treated with heliotrine for 18 hr. (or more). There was no significant decrease in rate in the liver nuclei from the rats treated with dimethylnitrosamine (19 hr.) relative to nuclear count.

3. The observations are discussed in relation to present knowledge of nicotinamide–adenine dinucleotide metabolism in liver poisoning.

We wish to express our thanks to Dr L. B. Bull and Dr A. T. Dick of the Animal Health Research Laboratories, C.S.I.R.O., Parkville, Victoria, for supplies of crystalline heliotrine and of Wistar rats of the strain used for the previous studies with heliotrine carried out in this Department. This work was made possible by the financial support of the Anti-Cancer Council of Victoria.

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Selective Inhibition by 2-Heptyl-4-hydroxyquinoline *N*-Oxide of Certain Oxidation–Reduction Reactions

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(*Received* 20 *February* 1962)

The streptomycin antagonist, 2-heptyl-4-hydroxyquinoline *N*-oxide (Lightbown, 1954; Cornforth & James, 1956), was shown by Lightbown & Jackson (1956) to be a potent inhibitor of some cytochrome-mediated electron-transport systems. It inhibited the succinoxidase and $NADH_2$-oxidase activities of a Keilin–Hartree heart-muscle preparation at a locus which appeared to be the same as that inhibited by antimycin A, namely, it inhibited the oxidation of cytochrome *b* and the reduction of cytochrome *c*. Very similar effects were observed with *Bacillus subtilis* and *Bacillus pumilis*, whereas in *Staphylococcus aureus* it inhibited the oxidation of cytochrome b_1 and the reduction of cytochrome a_2. Experiments designed to localize the effect of the inhibitor showed that it did not inhibit the cytochrome-oxidase activity or the succinate-dehydrogenase activity of heart-muscle preparation, nor was the diaphorase activity of heart-muscle preparation or of bacterial extracts affected. In extracts of *Escherichia coli* and *Proteus vulgaris* the reduction of the component which, on spectroscopic evidence, is usually called cytochrome b_1 was inhibited by this material, though the extent of the inhibition was much less than in the systems mentioned above. Extracts from a fluorescent pseudomonad [strain KB1 (Kogut & Podoski, 1953)] which catalysed the oxidation of succinate and malate were relatively insensitive to the inhibitor.

The same compound inhibits light-induced phosphorylation in extracts of *Rhodospirillum*

rubrum though the same concentration of inhibitor has no effect on the respiratory activity of these extracts (Smith & Baltscheffsky, 1959). It also affects photo-oxidation and reduction by bacterial chromatophores (Vernon, 1959; Vernon & Ash, 1959) and inhibits the phosphorylation catalysed by spinach chloroplasts (Baltscheffsky, 1959; Avron, 1961).

A $NADH_2$ peroxidase which catalyses the peroxidation of $NADH_2$ in the presence of hydrogen peroxide or methyl hydrogen peroxide, either anaerobically or in the presence of air (Dolin, 1957), has been isolated and purified from *B. subtilis* (Lightbown & Kogut, 1959). This enzyme is also very sensitive to low concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide (Kogut & Lightbown, 1960). The present paper is concerned with a further study of this system and with an examination of the effects of 2-heptyl-4-hydroxyquinoline *N*-oxide on various model systems, in an attempt to elucidate its mode of action.

MATERIALS AND METHODS

The isolation from *B. subtilis* and purification of $NADH_2$ peroxidase and $NADH_2$ oxidase, and of a $NADH_2$ dehydrogenase, has been reported (Lightbown & Kogut, 1959; Kogut & Lightbown, 1959). The procedures for obtaining the enzyme preparations used in the work reported here were as follows: all three enzymes were extracted together from *B. subtilis* (N.C.T.C. 8236) grown on Wright's infusion broth (Mackie & McCartney, 1949) at 37° with forced aeration for 12–18 hr. After harvesting by centrifuging (at a yield of approx. 2 mg. dry wt./ml.), the packed cells were suspended in water to give a density of approx. 200 mg. dry wt./ml. and incubated for 2 hr. at 37° in the presence of sufficient crystalline lysozyme [prepared by the method of Alderton & Fevold (1949)] to ensure 90% breakage of cells, as shown by phase-contrast microscopy. The lysates were very viscous and the viscosity was reduced by the addition of approx. 10 μg. of deoxyribonuclease (Derinase; Bioindustria, Novi Ligure, Italy)/ml. before centrifuging for 20 min. at 10 000*g*. The sediment was resuspended in the original volume of water and the incubation and extraction were repeated—with addition of more lysozyme and Derinase if necessary—until the extracts were almost colourless. The pooled dark-brown supernatants were adjusted to pH 5·0 with 3M-acetic acid at room temperature and, after removal of the resulting precipitate by centrifuging for 20 min. at 20 000*g*, were adjusted to pH 7·0 with N-NaOH. The extract was then fractionated with ammonium sulphate, and the material which precipitated between 30 and 70% saturation was collected and dialysed against four changes of water (each at least 50 times the volume of the extract) at 4°. The catalase still present at this stage was removed by carefully adjusting the pH to 5·0 with 3M-acetic acid, and then to pH 4·5 by stepwise addition of more dilute acetic acid, the precipitate which formed at each step being rejected. The additions of acid and centrifuging were carried out at 4°. For success in separating the catalase from the other enzymes by this procedure, very thorough dialysis against water before the acid precipitation was essential. The pH 4·5 supernatant was adjusted to pH 7·0 with NaOH and freeze-dried. The freeze-dried material was dissolved in, and dialysed against, potassium phosphate buffer, pH 6·0, *I* 0·01 (to give a concentration of approx. 50 mg. of protein/ml.) and placed on a diethylaminoethylcellulose (DEAE-cellulose) column equilibrated with the same buffer (about 1 g. of DEAE-cellulose/100 mg. of protein). The column was developed with buffers of decreasing pH and increasing ionic strength in the following sequence: potassium phosphate buffer, pH 6·0, *I* 0·05; sodium acetate buffers, pH 5·0, *I* 0·05; pH 5·0, *I* 0·1; pH 4·6, *I* 0·1. The $NADH_2$ peroxidase and $NADH_2$ oxidase were eluted as a single peak in the acetate buffer, pH 4·6, and the $NADH_2$ dehydrogenase was eluted as a later peak with the same buffer. The fractions from each of these two peaks, for which the ratios of enzyme activity to E_{280} were about 300 times higher than the ratios for the original extracts, were collected and stored at −20°. They are referred to below as 'the most highly purified preparations'. 'Less highly purified extracts' were preparations collected after ammonium sulphate fractionation and acid precipitation, but before DEAE-cellulose chromatography.

$NADH_2$-peroxidase activity was measured spectrophotometrically at room temperature by following the disappearance of $NADH_2$ in the presence of either hydrogen peroxide or methyl hydrogen peroxide and enzyme. Enzyme solution (0·1–0·2 ml.) was added to 1 cm. cuvettes containing $NADH_2$, peroxide and 33 mM-potassium phosphate buffer, pH 6·4, at zero time, and the extinction at 340 mμ was measured at 15 sec. intervals. One unit of enzyme activity is defined as a change in E_{340} of 0·01/min. at room temperature (Dolin, 1957). When the peroxidase preparation was free from $NADH_2$-oxidase activity measurements were made aerobically; with less highly purified $NADH_2$ peroxidase measurements were made anaerobically (Jackson & Lightbown, 1958) with special cuvettes equipped with ground-glass joints to which were attached modified Thunberg tubes. The enzyme solution was placed in the hollow stopper of the Thunberg tube attachment and the whole assembly was evacuated at a high-vacuum pump, flushed four times with oxygen-free nitrogen and finally filled with oxygen-free nitrogen. The spectrophotometer compartment containing the cells and modified Thunberg tubes was covered with a light-tight box. At zero time, enzyme was tipped from the hollow stopper into the cell contents.

$NADH_2$-dehydrogenase activity was measured spectrophotometrically, at room temperature, by following the reduction of 2,6-dichlorophenol-indophenol in the presence of $NADH_2$ and enzyme in approx. 0·4M-potassium phosphate buffer, pH 7·8.

Protein concentrations were measured by the method of Warburg & Christian (1942).

Spectroscopic observations were made with Beck or Zeiss low-dispersion microspectroscopes and a 150 candle-power Pointolite lamp. Spectrophotometric observations were made with a Unicam SP. 500 or with a Hilger Uvispek spectrophotometer, with 1 cm. silica cells. All difference spectra were obtained with the latter instrument.

Pseudomonas cytochrome 553 was isolated from *Pseudomonas* KB1 (Kogut & Podoski, 1953) and purified by a method briefly described by Kogut (1957).

