53. THE SITE OF ACTION OF NARCOTICS IN RESPIRATORY PROCESSES

BY M. MICHAELIS AND J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(Received 7 March 1941)

WHILST it is well known that narcotics as a general rule inhibit respiratory processes, their effects being mostly confined to the dehydrogenases, little is known as yet of the mechanism by which narcotics, when used at the small concentrations which have biological significance, exert their inhibitory effects.

It has been shown [Quastel & Wheatley, 1932; Jowett & Quastel, 1937; Jowett, 1938] that narcotics, such as chloretone and the barbiturates, greatly reduce the oxygen uptake of brain tissue (whether in the form of a mince or of tissue slices), as well as that of other organs, when the tissue respire in media containing glucose, sodium lactate or sodium pyruvate. Definite inhibitions of oxidative processes occur at concentrations of narcotics which bring about deep narcosis, the inhibitions being reversible *in vitro* [Quastel & Wheatley, 1934; Jowett, 1938]. McClure *et al.* [1939] have demonstrated the existence of anoxia in the central nervous system during the anaesthesia brought about by barbiturates and other narcotics. The fact that glucose oxidation is quantitatively the most important respiratory process in brain *in vivo* [see Quastel, 1939] makes it likely that *in vivo*, as well as *in vitro*, narcotics exert inhibitory effects on glucose oxidation by the brain.

Narcotics do not inhibit all oxidative processes to the same extent. Urethane, for example, at concentrations which inhibit succinic dehydrogenase has no effect on xanthine oxidase [Sen, 1931]. Barbiturates, chloretone and hyoscine, at concentrations which strongly inhibit glucose oxidation in the brain, have little effect on the oxidations by the same tissue of sodium succinate or of *p*-phenylenediamine [Quastel & Wheatley, 1932].

Narcotics include a large variety of structural types, such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides etc., and it is evident that their common characteristic of inducing narcosis must depend upon certain physico-chemical characters which they have in common rather than upon the possession of any special chemical structure. The well-known Overton-Meyer theory suggests one important factor in the determination of narcotic activity, i.e. the relative solubilities in lipoid and non-lipoid substances. It is evident, however, that if the nonpolar-polar character of a substance may determine, or partly determine, its entry into a cell, it gives no obvious clue to the mechanism of action of the narcotic once it is within the cell.

It is well known that many enzymic reactions taking place in cell-free extracts are inhibited by narcotics if these are present at sufficiently high concentrations. The mechanism of this inhibition is usually held to be due to a non-specific adsorption on the enzyme surface. So far as respiratory processes are concerned, the relative lack of inhibitory action of narcotics on the indophenol (or cytochrome) oxidase has made it appear that the dehydrogenases are the most sensitive structures to narcotic inhibition.

A study [Jowett & Quastel, 1937; Jowett, 1938] of the inhibitions of respiration in intact brain tissue secured by narcotics has revealed the following facts.

(1) The inhibitions for a large class of narcotics vary little with time and are reversible, i.e. if the narcotic is washed away from the tissue the latter tends to respire again at the normal rate. Such a class includes urethane, chloral, chloretone, barbiturates such as luminal and evipan, avertin (tribromoethyl alcohol) and Mg ions. The narcotic appears to reach a mass action equilibrium with some component of the respiratory system, the inhibition being given by a simple mass action equation.

(2) The steady reversible inhibitions depend on cation activity and particularly on the potassium ion concentration of the medium bathing the brain tissue. If the potassium concentration becomes low, say one-third of that present in blood, the inhibitions increase with time, often considerably. The effect of higher potassium concentration is to stabilize the inhibition. This phenomenon would be explained if, in the presence of low concentrations of potassium ions, a factor important in influencing narcotic inhibitions diffuses from the brain slices into the outer medium.

(3) The inhibition of respiration depends on the concentration of narcotic and not upon the concentration of the substrate which is oxidized.

When, however, anaerobic oxidations accomplished by brain tissue are considered, e.g. the oxidation of lactic acid by methylene blue, it is found that the inhibition by a narcotic does depend on the substrate concentration [Davies & Quastel, 1932]. There exists an equilibrium between lactic acid and the narcotic in presence of the lactic dehydrogenase of brain, to which the mass action law is applicable. There is thus an apparent discrepancy between the two results—an equilibrium between the substrate and the narcotic under anaerobic conditions, and a lack of such equilibrium under aerobic conditions. Yet under suitable ionic conditions an equilibrium does exist under aerobic conditions between the narcotic and some component of the respiratory system.

A clue to the solution of this anomaly may be found in the fact that the low concentrations of narcotics which are highly inhibitory aerobically have but little inhibitory effect anaerobically. Thus chloretone is ten times less effective in securing an inhibition of lactic acid oxidation in presence of brain anaerobically than under aerobic conditions.

A possible explanation is that the narcotic, at the low concentrations effective under aerobic conditions, exerts its main effect on a part of the respiratory system which is inert or functionless under anaerobic conditions. That such a part of the respiratory system has the properties of an enzyme structure is evident from the fact that it is affected by surface-active narcotics of so many different chemical types. It must, however, be an enzyme with a much higher affinity for narcotics than the most sensitive enzymes—the dehydrogenases—with which we are so far familiar.

If this view is true the inhibitions of respiration of intact brain tissue obtained by low concentrations of narcotics under aerobic conditions are not due to competition of the narcotics with the substrates (e.g. lactic or pyruvic acid) for their dehydrogenases, but to the affinity of the narcotics to a special enzyme structure which plays a highly important part in the chain of reactions composing the complete aerobic respiratory process of the cell.

It is the purpose of this communication to throw some light on the site of the enzyme system which is highly sensitive to surface-active narcotics.

EXPERIMENTAL TECHNIQUE

Measurement of oxygen uptake

The Warburg respiration apparatus has been used exclusively in this investigation. The volume of fluid in the reaction vessel is always 3 ml., the *pH* being kept at 7.4 by means of sodium phosphate buffer. In anaerobic experiments sodium bicarbonate solution (0.025*M*) is used in an atmosphere of 95 % N_2 + 5 % CO_2 . The ferricyanide technique of Quastel & Wheatley [1938] has been used in all the anaerobic work and the reader is referred to their paper for full experimental details. Experiments are usually run for 1 hr. at 37°.

