CCII. NARCOTICS AND BRAIN OXIDATIONS. REVERSIBILITY OF NARCOTIC ACTION *IN VITRO*.

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In earlier work the authors [1932] have shown that narcotics in general have the property of inhibiting the oxidation by brain tissue of glucose, sodium lactate and sodium pyruvate but not that of sodium succinate or of *p*-phenylenediamine. Narcotics such as luminal, chloretone or hyoscine at concentrations of 0·12 % inhibit the oxidation of glucose by brain tissue by amounts varying from 79 to 94 %. The reversibility of this inhibitory action by the narcotics was not at the time examined. This has since been done, and it is the purpose of the authors to show, in this communication, that with narcotics possessing powerful inhibitory effects, such as those already mentioned, reversibility occurs *in vitro* to the extent of at least 75 %.

Technique.

Minced tissue has been previously used in the work of the authors on oxidations of the brain. It had been found desirable to use brain tissue minced or chopped to as homogeneous a condition as possible in order to compare the respiration of such tissue in presence of narcotics *etc.* with that in the absence of added substances.

To test the reversibility of the inhibitory action of narcotics, it is necessary to remove the brain tissue from a medium containing a narcotic to a medium free from the narcotic, and to observe whether an increase in the respiration of the tissue takes place. With minced brain tissue this involves centrifuging and transference of the centrifuged deposit to a fresh medium. Hence it becomes important to discover whether this procedure in itself affects the subsequent respiration of the tissue when placed in a fresh medium.

The following experiment was therefore carried out: 0·5 g. minced fresh guinea-pig brain was placed in a medium containing 1 ml. *M*/5 phosphate buffer, p_H 7·4, 1·5 ml. saline and 0·5 ml. of 0·5 % glucose (in saline), and the respiration followed in a Barcroft respirometer. Air in the Barcroft vessels was displaced by oxygen, the temperature at which the experiment was carried out was 37° and the manometers were allowed to shake 90 times per minute. Readings were taken every 15 minutes.

The tissue was allowed to take up oxygen for 2 hours, after which the contents of the manometer vessel were transferred to a centrifuge-tube as completely as possible, the vessel being washed out twice with 0·5 ml. of the phosphate-saline-

glucose medium. The mixture was then centrifuged for 20 minutes. By means of a pipette 3·5 ml. of centrifugate were removed and 2·5 ml. of the phosphate-saline-glucose medium were added to the centrifuged deposit. The contents of the tube were then stirred and transferred carefully to a dry manometer vessel; the air was displaced by oxygen and the rate of respiration was again measured.

The results are noted in Table I which gives the Q_{O_2} as measured for each successive interval of 15 minutes. The Q_{O_2} (μl. O_2 at N.T.P. absorbed per hour per mg. dry weight of tissue) is reckoned on the basis that the dry weight is approximately equal to one-fifth of the wet weight of the tissue. It will be seen from

Table I.

	Q_{O_2}.							
Time from commencement (mins.)	15	30	45	60	75	90	105	120
Before centrifuging	6·7	6·8	6·3	6·2	5·7	5·3	5·2	5·5
After centrifuging	3·6	3·5	3·4	3·3	3·1	—	—	—

this table that the procedure of centrifuging, together with discarding the centrifugate, has resulted in a large drop in Q_{O_2} of the minced brain tissue. The Q_{O_2} had been fairly constant with the minced tissue for the hour previous to centrifuging and was also constant for the hour succeeding this operation. The drop in Q_{O_2} was of the order of 40 %—a value far greater than the estimated experimental error of 5 %.

Whether the fall in Q_{O_2} accompanying centrifuging and transference of the centrifuged deposit to a fresh medium is due to the removal of a factor important for the respiration of the tissue, or whether the fall is due largely to a progressive decrease of Q_{O_2} with time is a matter for further investigation; so far as the present work is concerned, the conclusion is clear that the use of minced brain tissue is undesirable for demonstrating reversibility *in vitro* of narcotic action.