Extracts of *Pseudomonas* KB1 for studies of respiratory activity were prepared as follows: The cells were grown in 3% (w/v) Difco yeast-extract medium at 30° with forced aeration to give a density of 6 mg. dry wt./ml., harvested by centrifuging, washed twice, and finally suspended in 25 mM-Sørensen's phosphate buffer, pH 7·2, to give a density of 60 mg. dry wt./ml. They were then treated for 10 min. in a 1 kw Mullard oscillator (Mullard Ltd., London) in three batches of 20 ml. The resulting material was pooled, and centrifuged first for 15 min. at 12 000*g* to remove unbroken cells and large fragments and then for 30 min. in a refrigerated centrifuge at 22 000*g*. The supernatant fraction was collected and dialysed against running tap water overnight to minimize endogenous respiration. These extracts were light brown and showed the typical cytochrome spectrum of this organism (Kogut, 1957).

$NADH_2$ was prepared from NAD (C. F. Boehringer und Soehne) by reduction with crystalline alcohol dehydrogenase (C. F. Boehringer und Soehne) and ethanol according to a modification of the method of Pullman, Colowick & Kaplan (1952) (Dr P. Greengard, personal communication). Reduced 2,6-dichlorophenol-indophenol (Kodak Ltd.) was prepared according to Smith & Stotz (1949). 2-Heptyl-4-hydroxyquinoline *N*-oxide was synthesized by Dr J. W. Cornforth by the method of Cornforth & James (1956). It was made up as a stock solution in mN-NaOH at a concentration of approx. 50 μg./ml. The actual concentration was determined spectrophotometrically before use. Methyl hydrogen peroxide was prepared according to the method of Rieche & Hitz (1929) and its concentration determined iodometrically. Notatin (glucose oxidase) was obtained from Boots Pure Drug Co. Ltd.

Cytochrome *c* was purchased from C. F. Boehringer und Soehne as horse-heart cytochrome *c*, 85–95% pure, and when required was reduced either by hydrogen in the presence of palladium-treated asbestos or by ascorbic acid. Concentrations of total and reduced cytochrome *c* in solutions were estimated as described by Margoliash (1954*a*).

Table 1. *Effect of various concentrations of 2-heptyl-4-hydroxyquinoline* N*-oxide on the dihydronicotinamide–adenine dinucleotide-peroxidase from* Bacillus subtilis

Cuvette contents were (final concentrations): potassium phosphate buffer, pH 6·4, 0·55 mM; hydrogen peroxide, 4·0 mM; $NADH_2$, 0·12 mM; FAD, 23 μM; 2-heptyl-4-hydroxyquinoline *N*-oxide, as indicated, was dissolved in 1·0 mM-NaOH, and the final concentration of NaOH in all cuvettes was 0·1 mN. Enzyme preparation, free from catalase and oxidase activities, 10·2 μg./ml. Blanks contained all reagents except $NADH_2$. The total volume was 2·5 ml. Reactions were started by the addition of enzyme, and the decrease in E_{340} was measured at intervals of 15 sec., the rate being calculated from the first reading after addition of enzyme.

Concn. of inhibitor (μM)	Reaction rate (ΔE_{340}/min.)	Inhibition (%)
0	0·058	.
1·55	0·0217	59
3·87	0·018	69
7·74	0·012	79

Hydrogen peroxide was British Drug Houses Ltd. microanalytical reagent grade, 100 vol. soln., and was kept in a dark bottle in the cold room. Dilutions were freshly prepared as required, and the stock solution was standardized iodometrically.

Haemin containing 8·1% of iron (British Drug Houses Ltd.) was dissolved in a minimal volume of 0·1 N-NaOH and diluted with water to approx. 1 mM. The pH was then adjusted with 0·1 M-KH_2PO_4 to approx. 8·0 and the concentration of haematin adjusted to approx. 0·1 mM. It was further diluted for use as required and, when necessary, it was reduced by sodium tetrahydroborate (borohydride).

Crystalline horse-radish peroxidase was purchased from L. Light and Co. FAD was obtained from Sigma Chemical Co. (minimal purity 80%). FMN was obtained as the sodium salt from L. Light and Co. Ltd. Solutions of flavins were freshly prepared and protected from light. Their concentrations were determined spectrophotometrically. Antimycin A was kindly supplied by Dr D. E. Green, and was used as a solution in ethanol.

The following millimolar extinction coefficients, at the indicated wavelengths, were used for calculating concentrations: FAD at 450 mμ, 11·3; FMN at 450 mμ, 12·2 (Whitby, 1953); $NADH_2$ at 340 mμ, 6·22 (Horecker & Kornberg, 1948); 2-heptyl-4-hydroxyquinoline *N*-oxide at 345 mμ, 9·45; reduced cytochrome *c* at 550 mμ, 27·7 (Margoliash, 1954*a*); oxidized cytochrome *c* at 550 mμ, 9·2 (Margoliash, 1954*b*).

RESULTS

Oxidation of dihydronicotinamide–adenine dinucleotide by dihydronicotinamide–adenine dinucleotide-peroxidase

It has not been possible, so far, to separate the $NADH_2$ peroxidase from another flavoprotein with $NADH_2$-oxidase activity, but in some of the most highly purified preparations the $NADH_2$ oxidase became completely inactivated on dilution and standing at room temperature, leaving only the $NADH_2$-peroxidase activity. In these most highly purified preparations FAD was required for activity but it could be partially replaced by FMN.

Inhibition of enzyme activity. Kogut & Lightbown (1960) reported that 2-heptyl-4-hydroxyquinoline *N*-oxide inhibits $NADH_2$-peroxidase activity at concentrations similar to those which inhibit heart-muscle succinoxidase activity. Table 1 shows the effect of different concentrations of the inhibitor on this enzyme system. Fig. 1 shows Lineweaver–Burk plots of the results obtained with various concentrations of $NADH_2$ and hydrogen peroxide. The inhibitor is not competitive with respect to either $NADH_2$ or hydrogen peroxide, and K_i is approx. 1·6 μM. Table 2 shows the effect of various concentrations of FAD on the enzyme activity in the presence and absence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Three different enzyme preparations were used, and in no case did increasing concentration of FAD reverse the inhibition.

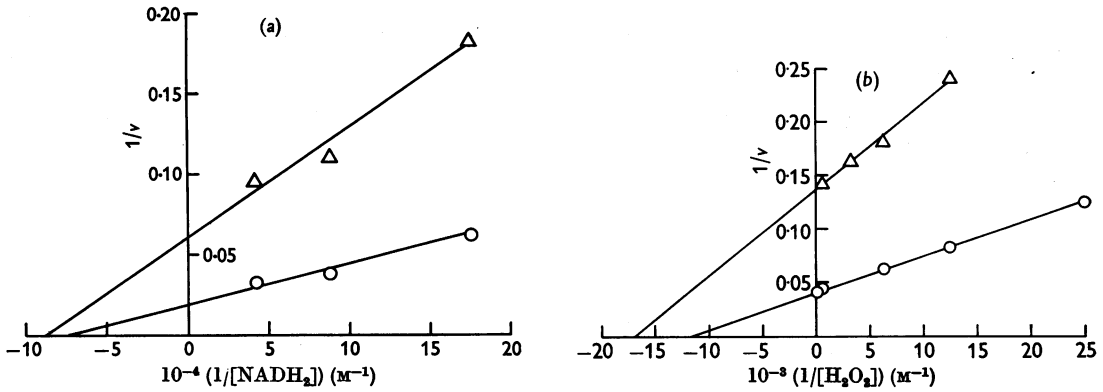

Fig. 1. Effect of dihydronicotinamide–adenine dinucleotide and hydrogen peroxide concentrations on the dihydronicotinamide–adenine dinucleotide-peroxidase enzyme from *Bacillus subtilis* in the presence and absence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Enzyme activity was assayed as described in the Materials and Methods section, and the results were plotted by the method of Lineweaver & Burk. Final concentrations of cuvette contents were: (*a*) potassium phosphate buffer, pH 6·4, 55 mM; FAD, 5·0 μM; H_2O_2, 2·0 mM; inhibitor, where indicated, 3·86 μM; NaOH, 0·1 mN; $NADH_2$, as indicated; enzyme preparation, 10·2 μg./ml.; (*b*) potassium phosphate buffer, enzyme, inhibitor and NaOH as in (*a*); $NADH_2$, 0·11 mM; FAD, 4·6 μM; H_2O_2, as indicated. Total vol. 2·5 ml. Measurements were under aerobic conditions. ○, Without inhibitor; △, with inhibitor.

Table 2. *Effect of various concentrations of flavin–adenine dinucleotide on dihydronicotinamide–adenine dinucleotide-peroxidase activity in the presence and absence of 2-heptyl-4-hydroxyquinoline* N*-oxide*

Different enzyme preparations were used in each of the three experiments. Those in Expts. 2 and 3 were not free from oxidase activity and measurements were therefore made under anaerobic conditions. The enzyme concentrations in Expts. 1–3 were 10, 50 and 266 μg./ml. respectively. The enzyme preparation of Expt. 3 had also some catalase activity, and methyl hydrogen peroxide (8·0 mM) was therefore used. Other conditions were as in Table 1.