Biological material

The biological material used in this work consists of thin slices of brain cortex (rat or guinea-pig), or of minced brain tissue, or of various preparations of tissue enzyme systems. The latter have been made by methods which are already fully described in the literature.

Narcotic investigated

Chloretone ($CCl_3.C(CH_3)_2OH$) is the narcotic extensively used in this investigation. This narcotic has been used because its solubility is such that it can be used conveniently in the Warburg apparatus, 0.2 ml. of a 0.5 % aqueous solution usually being placed in the side tube of the reaction vessel. This amount of chloretone on being tipped into the reaction vessel, after temperature equilibration, usually secures a large inhibition of the rate of O_2 uptake of the tissue (intact brain slices or minced brain). This drug is also used because it brings about, under suitable conditions, steady reversible inhibitions. It is also neutral in reaction, has no buffering value and is suitable for the anaerobic as well as the aerobic work. It appears to be quite stable, and dilute solutions can be kept at 0° for long periods without deterioration. It has the properties of a surface-active narcotic and has already been much investigated in this laboratory [see Jowett & Quastel, 1937; Jowett, 1938].

EFFECT OF CHLORETONE ON BRAIN RESPIRATION IN PRESENCE OF GLUCOSE

A few typical examples of the large inhibitory effects of chloretone on the respiration of brain cortex slices or of minced brain in a glucose-Locke medium are shown in Table 1. Under the conditions cited 0.033 % chloretone brings

Table 1. *Effect of chloretone on brain respiration in presence of glucose*

Exp. 1. Brain cortex slices in sodium phosphate (0.03*M*), *pH* 7.4-Locke medium ($K^+ = 0.004$ *M*; $Ca^{++} = 0.002$ *M*) containing glucose (0.016*M*); oxygen; 37°.

Animal	Chloretone concentration	Q_{O_2}	Inhibition
	%		%
Rat	Nil	11.8	—
Rat	0.033	4.0	66
Guinea-pig	Nil	11.0	—
Guinea-pig	0.016	5.3	52

Exp. 2. Minced rat brain, washed once with Locke solution and suspended in Locke solution (1 g./10 ml.); 1 ml. brain suspension in phosphate (0.03*M*) *pH* 7.4-Locke-glucose (0.016*M*) medium; air; 37°.

	μ l. O_2 uptake in 1 hr.	Inhibition %
No chloretone present	181	—
With chloretone 0.033 %	120	33.7
With chloretone 0.066 %	59	67

about an inhibition of 66 % in the respiration of rat brain cortex slices and an inhibition of 33 % in the respiration of minced rat brain tissue. The respiration of brain cortex slices in a glucose medium is usually more sensitive to narcotics than that of minced tissue, a fact already noted by Jowett [1938] using ethylurethane.

EFFECT OF CHLORETONE ON ANAEROBIC GLYCOLYSIS IN PRESENCE OF BRAIN SLICES

If the action of chloretone on glucose oxidation in brain cortex slices is due to an inhibitory effect upon dehydrogenases involved in the aerobic breakdown of glucose, it may be expected that this effect will be exerted also on the dehydrogenases involved in the anaerobic breakdown of glucose. The mechanism of anaerobic breakdown of glucose in brain is not yet clear, but if the reactions involved bear some resemblance to those taking part in the anaerobic breakdown of glucose in muscle or yeast, an interplay of dehydrogenase systems will be involved. Experiments (see Table 2) show that 0.033 % chloretone exerts no inhibitory effect whatever on the anaerobic breakdown of glucose to lactic acid in presence of rat brain cortex slices. The result shows that if a narcotic-sensitive dehydrogenase system plays an important role in the aerobic breakdown of glucose, it is either absent from, or is without influence in, the reactions involved in the anaerobic breakdown of glucose by brain.

Table 2. *Effect of chloretone on anaerobic glycolysis by brain*

Rat brain cortex slices in sodium bicarbonate (0.025 *M*)-Locke solution containing 0.025 *M* glucose and 0.003 *M* sodium pyruvate; 95 % $N_2 + 5$ % CO_2 ; 37°.

	$Q_M^{N_2}$		
Medium without added chloretone	15.2	19.8	16.5
Medium with chloretone 0.033 %	15.1	—	—
Medium with chloretone 0.05 %	—	17.8	16.4

ANAEROBIC OXIDATION OF GLUCOSE BY BRAIN TISSUE

A more direct approach to the problem as to whether a narcotic exerts large inhibitory effects on dehydrogenases involved in glucose breakdown was made in the following manner.

In the ferricyanide technique of Quastel & Wheatley [1938] use is made of the fact that in the reduction of 1 mol. ferricyanide 1 mol. acid is formed and in bicarbonate media this gives rise to 1 mol. CO_2 which is estimated manometrically. The relevant equations are:

The technique has been used for the study of cozymase-linked systems (since ferricyanide readily oxidizes reduced cozymase [Haas, 1937]), as well as for the study of other dehydrogenase systems (e.g. those involving succinic acid or choline) which do not depend for their activity on cozymase. The technique can be used in the investigations of anaerobic oxidations brought about by intact tissue slices as well as by minced tissue or by tissue extracts. Full experimental details may be found in the paper quoted.

The addition of ferricyanide solution containing a little bicarbonate (1 ml. 0.16 *M* $NaHCO_3$ to 5 ml. 10 % $Na_3Fe(CN)_6$) to minced brain tissue in a bicarbonate-glucose medium in an atmosphere of 95 % $N_2 + 5$ % CO_2 results in

but little evolution of CO_2 above that obtained, by glycolysis, in the absence of ferricyanide. The addition of pyocyanine to the medium, followed by that of ferricyanide, brings about a very large increase in the velocity of CO_2 output. Pyocyanine is reduced in the presence of brain tissue by glucose, or by a breakdown product of glucose, and the reduced pyocyanine is oxidized directly by ferricyanide. Pyocyanine in this way acts as a catalyst in the anaerobic oxidation of glucose (or of a breakdown product of glucose) by ferricyanide. A typical result with minced rat brain tissue is shown in Table 3. In this case a CO_2 output of $144.5 \mu\text{l.}$ in the presence of glucose and ferricyanide is increased to $520.2 \mu\text{l.}$ by the addition of pyocyanine.

