the system, was destroyed in the presence of the respiring organisms by reacting with the pyruvate which they produced.

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REFERENCES

- Altmann, S. M., Crook, E. M. & Datta, S. P. (1951). *Biochem. J.* **49**, lxiii.
- Brand, T. von, Tobie, E. J. & Mehlman, B. (1950). *J. cell. comp. Physiol.* **35**, 273.
- Cavallini, D. & Frontali, N. (1954). *Biochim. biophys. Acta*, **13**, 439.
- Chen, G. & Geiling, E. M. K. (1948). *J. infect. Dis.* **82**, 131.
- Cook, E. S., Perisutti, G. & Walsh, T. M. (1946). *J. biol. Chem.* **162**, 51.
- Fieser, L. F. & Fieser, M. (1944). *Experiments in Organic Chemistry*. New York: Reinhold.
- Fuller, A. T. & Maxted, W. R. (1939). *Brit. J. exp. Path.* **20**, 177.
- Fulton, J. D. & Stevens, T. S. (1945). *Biochem. J.* **39**, 317.
- Goodwin, T. W. & Williams, G. R. (1952). *Biochem. J.* **51**, 708.
- Harvey, S. C. (1949). *J. biol. Chem.* **179**, 435.
- Holtz, P. & Triem, G. (1937). *Hoppe-Seyl. Z.* **248**, 1.
- Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 293.
- Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
- Krijgsman, B. J. (1936). *Z. vergl. Physiol.* **23**, 663.
- Lipmann, F. (1944). *J. biol. Chem.* **155**, 55.
- MacLeod, J. (1951). *J. gen. Physiol.* **34**, 705.
- Mozingo, R. (1941). *Org. Synth.* **21**, 15.
- Mozingo, R. (1946). *Org. Synth.* **26**, 77 (procedure B).
- Proom, H., Woiwod, A. J., Barnes, J. M. & Orbell, W. G. (1950). *J. gen. Microbiol.* **4**, 270.
- Quastel, J. H. & Wheatley, A. H. M. (1932). *Biochem. J.* **26**, 2169.
- Randall, L. O. (1946). *J. biol. Chem.* **164**, 521.
- Reiner, L. & Leonard, C. S. (1932). *Proc. Soc. exp. Biol., N.Y.*, **29**, 951.
- Runnström, J. & Sperber, E. (1938). *Nature, Lond.*, **141**, 689.
- Schales, O. (1938). *Ber. dtsh. chem. Ges.* **71**, 447.
- Slater, E. C. (1949). *Biochem. J.* **45**, 14.
- Slater, E. C. (1952). *Nature, Lond.*, **170**, 970.
- Strangeways, W. I. (1937). *Ann. trop. Med. Parasit.* **31**, 405.
- Sumner, J. B. & Somers, G. F. (1943). *Chemistry and Methods of Enzymes*, p. 171. New York: Academic Press Inc.
- Thurlow, S. (1925). *Biochem. J.* **19**, 175.
- Tosic, J. (1947). *Nature, Lond.*, **159**, 544.
- Voegtlin, C., Dyer, H. A. & Leonard, C. S. (1923). *Publ. Hlth Rep., Wash.*, **38**, 1882.
- Voegtlin, C., Johnson, J. M. & Rosenthal, S. M. (1931). *Publ. Hlth Rep., Wash.*, **46**, 2234.
- Webb, E. C. & van Heyningen, R. (1947). *Biochem. J.* **41**, 74.
- White, I. G. (1954). *Aust. J. exp. Biol. med. Sci.* **7**, 379.
- White, I. G. (1955). *Aust. J. exp. Biol. med. Sci.* **33**, 359.
- Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 465.
- Yorke, W., Adams, A. R. D. & Murgatroyd, F. (1929). *Ann. trop. Med. Parasit.* **23**, 501.

Studies in Carotenogenesis

22. THE STRUCTURE OF ECHINENONE*

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The carotenoid echinenone was first obtained by Lederer (1935, 1938) from the gonads of the sea-urchin *Paracentrotus lividus*; it has now been shown to be widely distributed in marine invertebrates (see, for example, Goodwin, 1952). Lederer concluded that echinenone was a monoketo derivative of β -carotene, and Goodwin & Taha (1950) concluded, from spectrographic data on echinenone and the hydroxy- β -carotene derived from it by the action of aluminium isopropoxide, that it was probably 4-oxo- β -carotene. The present paper reports the application of new developments in carotenoid chemistry to this problem, which has made it possible to provide detailed confirmation of the proposed structure.