Expt. no.	Concn. of FAD (μM)	Concn. of inhibitor (μM)	Reaction rate (ΔE_{340}/min.) Without inhibitor	With inhibitor	Inhibition (%)
1	0	.	0·006	.	.
	4·5	23	0·025	0·002	92
2	0	3·7	0·020	0·005	75
	0·293	.	0·022	.	.
	0·586	.	0·025	.	.
	1·47	.	0·041	.	.
	2·93	3·7	0·039	0·007	82
	5·86	3·7	0·060	0·008	87
	11·72	3·7	0·065	0·004	94
	17·60	3·7	.	0·006	.
3	0	2·0	0·053	0·045	15
	0·8	.	0·004	.	.
	2·0	2·0	0·065	0·053	19
	2·0	2·0	0·067	0·054	20
	4·0	2·0	0·088	0·044	50
	4·0	2·0	0·080	0·042	48
	8·0	2·0	0·088	0·062	30
	8·0	2·0	.	0·057	35
	16·0	2·0	0·085	0·051	40

The least highly purified enzyme preparation (Expt. 3) had considerable $NADH_2$-peroxidase activity in the absence of added FAD. Though a concentration of 2·0 μM of the inhibitor had little effect on this activity it did abolish the increase in activity due to added FAD. In most of the experiments, $NADH_2$, FAD and inhibitor were added to the cuvettes first and enzyme was added at zero time. However, preincubation of enzyme with FAD for 15 min. gave no protection from inhibition.

A partially purified enzyme preparation which showed no activation by FAD was not inhibited by 23 μM-2-heptyl-4-hydroxyquinoline *N*-oxide.

Absorption spectra of dihydronicotinamide–adenine dinucleotide-peroxidase in the presence of dihydronicotinamide–adenine dinucleotide, flavin–adenine dinucleotide and 2-heptyl-4-hydroxyquinoline N-oxide

Effect of 2-heptyl-4-hydroxyquinoline N-oxide on dihydronicotinamide–adenine dinucleotide and flavin–adenine dinucleotide. Absorption spectra of $NADH_2$ and of $NADH_2$ plus the heptyl *N*-oxide (Fig. 2*a*) showed no evidence of interaction, but a comparison of absorption spectra of FAD and of FAD plus the inhibitor, carried out under anaerobic conditions, showed a decrease in absorption of the mixture in the 340–360 mμ region of the spectrum, which could be due to a change in the absorption of the heptyl *N*-oxide. Absorption spectra of $NADH_2$ plus FAD and of $NADH_2$ plus FAD plus the heptyl *N*-oxide under anaerobic conditions did not show evidence of interaction between $NADH_2$ and FAD, and no effect of the inhibitor on FAD plus $NADH_2$ was observed apart from the effect on FAD alone (Fig. 2*b*).

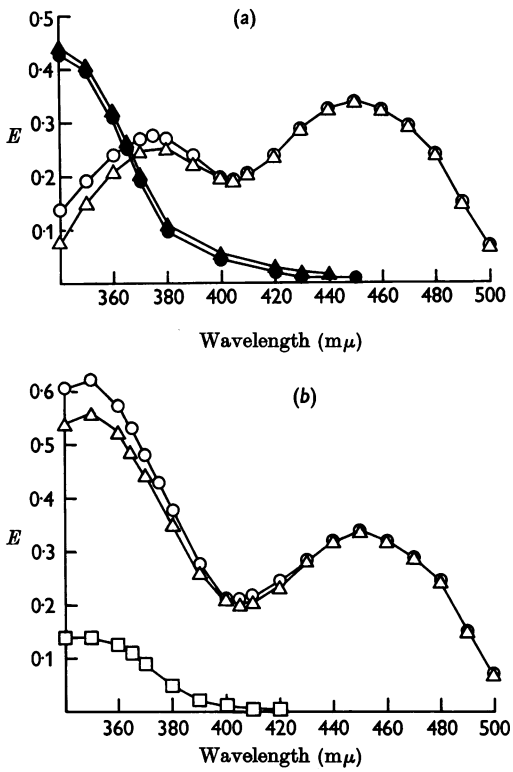

Fig. 2. Absorption spectra of dihydronicotinamide–adenine dinucleotide, flavin–adenine dinucleotide, and dihydronicotinamide–adenine dinucleotide plus flavin–adenine dinucleotide in the presence and absence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Final concentrations of cuvette contents were: potassium phosphate buffer, pH 7·0, 33 mM; inhibitor, where indicated, 14·8 μM; NaOH, 0·1 mN. (*a*) ●, 68 μM-$NADH_2$ (aerobic); ▲, 68 μM-$NADH_2$ in the presence of inhibitor, corrected for the absorption due to the inhibitor (aerobic); ○, 30 μM-FAD (anaerobic); △, 30 μM-FAD in the presence of inhibitor, corrected for the absorption due to the inhibitor (anaerobic). (*b*) ○, 68 μM-$NADH_2$ plus 30 μM-FAD (anaerobic); △, 68 μM-$NADH_2$ plus 30 μM-FAD in the presence of inhibitor corrected for the absorption due to the inhibitor (anaerobic); □, 14·8 μM-inhibitor (aerobic).

Effect of dihydronicotinamide–adenine dinucleotide and flavin–adenine dinucleotide on dihydronicotinamide–adenine dinucleotide-peroxidase in the absence and presence of 2-heptyl-4-hydroxyquinoline N-oxide. In these experiments the most highly purified enzyme, free from catalase and requiring FAD for activity, was used. In the first experiment, two 'anaerobic cuvettes' were set up, each containing $NADH_2$ peroxidase (10 μg. of protein/ml.) and FAD (52 μM). One cuvette contained 15 μM-2-heptyl-4-hydroxyquinoline *N*-oxide dissolved in sodium hydroxide (final concn. of sodium hydroxide, 0·1 mN), whereas the other cuvette contained only the equivalent concentration of sodium hydroxide. The inhibitor and sodium hydroxide solutions were added to the enzyme in the cuvette before the FAD. After evacuation the spectra of enzyme plus FAD, and of enzyme plus FAD in the presence of the heptyl *N*-oxide, were plotted, the absorption due to the inhibitor being subtracted (Fig. 3, curves 1*a* and 1*b*). There is a very slight diminution in extinction at 450 mμ (about 4%) and at 375 mμ (about 2%) in the sample containing the inhibitor.

In the second experiment, the cuvettes were set up as before, except that $NADH_2$ (104 μM) was added to the enzyme solution in the cuvette and FAD (52 μM) was placed in the hollow stopper of the Thunberg tube attachment to each cuvette. After evacuation of the cuvettes the absorption spectra of enzyme plus $NADH_2$ and of enzyme plus $NADH_2$, in the presence of the heptyl *N*-oxide, were first measured. Curves 2*a* and 2*b* of Fig. 3 show the plots of these spectra, with the absorption due to the heptyl *N*-oxide again subtracted. There is a significantly higher extinction in the presence of the inhibitor. This is due to the fact that the enzyme preparation used in this experiment contained some $NADH_2$-oxidase activity. The extinction measured after evacuation of the cuvettes differed from that due to the enzyme and $NADH_2$ originally added, owing to oxidation of some $NADH_2$ before the establishment of anaerobic conditions. It appears that this oxidation was in-

hibited by the heptyl *N*-oxide. The absorption spectra of enzyme and of the inhibitor alone were measured under similar conditions (curves 3 and 4 respectively). After completion of these spectra FAD from the stoppers of the Thunberg tubes was added to the cuvette contents, and, after incubation for 50 min. at room temperature in the dark, the spectra were again measured. Curves 2 in Figs. 4 (*a*) and 4 (*b*) show the plots of these spectra, with the absorption due to the inhibitor again being subtracted in Fig. 4 (*b*).

Analysis of absorption spectra. Curve 1 in Fig. 4 (*a*) is the calculated spectrum of enzyme plus $NADH_2$ plus FAD, and curve 1 in Fig. 4 (*b*) the calculated spectrum of enzyme plus $NADH_2$ plus FAD in the presence of the heptyl *N*-oxide (with correction for absorption of the inhibitor). These curves were obtained from the sums of the appropriate spectra in Fig. 3 by adjusting for dilution and for absorption of the enzyme solution which entered twice into the sum. They are thus the spectra one would obtain if no interaction occurred on mixing enzyme, $NADH_2$ and FAD. Comparison of curves 2 and 1 in Fig. 4 (*a*) shows a considerable decrease in absorption at the 450 mμ peak and in the region 330–360 mμ. If it is assumed that the decreases in absorption at 450 and 340 mμ represent reduction of FAD and oxidation of $NADH_2$ respectively, the quantity of FAD reduced is 14·1 μm-moles/ml. and that of $NADH_2$ oxidized 41·0 μm-moles/ml. From a comparison of curves 1 and 2 in Fig. 4 (*b*) it appears that no measurable amount of FAD has been reduced in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide. There is, however, some decrease in extinction at 340–360 mμ. If one assumes that this is due to oxidation of $NADH_2$, 14·2 μm-moles of $NADH_2$/ml. would have been oxidized without a concomitant reduction in FAD. However, 2-heptyl-4-hydroxyquinoline *N*-oxide itself has an absorption peak in

