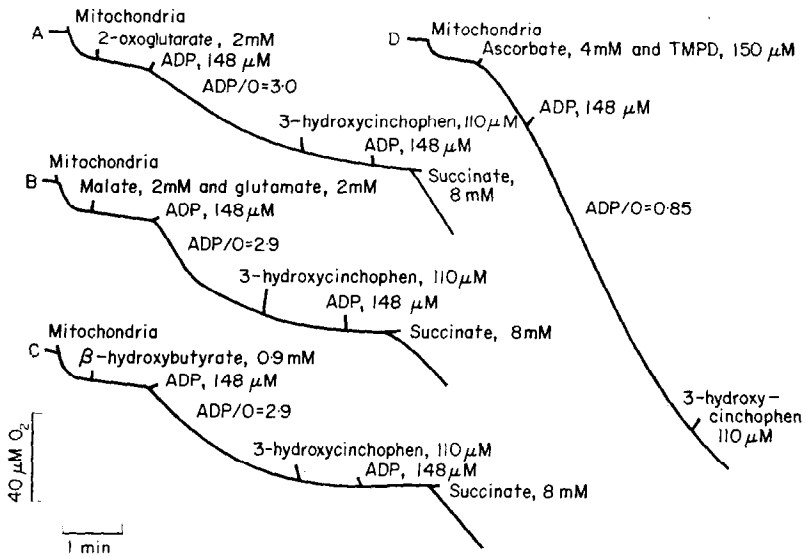

FIG. 4. The effect of 3-hydroxycinchophen on the respiration of isolated rat liver mitochondria (2·1 mg protein/ml) suspended in KClST-P_i-medium containing as substrate (a) 2-oxoglutarate, (b) malate-glutamate, (c) β-hydroxybutyrate or (d) ascorbate-*N,N,N′,N′*-tetramethyl-*p*-phenylendiamine (TMPD). The final concentrations of the reagents are shown at the point of addition.

Figure 4 shows the effect of 3-hydroxycinchophen on mitochondrial oxygen uptake when (a) 2-oxoglutarate, (b) glutamate-malate, (c) β-hydroxybutyrate and (d) ascorbate-TMPD is used as substrate. In all these cases 3-hydroxycinchophen (also cinchophen, not shown) acts as an inhibitor of respiration even at low concentrations. When succinate is added, the respiration is released (see a, b, c); in that case electrons bypass the dehydrogenases which used NAD^+ as coenzyme.

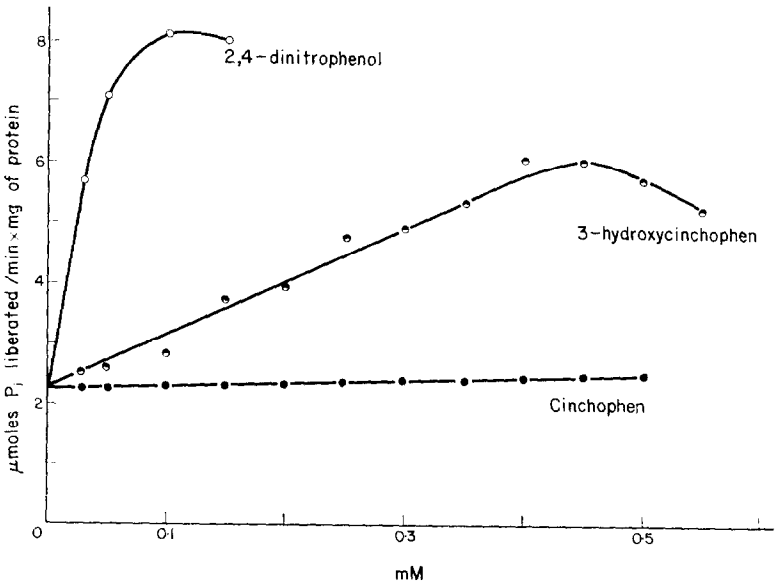

FIG. 5. The activation of the latent ATPase by cinchophen, 3-hydroxycinchophen and 2,4-dinitrophenol in isolated rat liver mitochondria (0·42 mg protein/ml) in KClST-medium containing 2·5 mM ATP.