The question then arose as to whether brain slices could be used. From the point of view of demonstrating reversibility it is immaterial as to how far the brain slice is composed of grey or of white matter, for clearly the purpose of the experiment is to compare the Q_{O_2} of a particular brain in presence of the narcotic with that after washing it as free as possible from the narcotic. It was necessary to carry out preliminary work to discover whether washing brain slices resulted in a fall in Q_{O_2}.

The following experiment was carried out. Small slices were cut, from the cortex as far as possible, of a fresh guinea-pig brain and placed in a medium composed of 1 ml. $M/5$ phosphate buffer solution, p_H 7·4, 1·5 ml. saline, 0·5 ml. of 0·5 % glucose (in saline). This was contained in the manometer vessel of a Barcroft respirometer. Air was displaced by oxygen and the oxygen uptake of the slices at 37° was followed, readings being taken each 15 minutes. At the termination of 2 hours, the slices were removed from the manometer vessel into a dish containing a phosphate-saline-glucose medium of the composition already given which had been warmed to 37°. The slices were gently stirred in the liquid and then removed to another manometer vessel containing 3 ml. of the same medium. The oxygen uptake was again followed for an hour or 75 minutes. The slices were then carefully transferred to a dish of distilled water, stirred gently, placed on a weighed drying-dish, dried at 105° and weighed.

This experiment was repeated many times, the slices being allowed to take up oxygen for varying intervals before washing. Typical results are given in Table II.

Table II.

Period of immersion of slices before washing (mins.)	Q_{O_2}. 120				60				30	
Time from commencement of exp. (mins.)	30	60	90	120	15	30	45	60	15	30
Before washing	15·8	16·3	16·7	14·3	14·0	15·6	15·3	15·6	13·7	14·7
After washing	13·8	13·0	—	—	13·2	15·2	15·0	13·8	14·1	14·7

The results of Table II show that it is possible to wash brain slices without seriously affecting the magnitude of the Q_{O_2}. As may be expected the largest drops in Q_{O_2} occur when the slices have been immersed for 2 hours before washing —there having occurred then the greatest disintegration of tissue—but the fall in Q_{O_2} does not exceed 20 % of the values obtained before washing. When the slices have been immersed 30 or 60 minutes before washing, the subsequent Q_{O_2} shows a fall varying from nil to 8 %.

Experience has shown that the most reproducible results occur when slices are taken which are not too thin. Slices of a thickness varying from 0·4 to 0·5 mm. are the most suitable. The slices should be trimmed, after cutting, into rectangular pieces whose areas can be readily estimated by carefully outlining them on squared paper in the dish of saline in which the slices are originally immersed. Knowing the weight of the slices and the area, the average thickness can be estimated with fair accuracy.

The preliminary results described above made it seem likely that brain slices could be used for demonstrating the reversibility of action of narcotics on oxygen uptake. The technique adopted was as follows.

Slices were cut, as far as possible from the cortex, of a fresh guinea-pig brain. About ten suitable small slices were chosen, their areas outlined and half of them were transferred to a manometer vessel containing 1 ml. $M/5$ phosphate buffer solution, p_H 7·4, 1·5 ml. saline, 0·5 ml. 0·5 % glucose (in saline) and the other half to a vessel containing 1 ml. phosphate buffer solution, p_H 7·4, 0·5 ml. 0·5 % glucose (in saline), 0·5 or 1 ml. of a saline solution of the narcotic under investigation together with sufficient saline to give a total volume of 3 ml. The respiration was followed in the usual way (in oxygen at 37°) for 30, 60 or 120 minutes after which the slices from each vessel were washed in a phosphate-saline-glucose medium of the composition already quoted and transferred to new manometer vessels each containing 3 ml. of the same medium. The respiration was again followed (in oxygen at 37°) for an hour or 75 minutes. The slices were then washed in distilled water, dried at 105° and weighed.