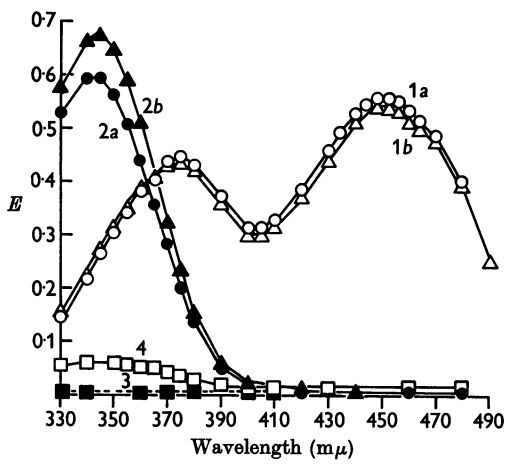

Fig. 3. Absorption spectra of dihydronicotinamide–adenine dinucleotide-peroxidase with flavin–adenine dinucleotide and with dihydronicotinamide–adenine dinucleotide in the absence and presence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Final concentrations of cuvette contents were: enzyme preparations, 10 μg./ml.; potassium phosphate buffer, pH 7·0, 33 mM; inhibitor, where indicated, 15·0 μM; NaOH, 0·1 mN. Incubations were under anaerobic conditions. ○ (1*a*), enzyme plus 52 μM-FAD; △ (1*b*), enzyme plus 52 μM-FAD plus inhibitor, corrected for the absorption due to the inhibitor; ● (2*a*), enzyme plus 104 μM-$NADH_2$; ▲ (2*b*), enzyme plus 104 μM-$NADH_2$ plus inhibitor, corrected for the absorption due to the inhibitor; ■ (3), enzyme alone; □ (4), inhibitor alone.

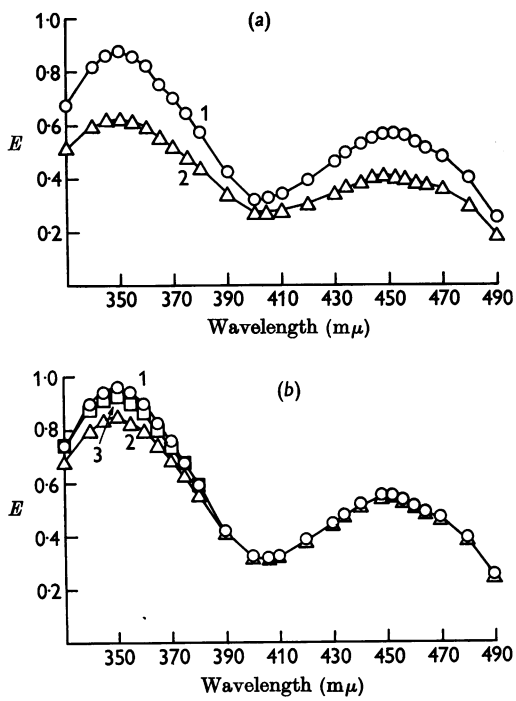

Fig. 4. Absorption spectra of dihydronicotinamide–adenine dinucleotide-peroxidase with flavin–adenine dinucleotide and dihydronicotinamide–adenine dinucleotide in the presence and absence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Conditions and concentrations were as in Fig. 3. (*a*) Spectra obtained in the absence of inhibitor; (*b*) spectra obtained in the presence of inhibitor corrected for the absorption due to the inhibitor. ○ (1), Arithmetical sums of curves 1*a* plus 2*a* and curves 1*b* plus 2*b* in Fig. 3, corrected for dilution and absorption of enzyme; △ (2), spectra observed on tipping FAD from the stopper into cuvettes containing enzyme and $NADH_2$ after completion of spectra 2*a* and 2*b* in Fig. 3, and incubation at room temperature for 50 min.; □ (3), spectrum calculated from that observed under 2*b* in Fig. 3, on the assumption that the inhibitor had been converted into a derivative with no extinction.

the region 340–350 mμ (curve 4, Fig. 3) and the possibility that it may have been converted into the deoxy compound which has negligible absorption in that region (or into some other derivative with no absorption at these wavelengths) cannot be excluded. Curve 3 in Fig. 4 (*b*) shows the absorption spectrum one would obtain from curve 2, if all the added 2-heptyl-4-hydroxyquinoline *N*-oxide had been converted into the deoxy compound. Thus the heptyl *N*-oxide completely inhibited the reduction of FAD, whereas the oxidation of $NADH_2$ was inhibited either by 43 %, if we assume that no change had occurred in the absorption due to the inhibitor (curve 2), or by 84 %, if we assume that all the added 2-heptyl-4-hydroxyquinoline *N*-oxide has been converted into a material without absorption at 340 mμ (curve 3). We cannot distinguish between these two possibilities at the moment and the true state of affairs may lie between these two assumptions. Fig. 2 (*a*) suggests that, on incubating FAD and the heptyl *N*-oxide together anaerobically, there is a decrease in the absorption at 340 mμ equivalent to 40 % of the absorption due to the latter at this wavelength. On the other hand, curve 1*b* of Fig. 3 does not show such an alteration of extinction at 340 mμ on incubating the heptyl *N*-oxide with FAD and enzyme anaerobically. However, with any of these assumptions concerning the extent of extinction change at 340 mμ which can be ascribed to the inhibitor, there remains a decrease in extinction at this wavelength which is presumably due to oxidation of $NADH_2$ without any decrease in extinction at 450 mμ. In other words 2-heptyl-4-hydroxyquinoline *N*-oxide appears to inhibit completely the reduction of FAD in this system without equally inhibiting the oxidation of $NADH_2$.

Non-enzymic model systems

Frisell & Mackenzie (1959) have described the coupled photochemical oxidation of $NADH_2$ and reduction of FMN obtained by illumination with a mercury-vapour lamp. We found that in such an experiment the heptyl *N*-oxide inhibited equally the oxidation of $NADH_2$ and the reduction of FMN (Table 3).

Isenberg & Szent-Györgyi (1958) have shown that FMN can form stabilized free radicals or charge-transfer complexes with proteins and certain amino acids. Using their technique, we could not detect a complex between FMN and 2-heptyl-4-hydroxyquinoline *N*-oxide. However, the conditions of our experiment differed from those used by Isenberg & Szent-Györgyi (1958) in that the maximum concentration of the inhibitor that could be used—because of its low solubility—was 35 μM, whereas the tryptophan concentrations used by the latter authors were 4–10 mM. We therefore examined the effects of 2-heptyl-4-hydroxyquinoline *N*-oxide on the complex-formation between FMN and tryptophan. Fig. 5 shows the reciprocals of the differences in extinction at 500 mμ between FMN and FMN plus tryptophan (○) and between FMN plus the inhibitor and FMN plus tryptophan plus the inhibitor (△) plotted against the reciprocals of the tryptophan concentration. Since the ex-

Table 3. *Effect of 2-heptyl-4-hydroxyquinoline* N*-oxide on oxidation of dihydronicotinamide–adenine dinucleotide and reduction of flavin mononucleotide by illumination*

(*a*) Cuvette contents were (final concentrations): potassium phosphate buffer, pH 7·0, 33 mM; $NADH_2$, 65 μM; FMN, 31·4 μM; 2-heptyl-4-hydroxyquinoline *N*-oxide, where indicated, 21 μM. The total volume was 3·0 ml. All measurements were made under anaerobic conditions. Extinctions at 340 and 445 mμ were measured in the Uvispek spectrophotometer, before and after illumination by a Hanovia mercury-vapour lamp, with a Woods glass filter, for 5 min. at a distance of 10 cm. from the lamp.

	E_{340}		E_{445}	
	Without inhibitor	With inhibitor	Without inhibitor	With inhibitor
Before illumination				
Complete system	0·602	0·769	0·388	0·366
Without $NADH_2$	0·193	0·361	0·384	0·362
Extinction of $NADH_2$	0·409	0·408	0·004	0·004
After illumination	0·328	0·553	0·154	0·181
Change on illumination	−0·274	−0·216*	−0·234	−0·185*

(*b*) Amounts of $NADH_2$ and FMN were calculated from the extinctions given above, the millimolar extinction coefficients being taken as 6·22 (at 340 mμ) for $NADH_2$ and 12·2 (at 450 mμ) for FMN.

	$NADH_2$ oxidized (μm-moles)	FMN reduced (μm-moles)	Ratio: $\frac{NADH_2 \text{ oxidized}}{FMN \text{ reduced}}$
Without inhibitor	132	57·6	2·29
With inhibitor	104·5	45·3	2·30

* Inhibition 21 %.

tinction at 500 mμ of FMN alone (i.e. the reference cuvette) is not depressed by the addition of the heptyl *N*-oxide, Fig. 5 indicates that the inhibitor increases the amount of, or the extinction of, material with absorption at 500 mμ which appears on addition of tryptophan. The fact that the two lines have the same intercept on the $1/\Delta E$ axis suggests that the same complex is formed, or at least that the same chromophoric group is involved. Isenberg & Szent-Györgyi (1958) showed that the two lines obtained from the extinction differences at 500 mμ (i) between FMN and FMN plus tryptophan and (ii) between FMN and FMN plus serotonin also have the same intercept on the $1/\Delta E$ axis. They suggested that the measured extinction increases are due to a flavin semiquinoid with a unique molar extinction coefficient at 500 mμ, whether in the presence of tryptophan or serotonin.

