Inhibition of Dihydrocozymase-Oxidase Activity of Heart-Muscle Preparations and of Certain Cell-Free Bacterial Preparations by 2-Heptyl-4-Hydroxyquinoline N-Oxide

By F. L. JACKSON AND J. W. LIGHTBOWN

King's College Hospital Medical School, Denmark Hill, London, S.E. 5 and the National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 9 July 1957)

The site of action of 2-heptyl-4-hydroxyquinoline N-oxide (heptyl N-oxide) as an inhibitor of heart-muscle succinic oxidase activity has been shown to be between succinic dehydrogenase and cytochrome *c* (Lightbown & Jackson, 1954, 1956). The type of heart-muscle preparation used also oxidizes dihydrocozymase, and the pathway involves at least cytochromes *c*, *a* and *a₃* (Slater, 1950*a*). In a preliminary communication (Jackson & Lightbown, 1956) we have reported the inhibitory effect of heptyl N-oxide on the dihydrocozymase-oxidase activities of heart-muscle succinic oxidase preparations and of certain cell-free preparations of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Bacillus pumilus*. The present paper includes a more detailed account of this work, together with some further observations.

METHODS

Heart-muscle and bacterial preparations. These were made as described by Lightbown & Jackson (1956). The micro-organisms used were strains of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Bacillus pumilus*. The fat-free dry weight of the heart-muscle preparations was approx. 20 mg./ml., and for visual spectroscopy 2 ml. volumes of the undiluted preparations were used, substrate and inhibitor being added as concentrated solutions so that the final volume did not exceed 2.1 ml.

Spectroscopic observations. These were made with Zeiss or Beck low-dispersion microspectroscopes and a 150 c.p. Pointolite.

Dihydrocozymase-oxidase activity. This was measured by following decrease in optical density at 340 m μ in a Hilger Uvispek or a Unicam SP. 500 spectrophotometer. To exclude non-oxidative inactivation of dihydrocozymase (DPNH), control experiments were done anaerobically. For this purpose, a Unicam 10 mm. fused silica cell (catalogue no. 523) was used. This cell, which is of rectangular cross-section, has at the open end a ground-glass socket. This socket was found to fit a C10 cone and a Thunberg tube was modified by addition of a C10 standard ground-glass cone, allowing the cell to be attached to the lower end of the tube. Greasing of this joint was unnecessary. After evacuation and flushing four times with oxygen-free nitrogen, the cell with Thunberg tube attached was placed in the spectrophotometer, and when time had been allowed

for equilibration of temperature, the substrate was added from the stopper of the Thunberg tube. The compartment of the spectrophotometer containing the cell and modified Thunberg tube was covered with a light-tight box.

The conditions for all spectrophotometric experiments were: DPNH concentration $0.1\text{M} \pm 10\%$; final phosphate concentration 0.143M ; pH 7.3; final concentration of heptyl N-oxide, when added, $4\text{ }\mu\text{M}$. Dilutions of bacterial and heart-muscle preparations were chosen to give reduction times of about 5 min. in the uninhibited preparations.

Diaphorase activity. This was examined by observing rates of reduction of methylene blue in Thunberg tubes anaerobically. Observations were at 37° and pH 7 in a final volume of 3 ml. Final phosphate concentration was 0.1M , and final methylene blue concentration was $1/40\,000$. Suitable dilutions of heart and bacterial preparations were found experimentally for each batch.

Materials. Cytochrome *c* and cozymase (DPN) were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. DPNH was prepared by reduction of cozymase with sodium dithionite as described by Slater (1950*a*).

2-Heptyl-4-hydroxyquinoline N-oxide was synthesized by Dr J. W. Cornforth by the method described by Cornforth & James (1956). In visual spectroscopic experiments it was used at a final concentration of $5\text{ }\mu\text{M}$.

Antimycin A was obtained from Dr D. E. Green. In visual spectroscopic experiments the final concentration used was approx. $1\text{ }\mu\text{M}$.

Phosphate buffer for all experiments was prepared with KH_2PO_4 and NaOH.

RESULTS

Reduction of bacterial cytochromes by dihydrocozymase. Direct visual spectroscopic examination of the cell-free bacterial extracts without added substrate showed no visual cytochrome bands, or at the most, very faint bands of reduced cytochromes. On addition of DPNH, the cytochromes of the preparations were rapidly reduced. The addition of heptyl N-oxide gave the same results as were observed when succinate was used as substrate (Lightbown & Jackson, 1956), i.e. the oxidation of the cytochrome *b₁* of *S. aureus* was inhibited, whereas in the *E. coli* preparations there was inhibition of reduction of the cytochrome *b₁*.