The narcotics chosen for the experiments about to be quoted were those which gave the most marked inhibitions of oxygen uptake and which belonged to different chemical types. It was felt that only by the use of such narcotics could reversibility be clearly tested. The narcotics chosen were chloretone, luminal and hyoscine, and results with these will be described in order.

Chloretone.

The effect of chloretone at a concentration of 0·04 % on the Q_{O_2} of guinea-pig brain slices is shown in Table III *a*.

The respirations of these brain slices after washing are shown in Table III *b*.

Table III *a*.

Time from commencement of exp. (mins.)	Q_{O_2} (before washing). 15	30	45	60	Average Q_{O_2}	% decrease in Q_{O_2} due to narcotic
In absence of narcotic	14·7	15·6	15·9	15·9	15·5	
In presence of 0·04 % chloretone	4·8	5·1	4·8	5·3	5·0	68

Table III *b*.

Time from commencement of exp. (mins.)	Q_{O_2} (after washing). 15	30	45	60	75	90	Av. Q_{O_2}	% decrease in Q_{O_2}
No narcotic previously administered	14·4	15·3	14·9	13·8	13·8	12·9	14·2	
0·04 % chloretone previously added	10·0	12·3	13·3	11·9	12·2	11·9	12·0	16

The following points are noteworthy.

1. The Q_{O_2} of the brain slices throughout an experiment remains very nearly constant.

2. The effect of the narcotic is rapid, the Q_{O_2} in presence of the narcotic reaching its equilibrium point within 15 minutes.

3. The Q_{O_2} does not fall progressively with time as the brain slice is kept exposed to the narcotic. If the narcotic were truly toxic, a "cell-poison," such a progressive fall in respiration would be expected.

4. The effect of washing brain slices previously immersed in the narcotic solution is to raise the Q_{O_2} to a level not far removed from that obtained with brain slices to which no narcotic had been added. With such washed brain slices a steady Q_{O_2} is rapidly reached showing the attainment of a new equilibrium.

Tables III *a* and III *b* show that chloretone (0·04 %) reduces the Q_{O_2} of guinea-pig brain slices 68 %; after washing the reduction is lowered to 16 % showing a reversibility of $\frac{52}{68} \times 100\ \% = 76\ \%$.

The effects of two different concentrations of chloretone to which brain slices were exposed for 1 hour, and the reversibilities produced by washing, are shown in Table IV. It will be observed from the results of the table that although chloretone at 0·04 % exercises a greater inhibition on Q_{O_2} than chloretone at 0·02 % the reversibility is about the same in both cases.

Table IV.

Concentration of chloretone	0·02 %	0·04 %
Q_{O_2} in absence of narcotic	15·7	15·5
Q_{O_2} in presence of narcotic	8·1	5·0
% decrease in Q_{O_2}	50	68
After washing:		
Q_{O_2}: no narcotic previously added	14·5	14·2
Q_{O_2}: chloretone previously added	12·3	12·0
% decrease in Q_{O_2}	15	16
% reversibility	70	76

Luminal.

Luminal was given as the sodium salt, a solution of which was brought to p_H 7·4 by the addition of HCl.

The effects of luminal at a concentration of 0·08 % (as the sodium salt) on the respiration of guinea-pig brain slices and the subsequent respiration after the

brain slices had been washed are shown in Table V. In this experiment the brain slices were exposed to the action of the luminal for 2 hours before washing.

Table V.

Time from commencement of exp. (mins.)	Q_{O_2}. 15	30	45	60	75	90	105	120	Av. Q_{O_2}	% decrease in Q_{O_2}
In absence of narcotic	14·7	15·8	16·8	16·2	15·8	16·7	14·7	14·3	15·6	
In presence of luminal	9·2	9·2	9·6	9·5	9·2	9·2	9·6	8·7	9·4	40
After washing:										
No narcotic previously added	14·6	13·8	13·8	12·9	12·3	—	—	—	13·5	—
Luminal previously added	13·1	13·1	12·2	12·1	11·3	—	—	—	12·4	8

Reversibility $= \frac{3\cdot2}{4\cdot0} \times 100 = 80$ %.