The same phenomenon is observed with brain cortex slices. A typical result is given in Table 4. The addition of pyocyanine increases the $Q_{\text{CO}_2}^{\text{N}_2}$ ($\mu\text{l. CO}_2$ produced per hr. per mg. dry weight of tissue) from 17.8 to 37.8.

Table 3. *Anaerobic oxidation of glucose, lactate and pyruvate by minced brain*

Minced brain tissue washed with saline and suspended in saline; 1 ml. suspension in bicarbonate ($0.025 M$)-saline; glucose, $0.016 M$; sodium lactate, $0.02 M$; sodium pyruvate, $0.015 M$; pyocyanine chloride, 1.6 mg. per 100 ml. 0.2 ml. 8.3 % sodium ferricyanide solution added where indicated; 95 % $\text{N}_2 + 5 \%$ CO_2 ; 37° .

Substrates etc. added	$\mu\text{l. CO}_2$ output		
	Rat 0.1 g. wet wt. tissue (1 hr.)	Rat 0.15 g. wet wt. tissue (1 hr.)	Guinea-pig 0.2 g. wet wt. tissue (2 hr.)
Ferricyanide	45.0	86.8	51.5
Pyocyanine + ferricyanide	175.5*	113.3	61.7
Glucose	105.0	—	—
Glucose + ferricyanide	144.5	—	—
Lactate + ferricyanide	—	107.2	—
Pyruvate + ferricyanide	—	—	68.4
Glucose + pyocyanine + ferricyanide	520.2*	—	—
Lactate + pyocyanine + ferricyanide	—	536.4	—
Pyruvate + pyocyanine + ferricyanide	—	—	248.0

* Pyocyanine chloride = 3.2 mg. per 100 ml.

Table 4. *Effect of chloretone on anaerobic oxidation of glucose by brain*

Rat brain cortex slices in bicarbonate ($0.025 M$)-Locke medium; 0.2 ml. 8.3 % sodium ferricyanide solution added where indicated; pyocyanine chloride, 3.2 mg. per 100 ml.; glucose, $0.01 M$; 95 % $\text{N}_2 + 5 \%$ CO_2 ; 37° .

	$Q_{\text{CO}_2}^{\text{N}_2}$	
	17.8	13.7
Glucose + ferricyanide	8.5	
Pyocyanine + ferricyanide	12.1	13.7
Glucose + pyocyanine	37.8	31.2
Glucose + pyocyanine + ferricyanide	29.5	26.8
Glucose + pyocyanine + ferricyanide + chloretone (0.083 %)	21.9 %	14.1 %
Inhibition by chloretone		

EFFECT OF CHLORETONE ON THE ANAEROBIC OXIDATION OF GLUCOSE BY BRAIN CORTEX SLICES

The presence of small concentrations of chloretone which inhibit vigorously the aerobic oxidation of glucose has no effect on the anaerobic oxidation of glucose by brain tissue in the presence of pyocyanine and ferricyanide. Even so high a concentration as 0.083 % chloretone, which inhibits the O_2 uptake of rat brain cortex slices in a glucose medium by over 80 %, only inhibits the anaerobic oxidation of glucose by pyocyanine and ferricyanide by about 20 %. Typical

results are noted in Table 4. This result indicates the relative insensitivity to chloretone of the dehydrogenase system responsible for the reduction of pyocyanine by brain tissue in presence of glucose.

It is possible that the substance which brings about the reduction of pyocyanine in the experiment just quoted is lactic acid, produced from glucose by glycolysis, and that the dehydrogenase system involved is that mainly concerned with the oxidation of lactic acid.

The effects of chloretone on the aerobic and anaerobic oxidations of lactic acid in the presence of brain will, therefore, now be considered.

EFFECTS OF CHLORETONE ON THE AEROBIC AND ANAEROBIC OXIDATIONS OF LACTIC ACID

As with the oxidation of glucose, narcotics exercise a large inhibition on the aerobic oxidation of lactic acid in the presence of brain. A few typical results, obtained both with brain cortex slices and minced brain tissue, are shown in Table 5. The inhibition by chloretone is of the same order of magnitude as with glucose oxidation.

Table 5

I. Guinea-pig brain cortex slices in Na phosphate (0.03 *M*) pH 7.4-Locke solution; sodium lactate, 0.02 *M*; O₂; 37°.

	Q_{O_2}	
	13.4	16.5
Lactate	6.9	7.5
Lactate + chloretone 0.016 %	49 %	54 %
Inhibition by chloretone		

II. Minced washed rat brain suspension (1 g. to 10 ml.) in Na phosphate (0.03 *M*) pH 7.4-saline; 1 ml. suspension taken; sodium lactate, 0.01 *M*; air; 37°.

	$\mu\text{l. O}_2$ uptake in 1 hr.	
Lactate	249.1	194
Lactate + chloretone 0.083 %	60.0	—
Lactate + chloretone 0.033 %	—	129

Pyocyanine greatly accelerates the anaerobic oxidation by ferricyanide of lactic acid in presence of brain tissue, as may be seen by a glance at Table 3. Chloretone, however, even at the concentration of 0.083 % exerts no inhibitory effect on the anaerobic oxidation of lactic acid by pyocyanine and ferricyanide in presence of rat brain cortex slices (see Table 6).

Table 6. *Effect of chloretone on anaerobic oxidation of lactate*

Rat brain cortex slices in bicarbonate (0.025 *M*)-Locke medium; 0.2 ml. 8.3 % Na ferricyanide solution added; sodium lactate, 0.016 *M*; pyocyanine chloride, 3.2 mg. per 100 ml.; 95 % N₂ + 5 % CO₂; 37°.