Thus 2-heptyl-4-hydroxyquinoline *N*-oxide apparently interacts with FMN so as to prevent its functioning as an electron acceptor for $NADH_2$ in the non-enzymic light-activated system, and to facilitate complex-formation between FMN and tryptophan. In the $NADH_2$-peroxidase system, it apparently inhibits the transfer of electrons between $NADH_2$ and FAD. It seems likely, therefore, that, by reacting with flavins, the inhibitor can alter their properties so that their behaviour in oxidation–reduction reactions is different.

This evidence for interaction between flavins and 2-heptyl-4-hydroxyquinoline *N*-oxide does not, however, satisfactorily explain the selective inhibitory action of the latter compound on a large variety of biological oxidation–reduction systems. Its effect on flavoprotein enzymes has been studied in the following instances: (i) $NADH_2$ dehydrogenase isolated from *B. subtilis* (Kogut & Lightbown, 1959), which is a flavoprotein on the evidence of its absorption spectrum and sensitivity to atebrin, although it is not activated by either FAD or FMN; (ii) succinate dehydrogenase and diaphorase of heart-muscle preparations (Lightbown & Jackson, 1956, Jackson & Lightbown, 1958); (iii) glucose oxidase (notatin) prepared by the method of Coulthard *et al.* (1945) (M. Kogut & J. W. Lightbown, unpublished work). None of these was sensitive to 2-heptyl-4-hydroxyquinoline *N*-oxide. In early work all systems that were sensitive to the heptyl *N*-oxide involved cytochrome catalysis. Experiments with non-enzymic model systems suggest that the inhibitor can affect the properties of certain cytochrome and other haem compounds so as to alter their behaviour in oxidation–reduction reactions.

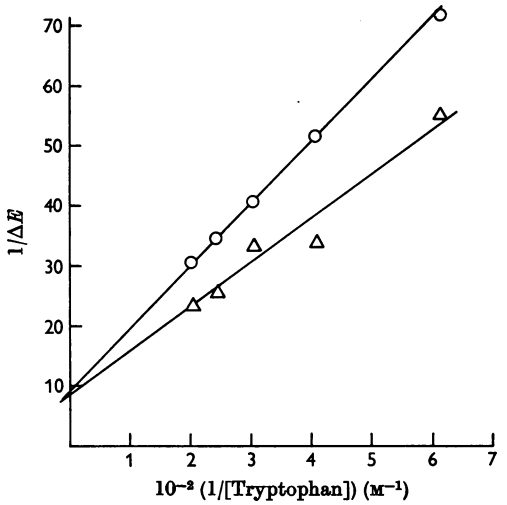

Fig. 5. Reciprocals of extinction difference between flavin mononucleotide and flavin mononucleotide plus tryptophan against reciprocals of tryptophan concentration, in the presence and absence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Final concentrations of cuvette contents were: potassium phosphate buffer, pH 7·0, 33 mM; FMN, 0·05 mM; inhibitor, where indicated, 35 μM; NaOH, 0·1 mN; tryptophan, as indicated. The total volume was 3·0 ml. ○, Without inhibitor; △, with inhibitor present in both cuvettes.

Peroxidative oxidation of reduced 2,6-dichlorophenol-indophenol catalysed by haem compounds

When hydrogen peroxide, or methyl hydrogen peroxide, is added to reduced 2,6-dichlorophenol-indophenol, the dye is slowly oxidized. This oxidation is greatly accelerated by very low concentrations of certain haem compounds, including mammalian cytochrome *c*, *Pseudomonas* cytochrome 553, haematin and horse-radish peroxidase. The reaction has been followed spectrophotometrically by observing the increase in extinction at 630 mμ. By having reduced dichlorophenol-indophenol and peroxide in the blank cuvette allowance is made for auto-oxidation. Table 4 shows the effect of hydrogen peroxide and cytochrome *c* concentrations on the rate of dye oxidation in the presence and the absence of the inhibitor, and the effects of different concentrations of the inhibitor in the presence of constant concentrations of reduced dye, cytochrome *c* and hydrogen peroxide. The rate of reaction is proportional to concentrations of cytochrome *c* and of hydrogen peroxide, both in the absence and presence of the inhibitor, whereas the percentage inhibition produced at different concentrations of these reactants is constant. The reaction rate was independent of the concentration of reduced dye over the concentration range that was practicable. Antimycin A, at concentrations which inhibited the succinoxidase activity of heart-muscle preparations by 90 %, had no effect on this

Table 4. *Effects of concentrations of cytochrome* c, *hydrogen peroxide and 2-heptyl-4-hydroxyquinoline* N*-oxide on the peroxidation of 2,6-dichlorophenol-indophenol*

Concentrations are final concentrations in the cuvettes. Cytochrome *c* was added last at zero time. Extinctions were measured at 630 mμ. The reaction rate is expressed as ΔE/min. Blank cuvettes contained all reagents except cytochrome *c*.

	Expt. A			Expt. B			Expt. C		
Concn. of potassium phosphate buffer, pH 7·0 (mM)	30			45			30		
Concn. of H_2O_2 (mM)	Varied (see below)			0·4			0·53		
Concn. of cytochrome *c* (μM)	3·8			Varied (see below)			1·9		
Concn. of dichlorophenol-indophenol (μM)	0·053			0·08			0·053		
Concn. of 2-heptyl-4-hydroxyquinoline *N*-oxide (μM)	26			27			Varied (see below)		
	Reaction rate			Concn. of cytochrome *c* (μM)	Reaction rate		Concn. of inhibitor (μM)	Reaction rate	Inhibition (%)
Concn. of H_2O_2 (mM)	Without inhibitor	With inhibitor	Inhibition (%)		Without inhibitor	With inhibitor	Inhibition (%)		
0·16	0·025	0·015	40	1·75	0·035	0·023	34	0	0·095	—
0·26	0·042	0·027	36	3·50	0·070	0·047	33	6·6	0·078	18
0·52	0·087	0·078	45	5·35	0·105	0·072	32	12·8	0·071	25
1·06	0·130	0·095	27	7·25	0·143	0·096	33	25·8	0·057	40
				9·00	0·177	0·120	32	51·4	0·052	45
				11·00	0·212	0·142	33			
				12·50	0·247	0·165	33			

reaction. Measurements of the extinction of hydrogen peroxide at 230 mμ in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide showed that there was no interaction between these two substances, nor was the absorption of dye at 630 mμ affected by the presence of the inhibitor.

Replacement of cytochrome *c* in the above system by haematin gave similar results. However, when crystalline horse-radish peroxidase (at 1/10 000 concentration) was used the activity was proportional to the concentrations of reduced dye, of hydrogen peroxide and of enzyme, but the inhibitor was without any effect over the concentration ranges of reactants which were practicable.

Pseudomonas cytochrome 553 is spectroscopically similar to mammalian cytochrome *c* but differs from it in some important respects (Kogut, 1957). This cytochrome could also promote a peroxidative oxidation of reduced dichlorophenol-indophenol. In this case, however, methyl hydrogen peroxide had to be used because the cytochrome preparation still contained some catalase. Fig. 6 shows the effect of 2-heptyl-4-hydroxyquinoline *N*-oxide on this reaction, compared with the same reaction in which a similar concentration of cytochrome *c* was used. (The concentration of the *Pseudomonas* cytochrome was adjusted on the basis of its absorption at 553 mμ, assuming the same molar extinction coefficient as cytochrome *c* at 550 mμ.) There was much less inhibition with the *Pseudomonas* cytochrome than with the cytochrome *c*. Further, when

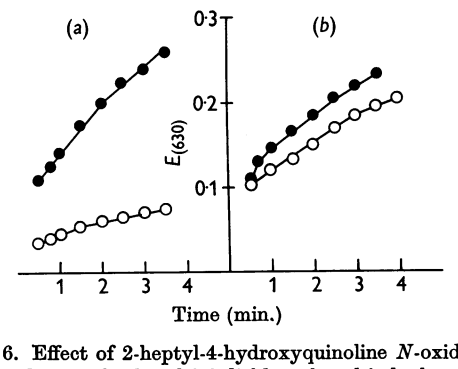

Fig. 6. Effect of 2-heptyl-4-hydroxyquinoline *N*-oxide on the oxidation of reduced 2,6-dichlorophenol-indophenol by methyl hydrogen peroxide in the presence of cytochromes. Final concentrations of cuvette contents were: potassium phosphate buffer, pH 7·0, 30 mM; methyl hydrogen peroxide, 1·0 mM, reduced 2,6-dichlorophenol-indophenol, 71 μM; NaOH, 0·1 mN; inhibitor, where indicated, 30·0 μM. (*a*) Mammalian cytochrome *c* (5·0 μM); (*b*) *Pseudomonas* cytochrome 553 (approx. 5·0 μM). The total volumes were 3·0 ml., and measurements were made under anaerobic conditions. Blanks contained all reagents except cytochromes, and reactions were started by addition of cytochrome from the hollow stopper of the Thunberg tube attachment. ○, With inhibitor; ●, without inhibitor.