As in the case of chloretone it will be noticed that a constant Q_{O_2} is obtained in the presence of luminal, and after washing, the Q_{O_2} is raised to a new equilibrium-point. The luminal effected a 40 % decrease in the Q_{O_2} of guinea-pig brain slices which was reduced to 8 % by washing; a reversibility of 80 % was thus effected.

In an experiment where the brain slices were in contact with luminal (0·08 % as sodium salt) for only 30 minutes, complete reversibility was effected. The luminal caused a drop in Q_{O_2} from 15·5 to 11·5 (33 %); after washing, the Q_{O_2} of the brain slices to which no narcotic had been added was 15·4 and that of the slices to which luminal had been added was 15·5.

Hyoscine.

Hyoscine was added as the hydrobromide, a solution of which was brought to p_H 7·4. Results with hyoscine (0·16 %) with guinea-pig brain slices are shown in Table VI. In this experiment the slices were exposed to the hyoscine for 1 hour before washing.

Table VI.

Time from commencement of exp. (mins.)	Q_{O_2}. 15	30	45	60	Av. Q_{O_2}	% decrease in Q_{O_2}
In absence of narcotic	14·0	15·6	15·3	15·6	15·1	
In presence of hyoscine	9·2	8·5	8·5	8·0	8·5	44
After washing:						
No narcotic previously added	13·2	15·2	15·0	13·9	14·3	
Hyoscine previously added	12·6	14·6	13·2	13·2	13·4	6

Reversibility $= \frac{3\cdot8}{4\cdot4} \times 100 = 86$ %.

Reversibility is effected by washing brain slices previously exposed to hyoscine just as in the cases of chloretone and luminal. In the experiment quoted in Table VI the reversibility was 86 %, the hyoscine having originally produced 44 % inhibition of the rate of oxygen uptake. This is typical of many experiments.

The results of exposing brain slices to hyoscine hydrobromide (0·16 %) for varying lengths of time and the effects of washing are shown in Table VII.

Table VII.

Duration of exposure of brain slices to hyoscine before washing (mins.)	30	60	120
% decrease in Q_{O_2} due to the hyoscine	44	44	49
% reversibility produced by washing	88	86	90

These results illustrate the fact already mentioned that the brain slices come rapidly into equilibrium with the narcotic; this occurs with chloretone and luminal as well as with hyoscine. The fact that there is no marked increase in the reduction of Q_{O_2} due to hyoscine with length of exposure to the drug shows that the phenomenon under consideration (inhibition of respiration) is not due to irreversible cell poisoning, a fact confirmed by the marked increase in Q_{O_2} of the brain slices after washing.

It is natural to enquire why reversibility is not in all cases of the order of 100 %. It has been shown that with luminal complete reversibility can be obtained, but with chloretone and hyoscine it has not been possible so far to exceed 90 %. It has been felt by the authors that this result is due to the incomplete washing of the slices. It is difficult to remove all traces of the narcotic by washing for a few minutes and it is conceivable that failure to arrive at reversibility greater than 90 % is due to remaining traces of the narcotic in the brain slices.

Mescaline.

Mescaline is a drug which gives rise to visual hallucinations. The authors [1933] have shown that it depresses the respiration of guinea-pig brain. It will now be shown that this inhibitory effect is largely reversible.

Mescaline hydrochloride (0·16 %) was added to guinea-pig brain slices in presence of the phosphate-saline-glucose medium and its effect on the Q_{O_2} measured. The slices were washed after an hour's exposure to the drug and the rate of respiration of the washed slices was determined. The results are given in Table VIII.