Goodwin & Taha (1950) concluded that the presence of a conjugated keto group in echinenone was strongly indicated by (a) the damping down of the fine structure of its absorption spectrum, (b) the shift of the main maximum (in light petroleum) from 448–449 m μ . in β -carotene to 453–454 m μ . in echinenone [453–454 m μ . is a more accurate value for λ_{max} . than 458 m μ . previously recorded by Goodwin & Taha (1950)], and (c) the production of a compound with an absorption spectrum indistinguishable from that of either β -carotene or cryptoxanthin (3-hydroxy- β -carotene) on reduction of the keto group of echinenone to a hydroxyl group. As cryptoxanthin and reduced echinenone were not separable chromatographically, it was suggested that the two were identical and that cryptoxanthin was 4-hydroxy- β -carotene. Karrer

* Part 21: van Niel, Goodwin & Sissins (1956).

(1951), however, found that cryptoxanthin, when treated with chloroform saturated with hydrogen chloride, did not lose water with the introduction of an additional conjugated ethylenic band; this would have been expected if the hydroxyl were in position 4, i.e. was allylic with respect to the first ethylenic double bond of the conjugated chain (Karrer & Leumann, 1951).

Wallcave & Zechmeister (1953) later synthesized 4-hydroxy- β -carotene (*isocryptoxanthin*) by treating β -carotene with boron trifluoride etherate and showed that with acid-chloroform it did lose water and that an additional double bond was introduced; furthermore, as the polyene produced had an even number (12) of conjugated double bonds, this was accompanied by a rearrangement of these ethylenic bonds to the 'retro' configuration described by Oroschnik, Karmas & Mebane (1952), the compound produced being dehydro- β -carotene.

Wallcave & Zechmeister (1953) also showed that *isocryptoxanthin* could be separated chromatographically from cryptoxanthin after prolonged development of the column. These experiments fully confirmed Karrer's conclusion that cryptoxanthin was not 4-hydroxy- β -carotene.

Fig. 1

The dehydro- β -carotene produced by Zechmeister & Wallcave (1953) was further characterized by the action of boron trifluoride etherate, which converts it into *isocryptoxanthin* with the concomitant production of β -carotene.

If echinenone were 4-oxo- β -carotene then its reduction product should be *isocryptoxanthin* and should undergo the very characteristic reactions reported by Wallcave & Zechmeister (1953), and shown in Fig. 1.

This series of reactions has now been demonstrated a number of times, starting with small amounts (200–500 μ g.) of crystalline echinenone.

Reduction of echinenone with lithium aluminium hydride proceeds readily and almost quantitatively at room temperature, and is much superior to the reaction with aluminum *isopropoxide* used previously (Goodwin & Taha, 1950). The reduced compound, which has spectral and chromatographic properties very similar to those of cryptoxanthin and *isocryptoxanthin*, was shown to be the latter by demonstrating (a) the allylic nature of the hydroxyl group, and (b) that the product of acid-chloroform treatment, when the hydroxyl group is removed, is dehydro- β -carotene.

There is therefore no doubt that reduced echinenone is *isocryptoxanthin*, and that echinenone itself must be 4-oxo- β -carotene.

EXPERIMENTAL

Isolation of echinenone. Gonads of the sea-urchin *Paracentrotus lividus* collected off Palermo, Sicily, were stored, immediately after removal from the animals, at -20° until required. The crude lipids were extracted at Palermo by the method of Goodwin & Taha (1950), sealed under N₂ and transported to Liverpool. The lipids (approx. 50 g.) were then dissolved in light petroleum (the fraction having b.p. 40 – 60° was used throughout this investigation) and a preliminary separation of the pigments was effected on alumina (P. Spence and Co., Widnes, Grade O) deactivated with methanol (Goodwin & Srisukh, 1949). The major fraction, containing predominantly echinenone, moved off the column when the chromatogram was developed with light petroleum containing 4% (w/v) of diethyl ether. A fraction containing β -carotene ran before echinenone, and a third, more strongly adsorbed fraction, which remained behind, was stripped off the column with ether and stored for further investigations.