Inhibition of dihydrocozymase-oxidase activity. Figs. 1 and 2 show the inhibition of DPNH-oxidase activity of heart-muscle preparations and of cell-free preparations of *S. aureus* produced by $4\ \mu\text{M}$ -heptyl *N*-oxide. The sensitivity of preparations of *P. vulgaris* and of *E. coli* to the inhibitor was rather less than that of the staphylococcal preparations, a concentration of $6\ \mu\text{M}$ -heptyl *N*-oxide producing about 50% inhibition. Similar

Fig. 1. Inhibition of DPNH-oxidase activity of heart-muscle preparation by heptyl *N*-oxide. ●, No inhibitor present; ○, with heptyl *N*-oxide ($4\ \mu\text{M}$ final concentration; readings corrected for slight absorption due to heptyl *N*-oxide). Final phosphate concentration, 0.143M ; pH 7.3. Final concn. of heart muscle, $45\ \mu\text{g}$. fat-free dry wt./ml. Final volume in cuvette, 2.8 ml.

Fig. 2. Inhibition of DPNH-oxidase activity of *Staphylococcus aureus* cell-free preparation by heptyl *N*-oxide. ●, No inhibitor present; ○, with heptyl *N*-oxide ($4\ \mu\text{M}$ final concentration; readings corrected for slight absorption by heptyl *N*-oxide). Final phosphate concentration, 0.143M ; pH 7.3. Final dilution of bacterial preparation 1/60. Final volume in cuvette, 2.8 ml.

concentrations produced about 70% inhibition with preparations of *B. pumilus*.

As crude bacterial preparations were used, the disappearance of DPNH might have been due to non-oxidative inactivation, as distinct from DPNH-oxidase activity. Observations on preparations of *S. aureus* and *E. coli* under anaerobic conditions with added DPNH were therefore made, but no measurable decrease in DPNH concentration was found.

Diaphorase activity. Since it was possible that the inhibition observed might be due to inhibition of diaphorase, the diaphorase activity of heart-muscle preparations and the similar activity of bacterial preparations were examined with and without added heptyl *N*-oxide. No inhibition was found, even with $10\ \mu\text{g}$. of heptyl *N*-oxide/ml.

Visual observations of reduction of cytochrome of heart-muscle preparations by dihydrocozymase. Slater (1950a) found that, after addition of DPNH to heart-muscle preparations anaerobically, there was rapid appearance of strong bands of reduced cytochromes *c* and *a*, but the band of reduced cytochrome *b* which appeared was faint and did not approach a density corresponding to full reduction even after 30 min. In similar experiments, with the same concentration of DPNH that Slater used (0.33 mM final), we have obtained the same result. When, however, either antimycin A (approx. $1\ \mu\text{M}$ final) or heptyl *N*-oxide ($5\ \mu\text{M}$ final) was added to the heart-muscle preparations before the DPNH, the addition of DPNH was followed by rapid and apparently complete reduction of cytochrome *b*, the reduction being complete within a second or two after mixing.

Further experiments, in the absence of any inhibitor, showed a striking effect of increasing concentration of added DPNH on the density of the band of reduced cytochrome *b*. When DPNH (0.66 mM final) was used there was rapid and simultaneous appearance within 2 or 3 sec. of mixing, of the bands of cytochromes *a*, *b* and *c*, the reduction of cytochrome *b* being comparable with that of cytochromes *a* and *c*.

Addition of DPNH (0.66 mM final) to heart-muscle preparations under aerobic conditions was followed by appearance of bands of reduced cytochromes *a* and *c*, and a faint *b* band was also visible. The DPNH added was rapidly oxidized by the preparation, so that the bands of the reduced cytochromes were visible for only a short period unless the DPNH was replenished. When heptyl *N*-oxide or antimycin A was added before the DPNH, an intense cytochrome *b* band appeared promptly on addition of DPNH, the bands of cytochromes *c* and *a* remaining faint.

When DPNH (0.66 mM) was added to heart-muscle preparations containing 0.02M -cyanide,

intense bands of reduced cytochromes *c* and *a* were seen, but the cytochrome *b* band was relatively weak. In the presence of heptyl *N*-oxide or antimycin A, however, DPNH produced, within 2 or 3 sec., appearance of an intense band of reduced cytochrome *b*.