Table VIII.

Average Q_{O_2} in absence of mescaline	16·7
Average Q_{O_2} in presence of mescaline	11·7
% decrease in $Q_{O_2}=30$.	
After washing:	
Average Q_{O_2}: mescaline not previously added	15·3
Average Q_{O_2}: mescaline previously added	14·6
% decrease in $Q_{O_2}=5$.	
Reversibility $=\frac{25}{30}\times 100=83$ %.	

Mescaline produced an inhibition of 30 % of the rate of oxygen uptake of guinea-pig brain slices. This inhibition was reduced by washing to 5 %, showing reversibility to the extent of 83 %.

Mescaline is 3 : 4 : 5-trimethoxy-β-phenylethylamine and it was of interest to observe whether the basic amines, whose effects on brain oxidations have already been noted [Quastel and Wheatley, 1933], behave in a reversible manner similarly to the narcotics. It was shown [1933] that many amines, including β-phenylethylamine and mescaline, and also indole resemble the narcotics in depressing the oxidation by the brain of glucose and sodium lactate and pyruvate, and in not affecting the action on sodium succinate. The inhibitory action of mescaline on brain oxidation is, as has already been shown, largely reversible.

The inhibitory action of β-phenylethylamine is also largely reversible as will be seen from the results quoted in Table IX. The brain slices in this experiment were exposed to β-phenylethylamine hydrochloride (0·16 %) for one hour.

Table IX.

Time from commencement of exp. (mins.)	Q_{O_2}. 15	30	45	60	Av. Q_{O_2}	% decrease in Q_{O_2}
In absence of amine	15·2	15·8	15·5	15·5	15·5	
In presence of β-phenylethylamine	9·5	10·4	9·9	10·2	10·0	36
After washing:						
No amine previously added	13·4	14·8	14·3	14·1	14·1	
β-Phenylethylamine previously added	12·3	12·4	2·2	12·4	12·3	13

Reversibility $= \frac{2}{3}\frac{3}{6} \times 100 = 64$ %.

The reversibility is apparently not as complete as with the narcotics or as with mescaline.

On testing indole it was found that no reversibility whatever occurs. Typical results with indole are shown in Table X. In this experiment guinea-pig brain slices were exposed to indole (0·03 %) for 2 hours before washing.

Table X.

Time from commencement of exp. (mins.)	Q_{O_2}. 15	30	45	60	75	90	105	120	Av. Q_{O_2}	% decrease in Q_{O_2}
In absence of indole	16·7	17·1	17·3	18·3	17·4	15·8	16·7	14·9	16·7	
In presence of indole	11·2	10·9	10·9	10·6	10·0	8·2	6·9	7·5	9·6	43
After washing:										
No indole previously added	14·3	14·0	14·0	13·5	12·0	—	—	—	13·9	
Indole previously added	7·7	8·3	8·0	8·3	6·6	—	—	—	8·1	42

Reversibility = nil within experimental error.

Now it was quite evident by perception of the odour alone in the vessel in the second part of the experiment that some of the indole had not been removed from the slices by washing; this would probably account for much of the lack of reversibility. It is to be noted, however, that the Q_{O_2} of the brain slices in presence of the indole steadily falls so that it is possible that indole, in contrast to the substances already investigated, has some progressively toxic effect on brain tissue. It is impossible, however, to decide this until more experiments have been carried out for longer periods to determine whether the Q_{O_2} drops continuously or whether an equilibrium is eventually attained. Conceivably the diffusion of indole into the cells of a brain slice is a slow process. It is significant that the Q_{O_2} of the washed brain slices, which had been in indole solution and which undoubtedly still contained indole, was fairly constant.

It is possible that the simple process of washing by immersing brain slices for a few minutes in a fresh phosphate-saline-glucose medium, which is effective with such narcotics as chloretone, luminal or hyoscine, is not so effective with the basic amines. This is a matter for further investigation.