Table 5. *Effect of 2-heptyl-4-hydroxyquinoline* N-*oxide on respiration of extracts from* Pseudomonas *KB* 1

Respiration was measured as oxygen uptake by standard Warburg techniques. Warburg vessels contained (final concentrations): Sørensen's phosphate buffer, pH 7·4, 12 mM; $MnSO_4$, 0·4 mM; $MgSO_4$, 0·4 mM; bacterial extract, 1·0 ml.; substrate, 8·0 mM; inhibitor, as indicated; flasks without inhibitor had equivalent volumes of 1·0 mN-NaOH. The total volume was 2·5 ml. Centre wells contained 0·2 ml. of 10% (w/v) NaOH and folded filter paper. Substrates were tipped from the side arm after temperature equilibration for 10 min. and oxygen uptakes were measured for 40–60 min. The temperature was 30° and the gas phase air; rates are expressed as μl. of O_2 uptake/hr. and were calculated from the linear portions of the oxygen-uptake curves, usually 10–40 min. after the addition of substrates.

Expt. no.	Concn. of inhibitor (μM)	O_2 uptake (μl./hr.) No substrate	Succinate	DL-Malate
1	0	36·4	162	214
	15·8	35·0	163	.
	47·4	.	.	174
2	0	37·6	99·0	212
	47·4	40·6	96·0	.
	79·0	41·6	83·4	180

ultrasonic extracts of *Pseudomonas* KB 1 catalysed the oxidation of malate or succinate, the heptyl *N*-oxide had only negligible effect on oxygen uptake at concentrations which completely inhibited the succinoxidase activity of heart-muscle preparations (Table 5). This is in contrast with the marked effect of the heptyl *N*-oxide on the respiration of *B. subtilis*, *B. pumilis*, *Staph. aureus*, and extracts of *E. coli* and *P. vulgaris* (Lightbown & Jackson, 1956). It may perhaps be significant that the heptyl *N*-oxide was discovered and isolated as a product of a *Pseudomonas* species (Lightbown, 1954).

Analysis of the inhibition by 2-heptyl-4-hydroxyquinoline N-*oxide of the cytochrome* c*-catalysed peroxidation of 2,6-dichlorophenol-indophenol*

When the oxidation of reduced dichlorophenol-indophenol with hydrogen peroxide was catalysed by cytochrome *c*, the latter remained at a constant level of reduction until most of the reduced dye had been oxidized (Fig. 7). In the presence of the heptyl *N*-oxide, the cytochrome *c* gave a higher extinction at 550 mμ, i.e. remained at a more reduced level, for longer than in the control. When reduced dye was added to oxidized cytochrome *c*, the reduction of the cytochrome was too fast to measure, both in the absence and presence of the heptyl *N*-oxide. However, when sodium ascorbate was used as the reducing agent, the rate of reduction of cytochrome *c* could be measured, and this was unaffected by the inhibitor. On the other hand the oxidation of reduced cytochrome *c* by hydrogen peroxide was inhibited by the heptyl *N*-oxide, although to a less extent than the cytochrome-mediated peroxidation of reduced dye. Lightbown & Jackson (1956) showed that dichlorophenol-indophenol can mediate the oxygen uptake of heart-muscle succinoxidase in the presence of

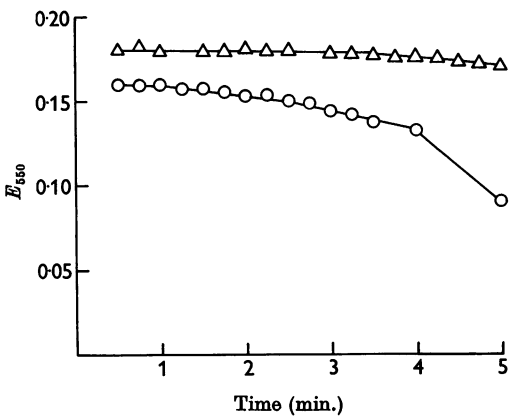

Fig. 7. Effect of 2-heptyl-4-hydroxyquinoline *N*-oxide on the level of reduction of cytochrome *c* in the presence of reduced 2,6-dichlorophenol-indophenol and hydrogen peroxide. Final concentrations of cuvette contents were: potassium phosphate buffer, pH 7·0, 30 mM; hydrogen peroxide, 0·29 mM; reduced 2,6-dichlorophenol-indophenol, 57 μM; cytochrome *c*, 9·0 μM; NaOH, 0·1 mM; inhibitor, where indicated, 27·0 μM. The total volume was 3·0 ml. Blank cuvettes contained all reagents except cytochrome *c*. Readings of extinctions were taken at 550 and 630 mμ. Recorded values are extinctions at 550 mμ due to cytochrome *c*; the extinctions at 550 mμ due to 2,6-dichlorophenol-indophenol, calculated from the readings at 630 mμ, have been subtracted. △, With inhibitor; ○, without inhibitor.

the heptyl *N*-oxide and that under these conditions cytochrome *c* can be reduced and oxidized. This suggests that the dye might act as a shunt for the inhibited reaction steps between cytochromes *b* and *c*. Although, therefore, in the heart-muscle preparations neither the oxidation of cytochrome *c* by cytochrome oxidase nor its reduction by either *p*-phenylenediamine or reduced dichlorophenol-

indophenol are inhibited by the heptyl *N*-oxide, the above experiments show that some interaction or combination between the latter and cytochrome *c* can occur.

Visual spectroscopy, either at room temperature or by the liquid-air technique of Keilin & Hartree (1949), showed no effect of the inhibitor on cytochrome *c* whether in the state of oxidation as received from the manufacturer, reduced by hydrogen or by sodium dithionite, or in the presence of hydrogen peroxide. Absorption spectra of almost completely reduced cytochrome *c* and of the same cytochrome *c* solution in the presence of the heptyl *N*-oxide did, however, show small differences, and we therefore obtained difference spectra of cytochrome *c* against cytochrome *c* plus 2-heptyl-4-hydroxyquinoline *N*-oxide with a Hilger Uvispek spectrophotometer with a carefully calibrated wavelength scale and specially matched cuvettes. The sample of cytochrome *c* used in this experiment was partially reduced (26·5%). Curves 1 and 1*a* in Fig. 8 show the difference spectra observed with two different concentrations of the heptyl *N*-oxide and the one concentration (10 μM) of cytochrome *c*. (Thus the concentration of reduced cytochrome *c* in the cuvette was 2·65 μM.) Both curves show the three absorption peaks of cytochrome *c*, and the heights of the peaks in the two difference spectra are proportional to the concentrations of the heptyl *N*-oxide present. Such difference spectra indicate changes in the concentration of reduced cytochrome *c* due to the presence of the inhibitor. From the height of the α-peak in curve 1, one can calculate that either 1·15 μm-moles of reduced cytochrome *c*/ml. could have disappeared from the test cuvette, or that 1·73 μm-moles of reduced cytochrome *c*/ml. could have become oxidized in the presence of the heptyl *N*-oxide. Curves 2 and 3 are difference spectra calculated on the basis of these two assumptions respectively. Of these, curve 2 most closely resembles the observed difference spectrum (curve 1), but it differs from this, too, in the following respects: If the heptyl *N*-oxide had removed all the α-peak absorption of a given amount of reduced cytochrome *c*, and our calculation is based on this assumption, considerably less than the total absorption of this amount of reduced cytochrome *c* at the γ-peak has disappeared, as well as considerably more than the absorption of the calculated amount of reduced cytochrome *c* at the β-peak and in between. This would suggest that the heptyl *N*-oxide removes, or alters, some of the oxidized cytochrome *c* as well as some of the reduced pigment. The difference spectrum which one would obtain if 1·15 μm-moles/ml. had been removed from the oxidized portion of cytochrome *c* present was also calculated, but this did not fit the observed difference spectrum and was therefore not included in Fig. 8. The height of the α-peak of the difference spectrum shows that about 1·15 μm-moles of cytochrome *c*/ml. were affected in the presence of 117 μm-moles of the heptyl *N*-oxide/ml., and the heights of the peaks of two different spectra measured in the presence of the same concentration of cytochrome *c* and different concentrations of the inhibitor were proportional to the latter. This apparent 100-fold excess of the inhibitor concentration over the amount of cytochrome *c* affected could be due to a very low affinity of the heptyl *N*-oxide for cytochrome *c*, or to the formation of a product which still had absorption at the α-peak, but of a lower extinction coefficient. Similar considerations hold for the γ-peak. Margoliash (1954*b*) reported the presence in highly purified cytochrome *c* preparations of a component which is spectroscopically indistinguishable from cytochrome *c*, but has altered properties. The possibility that such material was present in the cytochrome *c* preparations which we used and reacted differently with the heptyl *N*-oxide from ordinary cytochrome *c* must be borne in mind.