DISCUSSION

The DPNH-oxidase activity of the heart-muscle and bacterial preparations was inhibited by micromolar concentrations of heptyl *N*-oxide, whereas the diaphorase activity was unaffected. The site of action of heptyl *N*-oxide was found by Lightbown & Jackson (1956) to be between succinic dehydrogenase and cytochrome *c*. Slater (1950*a*) has suggested that diaphorase links with the cytochrome system through a factor between cytochrome *b* and cytochrome *c* in the heart-muscle system. An inhibitor acting on a factor in this position might therefore be expected to inhibit both succinate and DPNH oxidation (Fig. 3). Slater excluded cytochrome *b* from the main pathway for oxidation of DPNH in this type of preparation because of the observed slow and incomplete reduction of cytochrome *b* after addition of DPNH under anaerobic conditions. Any direct linkage between diaphorase and cytochrome *b* was regarded by Slater as insignificant as compared with the diaphorase-factor path, the observed slight reduction of cytochrome *b* by DPNH being ascribed to a back-reaction, the rate of which was not more than about 2% of the rate of the forward reaction through cytochrome *c*. In intact liver mitochondria, however, according to Chance & Williams (1955) it is certain that cytochrome *b* is on the main pathway for oxidation of DPNH.

The slow appearance and weakness of the band of reduced cytochrome *b* seen in certain aerobic and anaerobic experiments might have some explanation other than Slater's suggestion that cytochrome *b* is not on the main pathway for oxidation of DPNH, but is taking part in a slow back-reaction. It is known (Slater, 1950*b*) that added cytochrome *c* is reduced at a rate which is only about 0.1% of the rate of reduction of the intrinsic

cytochrome *c* of the preparation, and by the same method we have found a similar relationship of the rates with our preparation. However, concentrations of added cytochrome *c* in excess of the intrinsic cytochrome *c* are reduced in less than 1 sec. by the heart-muscle preparation used for visual spectroscopy. If this is so for a reaction with a rate which is 0.1% of that of the rate of the normal forward reaction to cytochrome *c*, it can be argued by analogy that a back-reaction to cytochrome *b*, with a rate of 2% of that of the forward reaction to cytochrome *c*, should lead to reduction of the cytochrome *b* more quickly than is observed, if there are no other complicating side-reactions. It is probable (Slater, 1949) that the molarity of cytochrome *b* in the preparations is roughly similar to that of cytochrome *c*.

Slater (1950*a*) has also reported that full reduction of cytochrome *b* by DPNH could be obtained under anaerobic conditions in the presence of cyanide, although the reaction was slow. He calculated that if the factor had a potential of 0.11v, half-way between the potentials of cytochrome *b* and cytochrome *c*, then if only 1% of the factor were in the oxidized form the maximum reduction of cytochrome *b* would be 24%. As he pointed out, the complete removal of oxygen from the tubes is difficult, and residual traces of oxygen might keep the factor slightly oxidized. This might be so if DPNH concentration was limiting, but, with an excess of DPNH, it seems probable that any residual oxygen would rapidly be exhausted.

Under aerobic conditions, a very slight cyanide-stable oxidation would serve to prevent full reduction of cytochrome *b* in the presence of cyanide, and there is the possibility that the so called 'auto-oxidation' of cytochrome *b*, which might involve the Slater factor (Lightbown & Jackson, 1956), would tend to keep the cytochrome *b* oxidized. The 'auto-oxidation' of cytochrome *b* is prevented by heptyl *N*-oxide and by antimycin A, so that if either it or a residual oxidation through the cytochrome system were involved, both would be inhibited in the presence of either of these substances. The phenomenon of 'auto-oxidation' was first

Fig. 3. Possible pathways in heart-muscle system. Arrows indicate direction of electron transfer. The broken line encloses the possible site of action of heptyl *N*-oxide. X, Postulated factor between cytochrome *b* and cytochrome *c*; Y, auto-oxidizable materials which may be present in the preparation.