	$Q_{CO_2}^{N_2}$
Pyocyanine + ferricyanide + lactate	20.8
Pyocyanine + ferricyanide + lactate + chloretone 0.083 %	19.6

If, therefore, the lactic dehydrogenase system responsible for the reduction of pyocyanine by lactic acid is identical with that which brings about the activation of lactic acid under aerobic conditions, the latter cannot be the narcotic-sensitive component of the aerobic respiratory mechanism.

EFFECT OF CHLORETONE ON THE AEROBIC AND ANAEROBIC
OXIDATIONS OF PYRUVIC ACID BY BRAIN TISSUE

Pyruvic acid oxidation by brain, either in the form of cortex slices or of a mince, is as sensitive to the action of chloretone as the oxidation of glucose or of lactic acid. Typical results are shown in Table 7, in which it is also shown that the addition of pyocyanine under aerobic conditions has no appreciable effect upon the chloretone inhibition.

Table 7

I. Rat brain cortex slices in Na phosphate (0.03 *M*, pH 7.4)-Locke-Na pyruvate (0.016 *M*) medium; O₂; 37°.

	Q_{O_2}
No chloretone present	10.1
With chloretone 0.033 %	3.5
Inhibition by chloretone	66 %

II. Minced guinea-pig brain washed thrice with saline and suspended in saline (1 g. to 5 ml.); 1 ml. suspension in Na phosphate (0.03 *M*, pH 7.4)-saline; Na pyruvate, 0.015 *M*; pyocyanine chloride, 1.6 mg. per 100 ml.; air; 37°.

Substrates etc. added	μ l. O ₂ uptake in 1 hr.
Nil	30.1
Pyocyanine	36.6
Na pyruvate	105.3
Na pyruvate + pyocyanine	99.1
Na pyruvate + chloretone 0.083 %	6.9 (inhibition = 93 %)
Na pyruvate + pyocyanine + chloretone 0.083 %	10.8 (inhibition = 89 %)

Anaerobically, pyruvic acid is but feebly attacked by ferricyanide in presence of brain cortex slices. The addition of pyocyanine greatly accelerates the rate of oxidation by ferricyanide, a $Q_{CO_2}^{N_2}$ of 6.6 in the absence of pyocyanine being increased to 17.9 in its presence. The addition of a relatively high concentration of chloretone has but little inhibitory effect upon the anaerobic oxidation of pyruvic acid by rat brain cortex slices. A representative result is shown in Table 8.

Table 8. *Effect of chloretone on anaerobic oxidation of pyruvate*

Rat brain cortex slices in bicarbonate (0.025 *M*)-Locke medium; 0.2 ml. 8.3 % Na ferricyanide added where indicated; pyocyanine chloride, 3.2 mg. per 100 ml.; Na pyruvate, 0.016 *M*; 95 % N₂ + 5 % CO₂; 37°.

	$Q_{CO_2}^{N_2}$
Ferricyanide	4.1
Pyocyanine + ferricyanide	8.5
Pyruvate + ferricyanide	6.6
Pyruvate + pyocyanine + ferricyanide	17.9
Pyruvate + pyocyanine + ferricyanide + chloretone (0.083 %)	13.1
Inhibition by chloretone	26.8 %

It follows from these results that if a pyruvic dehydrogenase is common to both the aerobic and anaerobic oxidations of pyruvic acid in brain, this enzyme cannot be regarded as the narcotic-sensitive component of the aerobic system.

EFFECT OF CHLORETONE ON THE ANAEROBIC OXIDATION
OF LACTIC ACID IN PRESENCE OF CRUSHED YEAST

Lactic acid is vigorously oxidized in presence of a suspension of dialysed crushed baker's yeast by ferricyanide under anaerobic conditions in the absence of any added carrier such as pyocyanine. The crushing of the yeast was accom-

plished in a wet-crushing mill [Booth & Green, 1938] and the crushed yeast was dialysed against distilled water overnight in the ice chest.

The addition of potassium ferricyanide solution to a suspension of the crushed yeast in a bicarbonate medium results in an evolution of CO_2 , due to the oxidation of substances still present in the suspension. The further addition of sodium lactate results in a large acceleration of the rate of CO_2 output. It will be seen from Table 9 that an evolution of $210\mu\text{l. CO}_2$ in 10 min. was increased, by the presence of lactate, to $683.5\mu\text{l. CO}_2$ in the same time.

Oxidation of lactic acid, in presence of crushed yeast, by ferricyanide occurs also on addition of sodium cyanide. The rate is not materially affected by the addition of a cozymase preparation (a slight increase in the rate due to added cozymase is noted in Table 9).

Table 9

Baker's yeast (40 g. yeast and 20 ml. water) crushed in mill and dialysed against distilled water; 1 ml. suspension in bicarbonate (0.025 *M*)-saline; 0.2 ml. 8.3 % K ferricyanide solution added; 95 % N_2 + 5 % CO_2 ; 37° .

Substances added	$\mu\text{l. CO}_2$ output in 10 min.
I. Ferricyanide	210.0
Ferricyanide + chloretone (0.083 %)	200.1
Na lactate (0.015 <i>M</i>) + ferricyanide	683.5
Na lactate (0.015 <i>M</i>) + ferricyanide + chloretone (0.083 %)	604.9
	$\mu\text{l. CO}_2$ output in 30 min.
II. NaCN (0.03 <i>M</i>) + ferricyanide	86.0
Na lactate (0.01 <i>M</i>) + NaCN (0.03 <i>M</i>) + ferricyanide	638.3
Na lactate (0.01 <i>M</i>) + NaCN (0.03 <i>M</i>) + 1 mg. cozymase + ferricyanide	705.6

The addition of chloretone (0.083 %) depresses only slightly the rate of oxidation of lactic acid by ferricyanide in presence of yeast lactic dehydrogenase (Table 9).

It is evident, therefore, that yeast lactic dehydrogenase, like that of brain, is not highly sensitive to the narcotic.