(2) *Uncoupler activated ATPase*

3-Hydroxycinchophen activated, but less than 2,4-dinitrophenol, the latent ATPase activity in rat liver mitochondria (Fig. 5). When 3-hydroxycinchophen was added, the ATPase activity first increased linearly. After reaching the maximum value at about 0·4 mM concentration the ATPase activity was inhibited by further additions of the drug. 6-Methoxycinchophen increased ATPase activity in liver mitochondria and to a slight extent this was the case also with 6-hydroxy-, 8-hydroxycinchophen and cinchophen (Table 1). Again, 7-hydroxy-, 8-methoxy- and 4'-hydroxycinchophen had no activating effect. The activated ATPase was inhibited by oligomycin (Table 1).

(3) *Mitochondrial swelling*

Figure 6 shows the mitochondrial absorbance changes due to cinchophen and some of its derivatives. Each of these compounds was active in inducing swelling of mitochondria. Again 3-hydroxycinchophen had the most rapid and greatest effect. 8-Hydroxycinchophen and cinchophen also had more rapid action than the other derivatives.

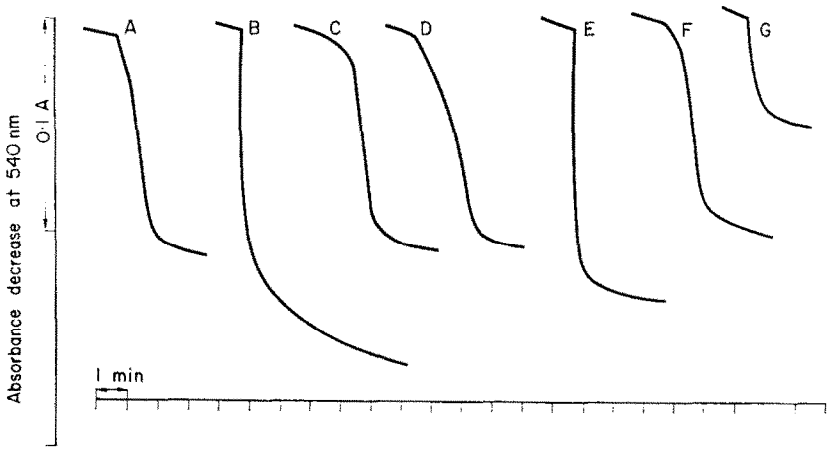

FIG. 6. The effect of cinchophen (a), 3-hydroxy- (b), 6-hydroxy- (c), 7-hydroxy- (d), 8-hydroxy- (e), 8-methoxy- (f) and 2,4-dinitrophenol (g) on the swelling of isolated rat liver mitochondria (1·8 mg protein/ml) in KCl ST-P_i-medium. The concentration of cinchophen and its derivatives was 0·5 mM, and the 2,4-dinitrophenol concentration was 0·1 mM.

DISCUSSION

3-Hydroxycinchophen was the most potent compound in the cinchophen series to increase oxygen consumption in the absence of a phosphate acceptor and to inhibit ATP synthesis in the isolated rat liver mitochondria respiring in the presence of succinate as an oxidizable substrate. It was also the most effective cinchophen derivative to activate the latent ATPase and to cause swelling of rat liver mitochondria. The parent compound, cinchophen, was less active in all these respects, and it activated little the mitochondrial ATPase. 8-Hydroxy-, 6-hydroxy- and 6-methoxy derivatives were also less effective than the 3-hydroxyderivative. 7-Hydroxy-, 8-methoxy- and 4′-hydroxycinchophen had no effects on mitochondrial oxygen consumption and the latent ATPase, although they caused mitochondrial swelling. The hydroxylation of the parent compound to positions 3, 8 and 6 apparently increases the mitochondrial toxicity of the compound. The masking of hydroxyl group at position 8 decreases the effects on mitochondrial respiration and swelling, but the 6-methoxyderivative is more active than free 6-hydroxyderivative. The inhibitory action of cinchophen on aminotransferases increases by hydroxylation in positions 3, 6, 7 and 8 in the case of aspartate aminotransferase and in positions 3, 6 and 7 in the case of alanine aminotransferase.[4] Thus the two aminotransferases and the mitochondrial functions are not inhibited in the same way by the different cinchophen derivatives.