observed in the presence of cyanide (Keilin, 1928), but we have found that when azide is used instead of cyanide, 'auto-oxidation' appears more marked. It is possible therefore that 'auto-oxidation' is partly inhibited by cyanide. A rough estimate of the molarity of the cytochrome *c* in the heart-muscle preparations used was made from the optical density of the band of the reduced cytochrome, and a value of about $6 \mu\text{M}$ was found. If similar molarities are assumed for cytochromes *b*, *a* and *a₃*, and for the Slater factor, which might exist as an electron-transporting unit, a concentration of 0.33 mM-DPNH would be at least ten times that of the total cytochrome and Slater factor. However, by adding DPNH alone, full reduction of cytochrome *b* is not seen under anaerobic conditions unless twice this concentration is used. The heart-muscle preparations may contain small quantities of unidentified materials which can be reduced slowly by reduced factor. Under anaerobic or nearly anaerobic conditions materials of this kind might be completely reduced in the presence of higher DPNH concentrations, and this might perhaps account for the more complete reduction of cytochrome *b* seen with higher DPNH concentrations. If there is residual oxygen in the tubes, there will be some initial reoxidation of reduced cytochromes, and again the higher DPNH concentration will favour full reduction of cytochrome *b*. Heptyl *N*-oxide or antimycin A would inhibit residual oxidation of the factor, whether by oxygen or by other substances in the preparation. Wainio & Cooperstein (1956) have pointed out the neglect of 'auto-oxidation' of cytochrome *b* in kinetic studies and the possibility that reducible substances in heart-muscle preparations might affect the degree of reduction of cytochrome *b*. Lundegårdh (1955), in an investigation of the cytochrome system of wheat roots, has found that, in the presence of cyanide, cytochrome *b* can to some extent act as a substitute for cytochrome oxidase although it is relatively very inefficient.

It appears therefore that cytochrome *b* might be on the main pathway for oxidation of DPNH in this type of preparation. The same considerations apply to the suggestion by Chance (1952) that cytochrome *b* is not on the main pathway for oxidation of succinate. The 'auto-oxidation' is perhaps an artifact resulting from the mode of preparation of the heart-muscle system, materials being freed which can react with the factor between cytochrome *b* and cytochrome *c* and which are themselves auto-oxidizable. This would complicate the kinetic analysis, since a branch would have been produced in the electron-transport chain at the Slater factor. The preparations probably always contain myoglobin, and this might react with the Slater factor although we have not so far

been able to obtain evidence for this. Accurate measurements of the rates of reduction of cytochrome *b* by DPNH and by succinate in the presence of antimycin A or heptyl *N*-oxide would help to clarify the function of cytochrome *b* in these preparations. The nature of the factor between cytochrome *b* and *c* remains unknown.

The results with bacterial preparations and heptyl *N*-oxide show some similarities between the mammalian and bacterial DPNH-oxidase systems. As with the succinic oxidase systems, antimycin A is without effect in bacterial preparations and the same possibilities which we have discussed for the succinic oxidase systems (Lightbown & Jackson, 1956) apply equally to the DPNH-oxidase systems.

SUMMARY

1. The cytochromes of preparations of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Bacillus pumilus* were reduced by added dihydrocozymase (0.66 mM).
2. Oxidation of 0.66 mM-dihydrocozymase by the bacterial preparations was inhibited by heptyl *N*-oxide ($4 \mu\text{M}$).
3. In the presence of $4 \mu\text{M}$ -heptyl *N*-oxide with dihydrocozymase (0.33 mM) as substrate, the oxidation of reduced cytochrome *b₁* of *Staphylococcus aureus* preparations was inhibited, but in preparations of *Escherichia coli*, the reduction of oxidized cytochrome *b₁* was inhibited.
4. The diaphorase activity of heart-muscle preparations and of the cell-free bacterial preparations was not inhibited by heptyl *N*-oxide.
5. Anaerobically, the cytochrome *b* of the heart-muscle preparations was slowly and incompletely reduced by 0.33 mM-dihydrocozymase, but complete reduction occurred when the dihydrocozymase was 0.66 mM.
6. Under aerobic conditions when heptyl *N*-oxide or antimycin A was present, strong reduction of cytochrome *b* of heart-muscle preparations was observed, even with low concentrations of dihydrocozymase (0.33 mM).
7. It is suggested that cytochrome *b* is on the main pathway for electron transport in the dihydrocozymase-oxidase system of the heart-muscle preparation.
8. The site of action of heptyl *N*-oxide in the dihydrocozymase-oxidase system of heart muscle is between diaphorase and cytochrome *c*, and most probably between cytochrome *b* and cytochrome *c*.
9. Both heptyl *N*-oxide and antimycin A inhibit 'auto-oxidation' of cytochrome *b*. The significance of this phenomenon is discussed.