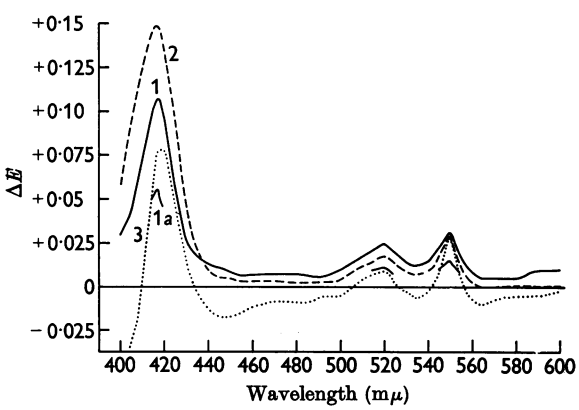

Fig. 8. Difference spectra of mammalian cytochrome *c* alone and in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide. Final concentrations of cuvette contents were: potassium phosphate buffer, pH 7·0, 45 mM; cytochrome *c*, 10·0 μm-moles/ml.; NaOH, 0·1 mN; inhibitor, as indicated. 1, Observed difference spectrum for cytochrome *c* minus cytochrome *c* in the presence of 117 μM-2-heptyl-4-hydroxyquinoline *N*-oxide; 1*a*, observed difference spectrum for cytochrome *c* minus cytochrome *c* in the presence of 58 μM-2-heptyl-4-hydroxyquinoline *N*-oxide; 2, calculated difference spectrum for 7·35 μm-moles of oxidized cytochrome *c*/ml. plus 2·65 μm-moles of reduced cytochrome *c*/ml. in cuvette one, and 7·35 μm-moles of oxidized cytochrome *c*/ml. plus 1·50 μm-moles of reduced cytochrome *c*/ml. in cuvette two; 3, calculated difference spectrum for 7·35 μm-moles of oxidized cytochrome *c*/ml. plus 2·65 μm-moles of reduced cytochrome *c*/ml. in cuvette one, and 9·08 μm-moles of oxidized cytochrome *c*/ml. plus 0·92 μm-mole of reduced cytochrome *c*/ml. in cuvette two.

DISCUSSION

The evidence presented here concerning the inhibitory action of 2-heptyl-4-hydroxyquinoline *N*-oxide on a variety of oxidation–reduction systems, together with that reviewed in the introduction, is summarized in Table 6. Inspection of this reveals no simple clue to the mode of action of this inhibitor. There are no obvious factors common to all the reactions which are sensitive to this agent but absent from those which are unaffected. Yet the selective action of this material, together with its very high activity in some of the reactions, must involve chemical or physical specificities in the reactions of sensitive systems, rather than general antioxidant properties. That such specificities are difficult to elucidate is not surprising with such complex systems as heart-muscle preparations, bacterial extracts or phosphorylating chloroplasts. The fact that all these do, or may, contain cytochromes has led certain authors to interpret inhibition by 2-heptyl-4-hydroxyquinoline *N*-oxide as positive evidence for participation of cytochromes in electron-transport systems (Iida & Tanigushi, 1959; Sadana & McElroy, 1957); this compound has even been called (without justification) 'the specific inhibitor for cytochrome b_1' (Tanigushi & Itagaki, 1960; Itagaki, Fujita & Sato, 1961). Any hypothesis that cytochromes might be the specific sites for the inhibition by heptyl *N*-oxide must now be discarded, because a system which is free from cytochromes, namely the purified $NADH_2$ peroxidase from *B. subtilis*, is as sensitive to the inhibitor as the heart-muscle or chloroplast preparations. Further, there is evidence that 2-heptyl-4-hydroxyquinoline *N*-oxide can interact or combine with flavins, under certain conditions, in such a way as to alter their functions as electron-carriers. Certain features of this interaction can be deduced: it is not a general inhibition of flavin-catalysed electron transport since several flavoprotein enzymes, including the unresolved $NADH_2$ peroxidase which does not respond to added FAD, are not inhibited. One possibility is that the heptyl *N*-oxide prevents the combination between enzyme protein and flavin, i.e. between the $NADH_2$-peroxidase apoenzyme and FAD, by combining with one of the two components. If such is the case one might expect that preincubation of FAD and apoenzyme would protect from the inhibitory action of the heptyl *N*-oxide, though this would depend on the kind and degree of binding between enzyme and FAD. This has not been observed after preincubation for 15 min. Provided

Table 6. *Sensitivities of various oxidation–reduction systems to* 2-*heptyl*-4-*hydroxyquinoline* N-*oxide*

The references quoted are as follows: 1, Lightbown & Jackson (1956); 2, Smith & Baltscheffsky (1959); 3, Baltscheffsky (1959); 4, Avron (1961); 5, Vernon (1959); 6, Vernon & Ash (1959); 7, J. W. Lightbown (unpublished work); 8, M. Kogut (unpublished work); 9, Kogut & Lightbown (1960).

		Inhibition by 2-heptyl-4-hydroxyquinoline *N*-oxide	Approx. concn. giving 50% inhibition (μM)	Ref.
Cytochrome electron-transport systems	Heart-muscle preparation	+	1·0	1
	B. pumilis	+	1·0	1
	E. coli	+	20·0	1
	Pseudomonas	Very slight	.	.
	R. rubrum	−	.	2
Photosynthetic systems	Phosphorylations			
	Spinach chloroplasts	+	1·0	3, 4
	R. rubrum	+	1·0	2
	Reductions			
	Spinach chloroplasts	+	1·0	3, 4
	R. rubrum	+	10·0	5, 6
Flavoprotein enzymes	Succinate dehydrogenase	−	.	1
	Diaphorase	−	.	1
	Glucose oxidase	−	.	7
	$NADH_2$ dehydrogenase (*B. subtilis*)	−	.	8
Peroxidative enzymes	$NADH_2$ peroxidase (*B. subtilis*)*	+	1·0	9
	Horse-radish peroxidase	−	.	.
	Catalase	−	.	.
Model systems	H_2O_2, dye	−	.	.
	Haematin, H_2O_2, dye	+	50·0	.
	Cytochrome *c*, H_2O_2, dye	+	50·0	.
	Pseudomonas cytochrome, methyl hydrogen peroxide, dye	−	.	.
	FMN, $NADH_2$, light	+	.	.

* Also a flavoprotein enzyme.

that there were reversible combination between enzyme and the heptyl *N*-oxide at the site of attachment of FAD, one would also expect the inhibition to be competitive with FAD. However, increasing concentrations of FAD do not reverse the inhibition due to the heptyl *N*-oxide.

The experiment represented in Fig. 4 (*a*) shows that $NADH_2$ peroxidase can catalyse the transfer of electons from $NADH_2$ to FAD [Fig. 2 (*b*) shows that no such transfer occurs in the absence of enzyme]. With an enzyme-protein concentration of 10 μg./ml., 41·0 μm-moles of $NADH_2$/ml. were oxidized and 14·1 μm-moles of FAD/ml. reduced. The purity of the enzyme preparation is not known but, assuming a molecular weight of about 100 000, its molar concentration can be taken to have an upper limit of approx. 0·1 μm-mole/ml. Since the enzyme can thus, in the absence of the heptyl *N*-oxide, catalyse the reduction of FAD in excess of its own concentration, there must be a dissociable combination between flavin and enzyme. If the inhibitor acted simply by preventing the combination between FAD and enzyme, no inhibition or only partial inhibition would be expected in the experiment depicted in Fig. 4, because the molar ratio FAD:2-heptyl-4-hydroxyquinoline *N*-oxide was 4. Further, on such an hypothesis, there should also be exactly equal inhibition of $NADH_2$ oxidation and FAD reduction, as was found in the non-enzymic light-catalysed reaction of $NADH_2$ with FMN.

It seems more likely that 2-heptyl-4-hydroxyquinoline *N*-oxide can interact with FAD in such a way that the latter can still combine with the enzyme protein, but that the resulting material has different properties from the complex formed in the absence of the inhibitor. Although there was no direct spectroscopic evidence for complex-formation between flavins and the heptyl *N*-oxide, such reactions cannot be ruled out (Ainsworth & Rabinowitch, 1960). The fact that the inhibitor enhanced the formation of a specific flavin derivative in the presence of tryptophan must mean either that it can itself form a complex of the kind described by Isenberg & Szent-Györgyi (1958), or that it alters the FMN in such a way that it becomes more susceptible to the action of tryptophan. When the oxidation of $NADH_2$ by enzyme and hydrogen peroxide in the presence of catalytic concentrations of FAD is inhibited by the heptyl *N*-oxide, the complex formed in the presence of the inhibitor must be inactive, i.e. not convertible into the original FAD or enzyme–FAD complex, and unable to accept further electrons from $NADH_2$. Dolin (1960) described the formation of a ternary complex of $NADH_2$, enzyme and FAD in a similar enzyme, namely the FAD-containing $NADH_2$ peroxidase from *Streptococcus faecalis*. The fact that preparations of $NADH_2$ peroxidase from *B. subtilis* which show no stimulation by FAD are not inhibited by the heptyl *N*-oxide suggests that the original enzyme-bound flavin is protected from interaction with the inhibitor. This may be due to differences in the steric configuration of different enzyme preparations, differences in the mode and degree of binding between protein and flavin, or to differences between added FAD and the real prosthetic group of the unresolved enzyme.