EFFECT OF CHLORETONE ON MUSCLE DEHYDROGENASE

A preparation of a dialysed extract of rabbit skeletal muscle was used as a source of muscle dehydrogenase. It was made according to the method of Green *et al.* [1937]. As shown by Quastel & Wheatley [1938], this extract contains dehydrogenases which, in the presence of cozymase and the appropriate substrates, rapidly reduce ferricyanide under anaerobic conditions.

Sodium hexosediphosphate and sodium α -glycerophosphate are examples of substrates which are oxidized rapidly by ferricyanide in the presence of rabbit skeletal muscle extract containing cozymase.

Chloretone (0.083 %) does not inhibit the anaerobic oxidation of hexosediphosphate by this system.

The oxidation of α -glycerophosphate is inhibited by chloretone (0.033 %) to the extent of 10 %, the inhibition being increased to 30 % when this concentration of chloretone is doubled. These facts are noted in Table 10.

It seems, therefore, that although the α -glycerophosphate dehydrogenase system of skeletal muscle shows some sensitivity to the narcotic, it is not nearly as sensitive as the aerobic respiratory systems of intact brain tissue in glucose media.

Table 10

Rabbit skeletal muscle extract dialysed; 1 ml. extract in 0.025 *M* bicarbonate-saline; 0.2 ml. 8.3 % K ferricyanide added where indicated; 1 mg. cozymase preparation added; Na hexosediphosphate, 0.23 %; Na α -glycerophosphate, 0.02 *M*; 95 % N₂ + 5 % CO₂; 37°.

	μ l. CO ₂ output in 15 min.
I. Na hexosediphosphate + cozymase	65.1
Na hexosediphosphate + cozymase + ferricyanide	409.2
Na hexosediphosphate + cozymase + ferricyanide + chloretone (0.083 %)	459.0
	μ l. CO ₂ output in 1 hr.
II. Na α -glycerophosphate + cozymase + ferricyanide	466.4
Na α -glycerophosphate + cozymase + ferricyanide + chloretone (0.016 %)	436.3
Na α -glycerophosphate + cozymase + ferricyanide + chloretone (0.033 %)	416.3
Na α -glycerophosphate + cozymase + ferricyanide + chloretone (0.05 %)	411.0
Na α -glycerophosphate + cozymase + ferricyanide + chloretone (0.066 %)	323.4

EFFECTS OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF RABBIT SKELETAL MUSCLE EXTRACT AND CRUSHED BAKER'S YEAST

The oxygen uptake obtained when sodium α -glycerophosphate or sodium hexosediphosphate is incubated at 37° with dialysed crushed baker's yeast is much increased when dialysed rabbit skeletal muscle extract is added to the mixture. This takes place although the incubation of sodium α -glycerophosphate or hexosediphosphate with the muscle extract alone leads to no consumption of oxygen. The increased oxygen uptake is doubtless due to the fact that the muscle extract supplies increased amounts of dehydrogenases, which in presence of cozymase and the cytochrome system provided by the crushed yeast, lead to oxidation of the α -glycerophosphate and hexosediphosphate. Typical results are shown in Table 11 (A and B) and Table 12 (A).

Table 11. *Rabbit skeletal muscle extract and crushed baker's yeast (20 %)*

A. 1 ml. dialysed muscle extract, 0.5 ml. crushed yeast in Na phosphate (0.03 *M*), pH 7.4-saline; Na α -glycerophosphate, 0.02 *M*; air; 37°.

	μ l. O ₂ uptake in 1 hr.
Muscle extract + α -glycerophosphate	0.0
Crushed yeast + α -glycerophosphate	89.3
Muscle extract + crushed yeast + α -glycerophosphate	198.5

B. 1.5 ml. dialysed muscle extract and 0.2 ml. dialysed crushed yeast in Na phosphate, pH 7.4-saline; Na α -glycerophosphate, 0.02 *M*; air; 37°.

	μ l. O ₂ uptake in 1 hr.
Crushed yeast + α -glycerophosphate	66.4
Crushed yeast + α -glycerophosphate + muscle extract	188.3
Crushed yeast + α -glycerophosphate + muscle extract + chloretone (0.05 %)	89.3
Inhibition by chloretone	51.7 %

C. 1.5 ml. dialysed muscle extract and 0.2 ml. dialysed crushed yeast in bicarbonate (0.025 *M*)-saline; Na α -glycerophosphate, 0.02 *M*; 0.2 ml. 8.3 % K ferricyanide; 95 % N₂ + 5 % CO₂; 37°.

	μ l. CO ₂ output in 1 hr.
Ferricyanide + muscle extract + crushed yeast + α -glycerophosphate	343.8
Ferricyanide + muscle extract + crushed yeast + α -glycerophosphate + chloretone (0.05 %)	260.4
Inhibition by chloretone	24.3 %

Table 12. *Rabbit skeletal muscle extract and crushed baker's yeast*

A. 1.5 ml. dialysed rabbit muscle extract and 0.2 ml. dialysed crushed baker's yeast in Na phosphate (0.03 *M*), pH 7.4-saline; Na hexosediphosphate, 0.005 *M*; air; 37°.

	$\mu\text{l. O}_2$ uptake in 1 hr.
Crushed yeast + hexosediphosphate	37.3
Crushed yeast + hexosediphosphate + muscle extract	129.9
Crushed yeast + hexosediphosphate + muscle extract + chloretone (0.05 %)	60.4
Inhibition by chloretone	53.4 %

B. 1.5 ml. dialysed rabbit muscle extract and 0.2 ml. dialysed crushed baker's yeast in Na bicarbonate (0.025 *M*)-saline; Na hexosediphosphate, 0.005 *M*; 0.2 ml. 8.3 % K ferricyanide; 95 % N₂ + 5 % CO₂; 37°.

	$\mu\text{l. CO}_2$ output in 1 hr.
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide	628.9
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide + chloretone (0.05 %)	620.1
Inhibition by chloretone	1.2 %

The addition of chloretone (0.05 %) to the system—skeletal muscle extract and dialysed crushed yeast—results in over 50 % inhibition of the oxygen uptake obtained in the presence of either sodium α -glycerophosphate (Table 11B) or of sodium hexosediphosphate (Table 12).