The echinenone fraction, which also contained colourless lipids, was saponified (Goodwin & Morton, 1946) and re-chromatographed on deactivated alumina; the homogeneous echinenone fraction was eluted with light petroleum containing 5–10% (v/v) of ether. The solvent was removed *in vacuo* and the residue transferred to a small centrifuge tube with about 5 ml. of light petroleum. The volume of solvent was then cautiously reduced at room temp. to 0.5 ml. in a stream of N₂ with constant swirling to avoid pigment deposition on the sides of the tube. The tube was stoppered under N₂ and placed at -20° until bright red crystals were formed, together with some white material. The latter was

removed by washing with cold light petroleum, and the echinenone was recrystallized from light petroleum; yield about 2 mg. A further small quantity was obtained from the mother liquor. The absorption spectrum of the echinenone ($\lambda_{\max}$, 453–454 m μ . in light petroleum) was recorded, but not the m.p. because of shortage of material.

Reduction of echinenone with LiAlH_4 . Echinenone (250 μg .) was dissolved in ether (10 ml.) in a 50 ml. Erlenmeyer flask and a small amount of LiAlH_4 added. The flask was allowed to stand at room temp. until no further evolution of gas occurred (about 15–20 min.). The contents were then transferred to a small separating funnel and the lemon-yellow insoluble complex was decomposed by the addition of ethanol; water was then added cautiously until two layers formed. The water was removed and the ether layer washed twice with water. The ether extract was dried over anhydrous Na_2SO_4 , which was washed three times with small volumes of ether and the solvent removed *in vacuo*; the residue was dissolved in light petroleum and chromatographed on deactivated alumina. Two zones were obtained: *A*, eluted with light petroleum containing 10–15% (v/v) of ether, was unchanged echinenone, and *B*, eluted with light petroleum containing 30% (v/v) of ether, was reduced echinenone; it had an absorption spectrum identical in shape and position ($\lambda_{\max}$, 448, 476 m μ . in light petroleum) with that of cryptoxanthin (Goodwin & Taha, 1950), and presumably very similar to that of *isocryptoxanthin*, for the absorption spectra of these two compounds were 'very similar' in hexane (Wallcave & Zechmeister, 1953). Absorption spectra of carotenoids in hexane have their absorption maxima shifted by about 2 m μ . to longer wavelengths, compared with those in light petroleum, and there is very little difference in shape of the spectra in the two solvents (Goodwin, 1955). The adsorptive properties of reduced echinenone also correspond with those of cryptoxanthin (Goodwin & Taha, 1950) and must be very little different from those of *isocryptoxanthin*, for this can be resolved from cryptoxanthin only on prolonged development (Wallcave & Zechmeister, 1953).

In all experiments the yield of reduced echinenone was about 75–80%, the remainder being mainly unchanged echinenone.

Reaction of reduced echinenone with acidified chloroform. To a solution of reduced echinenone (100 μg .) in CHCl_3 (10 ml.) previously washed with Na_2CO_3 were added a few drops of CHCl_3 previously shaken with conc. HCl ; the solution immediately darkened but was allowed to stand for 20 min. in diffuse daylight (Wallcave & Zechmeister, 1953). The CHCl_3 extract was then washed free from acid with 1.0% (w/v) aqueous Na_2CO_3 , dried with Na_2SO_4 and the solvent removed *in vacuo*. The residue was dissolved in light petroleum and chromatographed on deactivated alumina. Zone *B*₁ ran through with light petroleum containing 1% (v/v) of ether and zone *B*₂ was obtained by developing with light petroleum containing 30% (v/v) of ether. *B*₁ was purified by chromatography on a mixture (3:1) of activated and deactivated alumina; it was eluted with light petroleum containing 20–30% (v/v) of ether and had the characteristic adsorptive properties of dehydro- β -carotene (*isocarotene*) (Redfearn, 1954), being somewhat more strongly adsorbed than β -carotene. As could be expected, its absorption spectral maxima (466 and 492 m μ . in light petroleum) were always slightly lower than those

recorded by Wallcave & Zechmeister (1953) in hexane (471 and 502 m μ .). Part of this difference may be due to the presence of unresolved *cis*-isomers in our specimen, for this reaction does tend to produce *cis*-isomers (Wallcave & Zechmeister, 1953). Zone *B*₂ also probably contained a mixture of all *trans-isocryptoxanthin* and its *cis*-isomers; it had very similar adsorptive properties to the starting material, but its absorption maxima were shifted to shorter wavelengths and the definition of the bands was reduced (Zechmeister & Wallcave, 1953).