Although 3-hydroxycinchophen was the most potent cinchophen derivative it was less active than 2,4-dinitrophenol in all respects. The concentration which was needed for the maximal stimulation of the oxygen consumption was about 10 times higher than in the case of 2,4-dinitrophenol, and even at this concentration the consumption increased less than in the presence of 2,4-dinitrophenol. The maximal activation of ATPase was obtained at a concentration, which was about five times higher than in the case of 2,4-dinitrophenol. These findings are not in accordance with a previous report on the effects of these two compounds on the P:O ratios.[7]

3-Hydroxycinchophen was found to inhibit respiration (in the presence of succinate) and ATPase, if it was added in concentrations greater than 0·4–0·5 mM. This kind of

biphasic action has been found with a variety of uncoupling agents.[18–23] The inhibition of respiration at high concentrations is a general property of uncouplers. Several interpretations have been suggested,[24] of these at least the inhibition at the level of the primary dehydrogenation is in the present case possible, since 3-hydroxycinchophen has already previously been shown to inhibit succinate dehydrogenase.[5] The NAD-linked dehydrogenases seemed to be, however, much more sensitive to 3-hydroxycinchophen, because already low concentrations depressed the mitochondrial respiration. Cinchophen has been earlier shown to inhibit effectively some NAD- and NADP-linked dehydrogenases.[6] A similar inhibition of mitochondrial respiration was demonstrated ,when ascorbate-TMPD was used as an electron donor. The possible uncoupling of oxidative phosphorylation in mitochondria oxidizing other substrates than succinate is masked by the high inhibition of the respiration even at low drug concentrations.

The swelling effect of cinchophen derivatives may be related in the case of 3-hydroxycinchophen to the release of respiratory control and activation of ATPase, and it may indicate extensive structural disorganization. The inactive derivatives also caused, however, a mitochondrial swelling although less rapid and extensive than 3- and 8-hydroxyderivatives.

3-Hydroxycinchophen has the same partial structure as salicylic acid. This structure has been proposed by Whitehouse to be essential for the uncoupling activity among salicylic acid analogues.[25] He has also suggested that anionic anti-inflammatory drugs interact with amino groups of proteins, which take part in energy conservation.[26]

Serum albumin has a restoring effect on the oxidative phosphorylation uncoupled by many compounds.[27–30] It had a similar effect in the case of 3-hydroxycinchophen in the present study, too.

3-Hydroxycinchophen is much more active in producing gastric ulcers in dogs than cinchophen itself or other cinchophen derivatives, of which 7-hydroxy- and 8-hydroxycinchophen are not ulcerogenic at all in dogs.[17] The most potent ulcerogen appears thus to be the most potent uncoupler and a poison of mitochondrial functions. Salicylic acid having structural similarities with 3-hydroxycinchophen is also an uncoupler and a powerful ulcerogenic compound.[31] Although 8-hydroxycinchophen was found to be rather active in releasing mitochondrial respiration, it is not an ulcerogen. The effect of cinchophen and its derivatives on the respiration of tissue slices[32] and on the oxygen consumption in the presence of substrates oxidized by NAD-linked dehydrogenases suggests that the inhibition of respiration is probably more important *in vivo* than uncoupling.

When cinchophen is administered to animals, it is hydroxylated in many ways.[17] 3-Hydroxycinchophen cannot, however, be detected in the urine of cinchophen-treated dogs[17] and rats,[33] which probably indicates that this most toxic derivative is not formed *in vivo*.