Discussion.

The most important results arising from the work described above are as follows:

1. It is possible to wash brain slices by immersion in a phosphate-saline-glucose medium without seriously affecting the respiration of the tissue. Centrifuging minced brain tissue with transference of the centrifuged deposit to a fresh medium results in a greatly lowered respiration.

2. Brain slices rapidly come into equilibrium with narcotics such as chloretone, hyoscine and luminal, a constant Q_{O_2} being established. The fact that the Q_{O_2} does not fall progressively with time, even in presence of relatively high concentrations of the narcotics, shows that an irreversible destructive action on the brain cells is not taking place.

3. After washing brain slices which have been exposed to the narcotics mentioned above for intervals varying from 30 to 120 mins., the Q_{O_2} rises to a new equilibrium value.

4. The reversibility is greater than 70 %, complete reversibility having been found on several occasions.

5. Reversibility of inhibitory action on respiration of brain tissue is shown also by mescaline and by β-phenylethylamine. No reversibility, however, occurs with indole, where it is evident that washing has been inadequate. It is possible, however, that indole exerts some truly toxic action which increases with time.

The proof given above that reversibility of behaviour occurs *in vitro* with the narcotics gives greater biological significance to the results obtained by the authors on the action of narcotics on oxidations by minced brain tissue. It is evident that the narcotics, at the concentration used by the authors, are not acting as cell poisons, *i.e.* in the sense of substances which bring about irreversible damage to the tissue cells. That this was the case, *i.e.* that the narcotics did not act as general cell poisons, was evident from the lack of inhibitory action of narcotics on succinate oxidation by minced brain tissue. It is likely, as has already been suggested, that the narcotics inhibit respiration by competing with a substrate (probably lactic acid) at the active surfaces of the brain cells.

Bülow [1933] considers that because she failed to obtain reversibility after exposing minced brain tissue to an acetylene-oxygen mixture for 2 hours or more, that the inhibition of glucose oxidation effected by acetylene is due to an irreversible toxic effect. This may be true in the case of acetylene but such a deduction cannot be made on the basis of her experiments alone. In the first place it is evident that acetylene does not act as a general cell poison, for, as Bülow agrees, succinate oxidation is apparently unaffected by the gas. In the second place it is possible that her failure to obtain reversibility was due to the fact that the minced brain tissue which had been exposed to acetylene for 2 hours or more was still saturated—or partially saturated—with the gas, when the acetylene atmosphere in the manometer vessels was replaced by oxygen. Her results with minced brain tissue which had been washed by centrifuging in presence of distilled water are difficult to interpret in view of the results mentioned early in this paper. It is also difficult to understand the differences which she found between the actions of acetylene and ethylene; the results of Nord [1934] should be borne in mind in considering these results. The writers have exposed brain slices to an acetylene-oxygen atmosphere and have found no marked change in Q_{O_2} from that obtaining in a nitrogen-oxygen atmosphere of the same composition. It was obvious from the constancy of the Q_{O_2} that the acetylene was not exercising a progressively toxic action. It is a matter for

further experiment to discover why acetylene apparently does not inhibit the respiration of fresh brain tissue (either in the form of slices or of minced material) but behaves like narcotics in causing minced brain tissue, which has been exposed to the gas for 2 hours or more, to give a diminished oxidation of glucose and not of sodium succinate.

SUMMARY.

Using brain slices it is possible to show reversibility of the strong inhibitory actions of the narcotics, chloretone, luminal and hyoscine, on brain respiration. The reversibility is demonstrated by washing the slices in a phosphate-glucose-saline medium. It occurs to the extent of at least 70 % and complete reversibility has been found in a number of instances. Reversibility has also been demonstrated with mescaline and β-phenylethylamine which also inhibit brain oxidations. No reversibility has yet been found with indole which is a powerful inhibitor of brain respiration.

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