The system of muscle extract and crushed yeast can be investigated anaerobically using the ferricyanide technique. Under such circumstances the cytochrome system presumably plays no part, and the reduction of ferricyanide will be accomplished through the activities of the combined dehydrogenases of the muscle extract and crushed yeast, the cozymase necessary for the reduction being supplied largely by the yeast. Investigation of this system shows that the addition of chloretone (0.05 %) brings about little or no inhibition of the anaerobic oxidation of hexosediphosphate (Table 12B) and 24 % inhibition of the rate of anaerobic oxidation of α -glycerophosphate (Table 11C).

These results show that whilst the oxygen uptake of a respiratory system—muscle extract, crushed yeast and substrate—is considerably inhibited by a narcotic, the amount of inhibition by the same concentration of narcotic is greatly decreased when the system is examined anaerobically. Since the dehydrogenases therefore cannot be the narcotic-sensitive component of the respiratory system, it follows that the narcotic must act vigorously on some component occupying an intermediate position between the dehydrogenase and the cytochrome systems.

EFFECT OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF RABBIT SKELETAL MUSCLE EXTRACT, COZYMASE AND CYTOCHROME OXIDASE

Substantially the same results as those just recorded are obtained if crushed yeast be replaced by a mixture of cozymase and cytochrome oxidase, prepared from pig heart according to the method of Keilin & Hartree [1938]. A typical result given in Table 13A shows that oxygen consumption takes place only in the complete system—cytochrome oxidase, muscle extract, sodium α -glycerophosphate and cozymase. The cytochrome and flavoprotein necessary for the system are present in the cytochrome oxidase preparation.

Addition of chloretone (0.083 %) to this system brings about over 80 % inhibition of the rate of oxygen uptake (Table 13B). Thus a relatively narcotic-insensitive system—muscle extract, α -glycerophosphate, cozymase—is made narcotic-sensitive by the addition to it of a cytochrome oxidase preparation.

Table 13. *Rabbit muscle extract and pig heart muscle (cytochrome oxidase)*

1 ml. dialysed rabbit muscle extract and 0.5 ml. cytochrome oxidase preparation in Na phosphate (0.03 *M*) pH 7.4-saline; 1 mg. cozymase preparation; Na α -glycerophosphate, 0.02 *M*; air; 37°.

	μ l. O ₂ uptake in 1 hr.
A. Cytochrome oxidase preparation + muscle extract + α -glycerophosphate	0.0
Cytochrome oxidase preparation + cozymase + α -glycerophosphate	0.0
Cytochrome oxidase preparation + muscle extract + α -glycerophosphate + cozymase	96.1
B. Cytochrome oxidase preparation + muscle extract + α -glycerophosphate + cozymase	136.0
Cytochrome oxidase preparation + muscle extract + α -glycerophosphate + cozymase + chloretone (0.083 %)	16.7
Inhibition by chloretone	87 %

EFFECT OF CHLORETONE ON AN OXIDIZING SYSTEM COMPOSED OF GLUCOSE DEHYDROGENASE, COZYMASE AND CYTOCHROME OXIDASE

A water-soluble preparation of glucose dehydrogenase was made by acetone treatment of pig liver according to the method of Harrison [1931]. This preparation, as shown by Hawthorne & Harrison [1939], in presence of cozymase, cytochrome, cytochrome oxidase and glucose (probably with the co-operation of a flavoprotein) takes up oxygen, the glucose being oxidized to gluconic acid. Only the complete system takes up oxygen as shown by the results given in Table 14. Cytochrome was, in fact, not added in the experiment recorded in Table 14, as the cytochrome oxidase preparation contained sufficient quantities of it.

Table 14. *Glucose dehydrogenase (acetone pig liver) preparation and cytochrome oxidase (pig heart) preparation*

I. 1 ml. glucose dehydrogenase preparation (0.25 g. in 8 ml. saline), 0.5 ml. cytochrome oxidase preparation, 1 mg. cozymase preparation in Na phosphate (0.03 *M*), pH 7.4-saline; glucose, 0.016 *M*; air; 37°.

	μ l. O ₂ in 1 hr.
Glucose dehydrogenase + cozymase + glucose	0.0
Glucose dehydrogenase + cytochrome oxidase + glucose	6.0
Glucose dehydrogenase + cytochrome oxidase + cozymase	5.0
Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose	80.6
II. Glucose dehydrogenase (1 ml. of solution of 0.5 g. in 8 ml. saline) + cytochrome oxidase + cozymase + glucose (0.1 <i>M</i>)	341.2
Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose + chloretone (0.083 %)	8.4
Inhibition by chloretone	97 %

The complete system—glucose dehydrogenase, cytochrome oxidase, cozymase and glucose—is highly sensitive to chloretone. This narcotic at a concentration of 0.083 % brings about over 90 % inhibition of the rate of oxygen uptake (Table 14).

The system may be investigated anaerobically by the ferricyanide technique. Results in Table 15 (I) show that the addition of cozymase greatly increases the rate of CO₂ output obtained by incubating anaerobically the glucose dehydrogenase preparation with glucose and ferricyanide. The system is thus a typical cozymase-linked dehydrogenase system.

The addition of chloretone at a concentration of 0.083 % secures no inhibition within experimental error of the rate of anaerobic oxidation of glucose by ferricyanide in presence of glucose dehydrogenase and cozymase (Table 15).

Table 15. *Glucose dehydrogenase (acetone pig liver) preparation*

I. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0.025 *M* Na bicarbonate-saline; glucose, 0.016 *M*; 0.2 ml. 8.3 % K ferricyanide solution added; 95 % N₂ + 5 % CO₂; 37°.

	μ l. CO ₂ output in 1 hr.
Glucose dehydrogenase	88.9
Glucose dehydrogenase + cozymase	96.0
Glucose dehydrogenase + glucose	68.5
Glucose dehydrogenase + cozymase + glucose	233.3

II. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0.025 *M* Na bicarbonate-saline; glucose, 0.1 *M*; 0.2 ml. 8.3 % K ferricyanide added; 95 % N₂ + 5 % CO₂; 37°.