The yield of dehydro- β -carotene was usually of the order of 80%.

Reaction of dehydro- β -carotene with BF_3 . To a solution of dehydro- β -carotene (100 μg .) in light petroleum (10 ml.) three drops of boron trifluoride etherate were added (Wallcave & Zechmeister, 1953). The solution quickly turned blue and was shaken vigorously for 1 min. It was then poured into a separating funnel containing 50 ml. of acetone, and water was cautiously added until two layers were formed. The lower layer was removed and the light petroleum layer washed twice with a small amount of water, dried over anhydrous Na_2SO_4 and chromatographed on deactivated alumina. Three zones were obtained: *A* moved off with 1% (v/v) ether in light petroleum, *B* with 20% (v/v) of ether in light petroleum and *C*, into which *B* merged, with 30% (v/v) ether. *C* was indistinguishable spectroscopically and chromatographically from *isocryptoxanthin* obtained from the reduction of echinenone and this was confirmed by converting it into dehydro- β -carotene by the action of CHCl_3 – HCl . Zone *B* was probably mainly *cis-isocryptoxanthin* because of its somewhat decreased adsorptive properties and the slight shift of its maxima to shorter wavelengths and the reduced sharpness of its spectrum; it also reacted similarly with CHCl_3 – HCl ; the occurrence of considerable amounts of *cis*-isomers was also recorded by Wallcave & Zechmeister (1953). Zone *A* was purified by chromatography on a 1:1 mixture of activated and deactivated alumina. The major zone *A*₁ was eluted with light petroleum containing 5% (v/v) ether and was shown to be chromatographically and spectroscopically ($\lambda_{\max}$, 448, 474 m μ . in light petroleum) indistinguishable from β -carotene; *A*₂, which was slightly more strongly adsorbed, had an absorption spectrum without good definition with maxima at 426 and 449 m μ . in light petroleum. It corresponds to Wallcave & Zechmeister's (1953) 'intermediate zone' between β -carotene and *isocryptoxanthin* containing 'several pale yellow zones'.

SUMMARY

By application of the recently developed reactions of Wallcave & Zechmeister (1953), it has been demonstrated that echinenone is 4-oxo- β -carotene.*

I am very grateful to Professor A. Monroy for collecting the *P. lividus* gonads and providing facilities for their extraction; to the University of Palermo and The British Council for inviting me to visit Palermo, during which time the first part of this work was carried out; and to Miss B. M. Eales for skilled technical assistance.

* *Note added in proof.* While this paper was in the press, Ganguly, Krinsky & Pinchard (1956) also demonstrated this structure for echinenone.

REFERENCES

- Ganguly, J., Krinsky, N. I. & Pinchard, J. H. (1956). *Arch. Biochem. Biophys.* **60**, 345.
- Goodwin, T. W. (1952). *The Comparative Biochemistry of the Carotenoids*. London: Chapman and Hall.
- Goodwin, T. W. (1955). *Analysis of Carotenoids*. In *Handbook of Plant Analysis*, vol. 3, p. 272. Ed. by Paech, K. & Tracey, M. V. Heidelberg: Springer.
- Goodwin, T. W. & Morton, R. A. (1946). *Analyst*, **71**, 15.
- Goodwin, T. W. & Srisukh, S. (1949). *Biochem. J.* **45**, 263.
- Goodwin, T. W. & Taha, M. M. (1950). *Biochem. J.* **47**, 244.
- Karrer, P. (1951). *Helv. chim. acta*, **34**, 2160.
- Karrer, P. & Leumann, E. (1951). *Helv. chim. acta*, **34**, 445.
- Lederer, E. (1935). *C.R. Acad. Sci., Paris*, **201**, 300.
- Lederer, E. (1938). *Bull. Soc. Chim. biol., Paris*, **20**, 554.
- Oroshnik, W., Karmas, G. & Mebane, A. D. (1952). *J. Amer. chem. Soc.* **74**, 295.
- Redfearn, E. R. (1954). Ph.D. Thesis: University of Liverpool.
- van Niel, C. B., Goodwin, T. W. & Sissins, M. E. (1956). *Biochem. J.* **63**, 408.
- Wallcave, L. & Zechmeister, L. (1953). *J. Amer. chem. Soc.* **75**, 4495.
- Zechmeister, L. & Wallcave, L. (1953). *J. Amer. chem. Soc.* **75**, 5341.