We wish to thank Mr R. W. Warner, King's College Hospital Medical School, for technical assistance in this work.

REFERENCES

- Chance, B. (1952). *Nature, Lond.*, **169**, 215.
 Chance, B. & Williams, G. R. (1955). *J. biol. Chem.* **217**, 429.
 Cornforth, J. W. & James, A. T. (1956). *Biochem. J.* **63**, 124.
 Jackson, F. L. & Lightbown, J. W. (1956). *Biochem. J.* **62**, 16P.
 Keilin, D. (1928). *Proc. Roy. Soc. B.* **104**, 206.
 Lightbown, J. W. & Jackson, F. L. (1954). *Biochem. J.* **58**, xlix.
 Lightbown, J. W. & Jackson, F. L. (1956). *Biochem. J.* **63**, 130.
 Lundegårdh, H. (1955). *Physiol. Plant.* **8**, 95.
 Slater, E. C. (1949). *Biochem. J.* **45**, 1.
 Slater, E. C. (1950a). *Biochem. J.* **46**, 484.
 Slater, E. C. (1950b). *Biochem. J.* **46**, 499.
 Wainio, W. W. & Cooperstein, S. J. (1956). *Advanc. Enzymol.* **17**, 329.

The Effect of Digitonin on the Cytochrome *c* Oxidase Activity of Plant Mitochondria

By E. W. SIMON

Department of Botany, The Queen's University, Belfast

(Received 13 September 1957)

The activity of an enzyme is given by the rate at which it causes its substrates to react under defined conditions. To determine this activity it is necessary to create an experimental situation in which the substrates have free access to the enzyme, since any barrier between enzyme and substrates will appear to reduce the activity of the enzyme.

Cytochrome *c* oxidase promotes the aerobic oxidation of cytochrome *c*, a substrate of relatively large molecular dimensions with a molecular weight of about 12 000 (Wainio & Cooperstein, 1956). It is hardly to be expected that so large a molecule will penetrate freely into cells—indeed experiment has shown that exogenous cytochrome *c* does not penetrate into rat-tissue cells (Beinert & Reissmann, 1949)—and it is therefore necessary to break cells to measure the activity of their cytochrome oxidase. If the cells are ground in the appropriate buffered sucrose medium, centrifuging the resulting homogenate will yield mitochondria which bear cytochrome oxidase in addition to many other enzymes. Cytochrome oxidase-bearing particles can also be isolated from homogenates made by grinding cells in plain buffer solutions (Keilin & Hartree, 1947; Bhagvat & Hill, 1951). These particles, which are fragments of the original mitochondria, lack many of the activities of the intact mitochondria but they possess nevertheless several other enzymes besides cytochrome oxidase (Keilin & Hartree, 1947). The test system for measuring cytochrome *c* oxidase activity therefore consists of a substrate (cytochrome *c*) of large molecular size and a particulate enzyme system in which the oxidase is bound to other enzymes. It seems reasonable to suppose that the cytochrome *c* supplied exogenously can react only with oxidase molecules located at the surface of the particles

(Cleland & Slater, 1953), any oxidase molecules lying within the particles being inaccessible to the substrate. In support of this postulate there is evidence that procedures which disrupt the particles yield preparations having a higher cytochrome oxidase activity. Thus Wainio & Aronoff (1955) found that a variety of surface-active agents increased the cytochrome oxidase activity of a Keilin and Hartree heart-muscle preparation. Mackler & Green (1956) obtained increases in a heart preparation with deoxycholate and Simon (1957) described experiments in which the cytochrome oxidase activity of *Arum* mitochondria was increased after mechanical vibration or treatment with digitonin.

Little attempt has been made so far to determine how much cytochrome *c* oxidase activity can be revealed by such procedures, although this information may have an important bearing on our understanding of oxidation processes in those tissues which appear at the moment to be deficient or lacking in cytochrome oxidase. The action of digitonin was selected for further study as the work of Wainio & Aronoff (1955) and Simon (1957) suggested that it was particularly active. The present paper describes experiments on the action of digitonin in which particles isolated from bean shoots were used as a source of cytochrome oxidase. It is shown that incubation of the particles with digitonin for a short time increases cytochrome oxidase activity by a substantial amount.

MATERIALS AND METHODS

Plant material. Seeds of *Phaseolus vulgaris* (French bean) were sown in moist sand in covered glass containers and incubated for 5 days at 25° in the dark with occasional weak