	μ l. CO ₂ output in 30 min.
Glucose dehydrogenase + cozymase	75.4
Glucose dehydrogenase + cozymase + chloretone (0.083 %)	75.6
Glucose dehydrogenase + cozymase + glucose	335.8
Glucose dehydrogenase + cozymase + glucose + chloretone (0.083 %)	329.7
Inhibition by chloretone	2 %

The conclusion must be drawn that, as with the α -glycerophosphate system of muscle, the glucose dehydrogenase of liver is relatively narcotic-insensitive, and that the glucose-oxidizing system becomes highly narcotic-sensitive on the addition to it of a cytochrome oxidase preparation.

CHLORETONE AND CYTOCHROME OXIDASE

It is well known that cytochrome oxidase is relatively insensitive to narcotics. Quastel & Wheatley [1932] for example showed that chloretone and barbiturates, at concentrations which greatly inhibited the respiration of brain in glucose media, have little or no effect upon the oxidation of *p*-phenylenediamine or of Na succinate by brain tissue. It must be concluded that the narcotic inhibits neither the mechanism by which oxygen enters the cell nor that by which it is brought into a state of activity.

CHLORETONE AND THE AEROBIC RESPIRATORY SYSTEM

The observations which have been recorded so far may be summarized as follows:

1. Chloretone, at the small concentrations which inhibit the respiration of intact brain tissue, has but little inhibitory effect upon the dehydrogenase systems which may be expected to play a part in respiration. This insensitivity of the known dehydrogenases towards the small but biologically important concentrations of narcotic probably accounts for the fact that the inhibition of respiration by a narcotic is independent of the concentration of the substance burned [Jowett & Quastel, 1937].

2. Chloretone has little or no inhibitory effect on the activity of cytochrome oxidase.

3. A respiratory system composed of a mixture of a dehydrogenase system and a cytochrome oxidase preparation—both of which components are separately relatively narcotic-insensitive—is narcotic-sensitive.

The conclusion to be drawn from these observations is that the component in the complete respiratory system which is highly narcotic-sensitive must be a factor playing an intermediate role between the dehydrogenase and cytochrome

oxidase systems. This factor must be lacking in the respiratory system involving the aerobic oxidation of succinate in animal tissues, since this is relatively narcotic-insensitive.

The narcotic-sensitive factor in the system involving the oxidation of lactic acid by animal tissues must take part in the reactions between reduced cozymase and cytochrome (or one of the cytochromes). A number of substances may be involved in this intermediate section of the respiratory system and these are not yet clearly defined or identified.

The evidence points, however, to the fact that a flavoprotein plays an important role in this part of the respiratory system. A flavoprotein (diaphorase) is known to be reduced by reduced cozymase and there is some evidence that reduced flavoproteins may react with components of the cytochrome system [Theorell, 1936; Dewan & Green, 1938]. Haas *et al.* [1940] have reported the existence of a flavoprotein in yeast which completes the oxidation-reduction chain between hexosemonophosphate and cytochrome *c*.

Flavoprotein, together with the cytochrome components, is present in the cytochrome oxidase preparation used in the experiments of which the results are given in Tables 13 and 14.

An investigation, therefore, was made of the effects of chloretone on the accelerating action of flavoprotein in an oxidizing system.

CHLORETONE AND FLAVOPROTEIN

Heart muscle flavoprotein was prepared by the method of Straub [1939]. Its catalytic activity in oxidation has been demonstrated by Corran *et al.* [1939]. As shown by these authors the oxygen uptake of a system composed of rabbit skeletal muscle extract, cozymase, sodium lactate, sodium cyanide and methylene blue is much increased by the addition of flavoprotein. A typical result is shown in Table 16 (I). The flavoprotein is reduced by reduced cozymase, and methylene blue is in turn reduced by the reduced flavoprotein. The reduced methylene blue is autoxidizable, and hence a chain of reactions securing the oxidation of lactate is formed. Cyanide is added to the system to fix the pyruvate, which would otherwise inhibit the activity of the lactic dehydrogenase of the muscle extract.

Table 16

Constituents present in Na phosphate (0.02 *M*) pH 7.4-saline medium: 0.3 ml. dialysed rabbit skeletal muscle extract, 0.5 ml. flavoprotein preparation, 1 mg. cozymase preparation, Na cyanide (0.083 *M*), Na lactate (0.13 *M*) and methylene blue (0.017 %); air; 37°.

	$\mu\text{l. O}_2$ uptake in 1 hr.
I. Muscle extract + cozymase + Na lactate + NaCN + methylene blue	62.5
Flavoprotein + cozymase + Na lactate + NaCN + methylene blue	44.2
Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	303.2
II. Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	268.5
Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue + chloretone (0.083 %)	270.5
% inhibition by chloretone	Nil

Addition of chloretone at a relatively high concentration (0.083 %) has no inhibitory effect on this system (Table 16 (II)).

It follows from this fact that heart flavoprotein is not itself unduly affected by the narcotic. The narcotic appears to affect neither the reduction of flavo-

protein by cozymase nor the reduction of methylene blue by reduced flavo-protein. Yet in a respiratory system consisting of rabbit skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase, sodium lactate and semicarbazide (added to fix the pyruvate) the addition of chloretone (0.083 %) causes 52% inhibition of the velocity of oxygen uptake. This fact is noted in Table 17.

Table 17

Constituents present: 1 ml. dialysed rabbit skeletal muscle extract; 1 ml. flavoprotein preparation, 1 mg. cozymase preparation, 0.2 ml. cytochrome oxidase preparation, semicarbazide 0.03 *M*, Na lactate 0.13 *M* and saline; air; 37°.