Our work has also demonstrated interactions between 2-heptyl-4-hydroxyquinoline *N*-oxide and some, but not all, of a number of haem compounds, including mammalian cytochrome *c*. Quantitatively, the effect of the heptyl *N*-oxide on isolated mammalian cytochrome *c* is of a lower order than its effect on the respiratory system of heart muscle. Qualitatively, it is difficult to reconcile the evidence with any simple mode of action. In the cytochrome-mediated dye-peroxidation system, the inhibitor appears to increase the steady level of reduction of cytochrome *c* (increased absorption at 550 mμ); or, in other words, to block the oxidation part of the reaction. From the difference spectra, it appears to act by decreasing the absorption due to reduced cytochrome *c* (and some oxidized cytochrome *c* as well). It seems possible that in the dye-peroxidation system interaction between cytochrome, dye and peroxide may occur in such a way that the effect of the heptyl *N*-oxide on the oxidation–reduction properties of the cytochrome is then different from that on cytochrome alone. In the heart-muscle system, the site of inhibition by the heptyl *N*-oxide is between cytochromes *b* and *c*, and it is the reduction of cytochrome *c* that is inhibited, by concentrations of the inhibitor at least 10 times lower than those used in the above experiments. Some as yet unknown flavoprotein enzyme between cytochromes *b* and *c* may be the site of action.

The results reported above can therefore only be interpreted as evidence for the general possibility of interaction between cytochromes and the heptyl *N*-oxide. The question remains whether these interactions with some haem compounds and with flavins could have a common mechanism associated with the detailed electronic configuration of these compounds or certain portions of them. Considerable evidence for free-radical participation in a number of relevant biological oxidation–reduction systems has appeared in recent years (Ehrenberg, 1957; Ehrenberg & Ludwig, 1958; Commoner *et al.* 1957; Yamazaki, Mason & Piette, 1959; Bray, Malmström & Vänngård, 1959; Sands & Beinert, 1960; Beinert & Sands, 1960). Dr R. C. Smith and Miss M. J. Hill of Guy's Hospital Medical School (personal communication) have investigated the electron-spin-resonance spectrum of 2-heptyl-4-

hydroxyquinoline *N*-oxide and found no evidence for free radical formation in the solid material before and after ultraviolet irradiation for 4 hr. (less than 6×10^{-6} free radicals/molecule). They also found that it had no effect on the formation of the *p*-benzosemiquinone radical (Venkataraman & Fraenkel, 1955). Examination of the possible effects of the heptyl *N*-oxide on the electron-spin-resonance spectra of more biological systems would, however, be of interest.

An understanding of the mechanism of the inhibitory action of 2-heptyl-4-hydroxyquinoline *N*-oxide would lead to a better understanding of biological electron-transport mechanisms. Perhaps inhibition should not be thought of as an action on a specific intermediate in electron transport (e.g. cytochromes) but rather as an effect on specific intra- or inter-molecular interactions.

SUMMARY

1. The streptomycin antagonist 2-heptyl-4-hydroxyquinoline *N*-oxide inhibited the oxidation of dihydronicotinamide–adenine dinucleotide ($NADH_2$) by hydrogen peroxide or methyl hydrogen peroxide, catalysed by a $NADH_2$ peroxidase, isolated in purified form from *Bacillus subtilis*. This enzyme is a flavoprotein which, in its purified form, required flavin–adenine dinucleotide (FAD) for activity. The inhibition by 2-heptyl-4-hydroxyquinoline *N*-oxide was only observed in enzyme preparations which showed requirement for FAD.

2. The inhibition of $NADH_2$ peroxidase by the 2-heptyl-4-hydroxyquinoline *N*-oxide was non-competitive with respect to $NADH_2$, peroxide or added FAD.

3. Experiments in which the reduction of FAD by $NADH_2$ catalysed by $NADH_2$ peroxidase was measured indicated that, in the presence of enzyme, 2-heptyl-4-hydroxyquinoline *N*-oxide altered the oxidation–reduction properties of FAD.

4. Experiments with non-enzymic model systems indicated that 2-heptyl-4-hydroxyquinoline *N*-oxide also altered the oxidation–reduction properties of free flavins and could enhance the formation of a semiquinoid complex formed by flavin mononucleotide in the presence of tryptophan.

5. Experiments with model systems in which the oxidation of reduced 2,6-dichlorophenol-indophenol is catalysed by cytochrome *c*, haematin, horse-radish peroxidase or a bacterial cytochrome from *Pseudomonas*, showed that 2-heptyl-4-hydroxyquinoline *N*-oxide inhibited the reaction in the presence of the first two, but not with the other two. The inhibition appeared to be localized on the oxidation side of the haem compound.

6. Difference spectra of mammalian cytochrome *c* and cytochrome *c* in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide showed that some of the absorption of cytochrome *c* disappeared in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide. The effect, though proportional to concentrations of 2-heptyl-4-hydroxyquinoline *N*-oxide, was small and suggested either a very low affinity of cytochrome *c* for 2-heptyl-4-hydroxyquinoline *N*-oxide or, alternatively, the formation of a complex between 2-heptyl-4-hydroxyquinoline *N*-oxide and cytochrome *c* with a spectrum similar to that of cytochrome *c* but with different extinction coefficients.

We thank Dr F. A. Holton (now at the Royal Veterinary College) of the Experimental Radiopathology Research Unit, London, W. 12, for his helpful advice and use of his spectrophotometer in obtaining the difference spectra depicted in Fig. 8.

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The Existence of Two Routes for Incorporation of Amino Acids into Protein of Isolated Rat-Liver Mitochondria

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(*Received* 15 *January* 1962)

It has been demonstrated (Roodyn, Reis & Work, 1961; Roodyn, Suttie & Work, 1962) that isolated, washed, rat-liver mitochondria can incorporate amino acids into protein. Amino acid incorporation into mitochondrial protein is dependent on the maintenance of oxidative phosphorylation and, under optimum conditions (Roodyn *et al.* 1961), the rate of incorporation was linear for about 1 hr. Incorporation could, however, be maintained for longer periods if the medium were changed at regular intervals.

None of the many changes of incubation conditions which were tried led to any increase in the initial rate of synthesis; it seemed worth while, therefore, to try to extend the period of synthesis beyond the 3 hr. already achieved, in the hope of demonstrating net synthesis of protein. It was possible to extend the period of incorporation by frequent changes of medium; unfortunately, there was a progressive change in the characteristics of the incorporation system, so that it was no longer dependent on oxidative phosphorylation. This secondary process of amino acid incorporation was completely resistant to various inhibitors that had prevented incorporation in the primary, phosphorylation-dependent, system. Evidence is now presented which strongly suggests that the secondary, non-phosphorylative, incorporation is due to transpeptidation.

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METHODS

Isolation of mitochondria. Mitochondria were isolated from rat liver as described by Roodyn *et al.* (1961). They were washed with four changes of sucrose–EDTA–nicotinamide as described previously, and the pellet from the fourth wash was suspended either in 0·25 M-sucrose or in the sucrose–EDTA–nicotinamide mixture of Roodyn *et al.* (1961).

Incubation conditions. For experiments on the phosphorylation-dependent incorporation, the incubation conditions were those given by Roodyn *et al.* (1961) with 'medium B' [0·1 M-sucrose, 0·04 M-KCl, 8 mM-$MgSO_4$, 1·3 mM-EDTA (disodium salt), 0·02 M-nicotinamide, 0·01 M-potassium succinate, 0·016 M-potassium phosphate, 4 mM-AMP, 0·5 mM-NAD, 50 μg. of synthetic amino acid mixture/ml.; pH adjusted to 7–7·2 with KOH]. In the experiments where non-phosphorylative incorporation was examined, the mitochondria were incubated in a medium containing only sucrose (0·12 M) and tris–HCl buffer, pH 7·5 (0·05 M with respect to tris). In all experiments penicillin G (100–150 units/ml.) was present.

Bacterial contamination. After incubation a sample of the mitochondrial suspension was centrifuged at 8000*g* for 10 min. to sediment both bacteria and mitochondria. The pellet was resuspended in fresh 0·25 M-sucrose and samples were plated on blood–agar. Colony counts were made after 24 hr. at 37°.

Treatment of radioactive proteins

All proteins were washed and extracted by the standard method of Simkin & Work (1957) before radioactivities were determined.

Removal of non-peptide bound amino acid. As a check