Anticholinesterase Activity of Ethyleneimines and certain other Cytotoxic Agents

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It has previously been shown by Bullock (1955) that cytotoxic activity and anticholinesterase activity do not run parallel. Indeed certain epoxides and methanesulphonyloxy derivatives possessing cytotoxic activity were almost inactive as anticholinesterases. On the other hand, three ethyleneimines inhibited acetylcholinesterase (AChE) of nervous tissue, but more feebly than did *NN*-di-(2-chloroethyl)methylamine hydrochloride (HN2). Unlike HN2 they inhibited pseudocholinesterase of serum more strongly than AChE. In most cases the inhibition was progressive, the progress being in some cases stopped by the addition of the substrate acetylcholine. At the time it was concluded that the most interesting feature of the results might well be that these cytotoxic agents, designed to resemble the nitrogen mustards and their breakdown products, differ from HN2 in being more feeble inhibitors of AChE and in showing their greatest activity against pseudocholinesterase. Four considerations have led to the subject being pursued further: (1) Most of the ethyleneimines and especially 2:4:6-tri(ethyleneimino)-s-triazine (tretamine, TEM), a substance at present undergoing clinical trial in the treatment of malignant disease, are much more active against AChE at pH 6.3 than at 7.4, the pH customarily employed in Warburg determinations. (2) The ethyleneimines are irreversible progressive inhibitors, so that their inhibitory effects may be more important than if the reverse had been true. (3) At pH 6.3 the anti-AChE activity of TEM is so greatly enhanced that the kinetics of the inhibition can reasonably be studied. (4) The study of the anti-AChE activity of the ethyleneimines might throw some light on the

mode of their reaction with proteins. The epoxides and methanesulphonyloxy derivatives were examined for activity at pH 6.3. Diisopropyl phosphorofluoridate (DFP, an irreversible inhibitor, and neostigmine and eserine, two reversible inhibitors of AChE, were included in the tests for comparison.

Among other attempts which have been made to study the actions of cytotoxic agents on proteins may be mentioned those of Alexander, Fox, Stacey & Smith (1952) and Watkins & Wormall (1952). The general chemistry of the cytotoxic alkylating agents has been discussed by Ross (1953) and their behaviour as chemotherapeutic agents in malignant disease has been reviewed by Bergel (1955).

EXPERIMENTAL

Materials. Epichlorohydrin, propylene oxide, DFP, TEM, *NL*- β -acetoxypropyltrimethylammonium chloride (MeCh, methacholine chloride, B.P. 1953) and di-(2-chloroethyl)methylamine (HN2, mustine hydrochloride B.P.C. 1954) were purchased. The other substances examined for anti-AChE activity were very kindly supplied by Dr W. C. J. Ross, Mr J. L. Everratt and Mr G. M. Timmis of the Chester Beatty Cancer Research Institute, London.

pH values. These were determined by a glass electrode and valve potentiometer, except that the value of 7.4 was assumed for the contents of the Warburg flasks containing 0.025 M-NaHCO₃ after equilibration with N₂ + CO₂ (19:1).

Solutions of inhibitors. These were freshly prepared. 4-[*p*-Di-(2-chloroethyl)aminophenyl]butyric acid (CB1348) was dissolved in a slight excess of 0.1N-NaOH and the pH reduced to 7.4 with 0.1N-HCl. Sparingly soluble substances (methanesulphonyloxy derivatives) were directly weighed into the Warburg flask or triturated with the required amount of water, or dissolved in a minimum of ethanol.