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REFERENCES

1. T. P. CHURCHILL and F. H. VAN WAGONER, *Arch. Path.* **13,** 850 (1932).
2. K. HARTIALA, A. C. IVY and M. J. GROSSMAN, *Am. J. Physiol.* **162,** 110 (1950).
3. M. REUNANEN, O. HÄNNINEN and K. HARTIALA, *Nature, Lond.* **213,** 918 (1967).
4. O. HÄNNINEN and K. HARTIALA, *Biochem. Pharmac.* **14,** 1073 (1965).

5. C. LUTWAK-MANN, *Biochem. J.* **36,** 706 (1942).
6. O. HÄNNINEN, *Ann. Acad. Sci. Fenn.* **A2,** no. 142 (1968).
7. M. W. WHITEHOUSE and J. M. HASLAM, *Nature, Lond.* **196,** 1323 (1962).
8. C. J. PFEIFFER, N. UMEDA, J. WEIBEL and J. L. A. ROTH, Abstracts, 4th World Congress of Gastroenterology, Copenhagen, p. 372 (1970).
9. K. WILLMAN, O. HÄNNINEN and K. HARTIALA, *Ann. Med. exp. Fenn.* **45,** 235 (1967).
10. J. HYLGAARD-JENSEN and M. VALENTA, *Enzym. biol. clin.* **11,** 336 (1970).
11. B. CHANCE and G. HOLLUNGER, *J. biol. Chem.* **236,** 1534 (1961).
12. E. LAYNE, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN) Vol. 3, p. 447. Academic Press, New York (1957).
13. B. CHANCE, G. R. WILLIAMS and G. HOLLUNGER, *J. biol. Chem.* **283,** 439 (1963).
14. J. HEINONEN, *Ann. Acad. Sci. Fenn.* **A2,** no. 151 (1970).
15. G. HOLLUNGER, *Acta Pharmac.* **11,** Suppl. 1, 7 (1955).
16. C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* **66,** 375 (1925).
17. K. HARTIALA, T. TERHO and I. HÄKKINEN, *Biochem. Pharmac.* **16,** 843 (1967).
18. H. C. HEMKER, *Biochem. biophys. Acta* **63,** 46 (1962).
19. H. C. HEMKER, *Biochem. biophys. Acta* **81,** 1 and 9 (1964).
20. D. K. MEYERS and E. C. SLATER, *Biochem. J.* **67,** 558 (1957).
21. D. K. MEYERS and E. C. SLATER, *Biochem. J.* **67,** 572 (1957).
22. K. VAN DAM, *Biochem. biophys. Acta* **131,** 407 (1967).
23. D. F. WILSON and R. D. MERZ, *Archs Biochem. Biophys.* **119,** 470 (1967).
24. K. VAN DAM and E. C. SLATER, *Proc. natn. Acad. Sci.* **58,** 2015 (1967).
25. M. W. WHITEHOUSE, *Biochem. Pharmac.* **13,** 319 (1964).
26. M. W. WHITEHOUSE and I. F. SKIDMORE, *J. Pharm. Pharmac.* **17,** 668 (1965).
27. E. C. WEINBACH and J. GARBUS, *J. biol. Chem.* **241,** 169 (1966).
28. E. C. WEINBACH and J. GARBUS, *J. biol. Chem.* **241,** 3708 (1966).
29. L. VÁQUEZ-COLÓN, F. D. ZIEGLER and W. B. ELLIOTT, *Biochemistry* **5,** 1134 (1966).
30. K. UTSUMI and L. PACKER, *Archs Biochem. Biophys.* **122,** 509 (1967).
31. M. J. SMITH, in *The Salicylates* (Eds. M. J. SMITH and P. K. SMITH), p. 235. Interscience, London (1966).
32. A. AITIO, personal communication (1970).
33. O. HÄNNINEN and K. HARTIALA, *Acta physiol. scand.* **68,** Suppl. 277, 90 (1966).