	$\mu\text{l. O}_2$ uptake in 1 hr.
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate	135.1
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate + chloretone (0.083 %)	64.8
Inhibition by chloretone	52 %

Unless, therefore, there is still another enzyme which is highly narcotic-sensitive and which is playing a role between flavoprotein and one or more of the cytochrome components, it must be concluded that either

(a) There is present in animal tissues a special narcotic-sensitive flavoprotein which forms an important link between reduced cozymase and the cytochrome system. It should be noted that the cytochrome *c* reductase of Haas *et al.* [1940] is a flavoprotein composed of a specific protein and an alloxazine mononucleotide. It is very unstable compared with other flavoproteins and its activity is destroyed by acetone and dioxane. Conceivably such a flavoprotein may be highly narcotic-sensitive; or

(b) Some component of the cytochrome system itself is highly narcotic-sensitive (see e.g. Keilin & Hartree [1939]). This component would obviously play little part in the oxidation of either sodium succinate or of *p*-phenylenediamine in animal tissues.

The oxidation of pyruvate by tissues is greatly inhibited by a narcotic such as chloretone (Table 7) and this system is presumably independent of the action of cozymase. There is some evidence, however [Lipmann, 1939], that a flavoprotein is involved in pyruvate oxidation by bacteria and conceivably a flavoprotein is involved in the oxidation of pyruvate by animal tissues.

The oxidation of *dl*-alanine by rat kidney slices has been shown [Jowett & Quastel, 1937] to be inhibited by small concentrations of a narcotic. Since amino-acid oxidase has been identified as a flavoprotein, it is possible, from what has been said, that the narcotic acts here on the process intermediate between the flavoprotein and a cytochrome component.

With our present state of incomplete knowledge of the components of respiratory systems of animal tissues it would be unwise to speculate further. A solution of the problem of the precise site at which a narcotic acts, in biologically important concentrations, in respiratory systems, depends on a more complete elucidation of the details of these systems. It may be suggested, however, that the property of narcotics of inducing, at low concentrations, strong inhibition of respiratory activity may be used more extensively than hitherto in the investigation of the components of respiratory systems.

What is clear from these results is that, in the inhibition of respiratory activity effected by low concentrations of narcotics, the familiar dehydrogenases, which have been popularly supposed to be the narcotic-sensitive components of

the respiratory processes, are practically unaffected. The narcotic seems to act at a site involving a special flavoprotein or an intermediate between flavoprotein and the cytochrome oxidase system.

SUMMARY

1. The effects of chloretone on the oxidations of glucose, sodium lactate and sodium pyruvate in presence of brain tissue (cortex slices and minced brain), both under aerobic and anaerobic conditions have been investigated. Concentrations of chloretone which effect large inhibitions of the aerobic oxidations have but little effect upon the anaerobic oxidations (using the ferricyanide technique). The dehydrogenases involved in the oxidation of glucose, lactate and pyruvate in presence of brain tissue are (relatively) narcotic-insensitive.

2. Anaerobic glycolysis by brain is not inhibited by relatively high concentrations of chloretone.

3. Anaerobic oxidations of glucose, lactate and pyruvate in the presence of brain tissue and of a carrier such as pyocyanine can be followed by the ferricyanide technique.

4. Anaerobic lactate oxidation by ferricyanide, in presence of crushed yeast, can be followed manometrically in the absence of added carriers. It is but feebly inhibited by relatively high concentrations of chloretone.

5. The addition of crushed yeast to skeletal muscle extract containing sodium α -glycerophosphate produces a narcotic-sensitive respiratory system.

6. The following systems which are but feebly narcotic-sensitive become highly narcotic-sensitive on addition to them of a cytochrome oxidase preparation, viz. dialysed skeletal muscle extract, cozymase and α -glycerophosphate; liver glucose dehydrogenase, cozymase and glucose.

These systems which can be studied manometrically under anaerobic conditions by the ferricyanide technique are not inhibited appreciably by concentrations of chloretone which exercise large inhibitions of the oxygen uptake when pig heart cytochrome oxidase is added.

7. Chloretone at relatively high concentrations does not inhibit the reduction of heart flavoprotein by reduced cozymase, or the reduction of methylene blue by reduced flavoprotein. It exercises a large inhibition of the oxygen uptake of a system composed of skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase and sodium lactate.

8. The evidence suggests that chloretone at biologically important concentrations affects largely either a special flavoprotein or some component of the tissue respiratory system which plays an intermediate role between flavoprotein and cytochrome oxidase.

Our thanks are due to the Medical Research Council for a grant in aid of the equipment of this laboratory, to the Society for the Protection of Science and Learning for a grant to one of us (M. M.), and to Dr P. J. G. Mann for his help in some of the experimental work described in this paper. We are also grateful to the Ella Sachs Plotz Foundation for a grant for the purchase of animals.

REFERENCES

- Booth & Green (1938). *Biochem. J.* **32**, 855.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Davies & Quastel (1932). *Biochem. J.* **26**, 1672.
Dewan & Green (1938). *Biochem. J.* **32**, 626.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
Haas (1937). *Biochem. Z.* **291**, 79.
—— Horecker & Hogness (1940). *J. biol. Chem.* **136**, 747.
Harrison (1931). *Biochem. J.* **25**, 1016.
Hawthorne & Harrison (1939). *Biochem. J.* **33**, 1573.
Jowett (1938). *J. Physiol.* **92**, 322.
—— & Quastel (1937). *Biochem. J.* **31**, 565.
Keilin & Hartree (1938). *Proc. roy. Soc. B*, **125**, 171.
—— (1939). *Proc. roy. Soc. B*, **127**, 167.
Lipmann (1939). *Nature, Lond.*, **143**, 436; *Enzymologia* (1937), **4**, 65.
McClure, Hartmann, Schnedorf & Schelling (1939). *Ann. Surg.* **110**, 836.
Quastel (1939). *Physiol. Rev.* **19**, 135.
—— & Wheatley (1932). *Proc. roy. Soc. B*, **112**, 60.
—— (1934). *Biochem. J.* **28**, 1521.
—— (1938). *Biochem. J.* **32**, 936.
Sen (1931). *Biochem. J.* **25**, 849.
Straub (1939). *Biochem. J.* **33**, 787.
Theorell (1936). *Biochem. Z.* **288**, 317.