Indeed, the preferential utilization of NADH would be expected in part since the rate of the NADH microsomal peroxidase activity exceeds the NADPH mediated peroxidase activity [2–4, 8]. Then, as the CHP concentration decreases (*ca.* 80 per cent CHP reduced in 3 min), the remaining NADPH can re-reduce the cytochrome b_5 more efficiently to its reduced state.

In addition, Werringloer and Estabrook [21] have reported that the oxidation of NADH was increased in the presence of NADPH, both in the presence and absence of substrate. The oxidation appeared to be dependent on the concentration of NADPH as well. This effect would also increase the conversion of the NADH maintained reduced cytochrome b_5 steady state to the NADPH reduced cytochrome steady state in the presence of CHP.

Thus, cytochrome b_5, reduced by either NADPH or NADH, was oxidized in the presence of CHP. NADH maintained the reduced steady state of cytochrome b_5 more efficiently than did NADPH. Yet, in the presence of both pyridine nucleotides, the hydroperoxide initially oxidized the cytochrome to the NADH mediated steady state but was then oxidized further to the NADPH reduced steady state. From these redox changes, we conclude that NADH was used either preferentially or at a faster rate, sparing the NADPH which was then available to re-reduce cytochrome b_5 to the NADPH steady state.

More detailed experiments are in progress, quantitating each of the pyridine nucleotides and the CHP with time. These experiments should clarify the exact causes of the redox changes observed and may improve our understanding of the central role played by cytochrome b_5 in the microsomal electron transfer reactions, especially to the microsomal peroxidase activities.

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Effects of morphine and methadone on the isolated perfused rat brain

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The variable results obtained previously [1–4] in studies in rats of the effects of the administration of morphine on the intermediary metabolites and energy reserves of brain tissue may have resulted from differences in the techniques of sampling brain tissue for analysis. We have studied the direct effects of morphine and also methadone on the rat cerebral cortex, using an isolated perfused rat brain preparation [5]. With this intact brain preparation, there is no interference from other organs, no anesthetic agent is used, blood flow and blood gases are controlled, and reproducible samples of cerebral cortex are very rapidly frozen for analysis. We found that perfusion of this isolated brain with fluid containing morphine (50 μg/ml) caused significant decreases in the concentrations of ATP and creatine phosphate and a significant increase in lactate. In contrast, brains perfused with methadone (50 μg/ml) showed very little change in brain metabolites, only a small decrease in ATP, and an increase in lactate.

Methods

Preparation and perfusion of isolated brain. Unanesthetized male Sprague–Dawley rats (about 250 g) were adequately anesthetized for surgery by deep hypothermia (rectal temperature 16–18°) induced as described previously [6]. The isolated brain preparation, consisting of the detached skull and its contents, was made from the hypothermic rat without lapse of circulation and was perfused through the internal carotid arteries as described previously [5]. The perfusion fluid [7] contained the perfluoro compound FC-80 (3M Co., St. Paul, MN) as an erythrocyte substitute which was dispersed ultrasonically in an 8% (w/v) solution of bovine albumin (fraction V powder, Sigma

Table 1. Effects of morphine and methadone on metabolism of isolated, perfused rat brains*

	Glucose consumption (μmoles/hr)	Lactate production (μmoles/hr)
Control	26 ± 2	18 ± 2
Morphine (50 μg/ml)	35 ± 1†	27 ± 2†
Methadone (50 μg/ml)	34 ± 3‡	21 ± 1

* The values given are the mean ± S.E. for seven experiments in each group. Closed-circuit perfusion was done at 25° for 40 min with an artificial blood equilibrated with 95% O_2–5% CO_2.

† Significantly different from the control value ($P < 0.01$).

‡ Significantly different from the control value ($P < 0.05$).

Chemical Co., St. Louis, MO) in Krebs–Ringer bicarbonate buffer containing 2 mg/ml of glucose (11 mM). Closed-circuit perfusion (2 ml/min) was performed at 25° with continuous oxygenation of the perfusion fluid with 5% CO_2–95% O_2. The venous drainage during the first 10 min was discarded, recirculation was started, and then the drug was added to the perfusion fluid. Bipolar electroencephalograms (EEG) were recorded from silver electrodes placed in depressions made in the bone of the frontal and parietal regions on both sides. If the EEG was not normal during the first 10 min of perfusion, the preparation was discarded.

Sampling and analysis of fluid and tissue. Samples of perfusion fluid were collected at intervals from the venous drainage and analyzed for glucose by a commercial glucose oxidase reagent (Glucostat, Worthington Biochemical Co., Freehold, NJ) and for lactate [8] and pyruvate [9] with lactate dehydrogenase.

At the end of the perfusion period, the calvarium was removed while perfusion continued and the brain preparation was rapidly crushed between the jaws of heavy tongs which had been immersed in liquid nitrogen. This caused cerebral tissue to be extruded from the skull and very rapidly frozen. The whole preparation was immediately immersed in liquid nitrogen. The extruded, frozen cerebral tissue was separated from bone and stored in liquid nitrogen until it was analyzed. Extracts of the cerebral tissue were made and analyzed for adenine nucleotides, creatine phosphate and glycolytic intermediates by enzymic methods in which the oxidation or reduction of coupled pyridine nucleotides was measured fluorometrically as described previously [10].

Concentration of morphine and methadone in perfusion fluid. In previous studies of the effects of morphine *in vivo* in the rat, doses varying from 15 to 45 mg/kg have been used [1, 2]. If we assume that the space in which injected morphine is distributed is approximately the total body water of the rat (60% of body wt), then a dose of 30 mg/kg would yield a concentration of about 50 μg/ml in the body fluids. This calculation can yield only a rough order of magnitude value since the actual volume of distribution is not known and the sensitivity to morphine of the perfused brain, which has no neural input, may be very different from the brain of the intact rat. Therefore, we did preliminary experiments in which the isolated brain was perfused with 10, 50 and 250 μg/ml of morphine. The EEG of the perfused brain was not affected significantly by perfusion with 10 μg/ml of morphine, it was markedly depressed by 250 μg/ml, and with 50 μg/ml there was a definite but not severe effect. For this reason, we chose the value 50 μg/ml for the concentration of morphine in our perfusion experiments. In similar preliminary experiments with methadone, 50 μg/ml was the lowest concentration which yielded definite EEG effects, and therefore we used the same concentration of methadone in our perfusion studies.

The isolated brains were perfused with fluid containing morphine or methadone for 40 min. This period was chosen because the EEG was stable and the rate of consumption of glucose did not change significantly during the first hour of perfusion in control experiments. After more than 1 hr of perfusion, there was usually a gradual decrease in the amplitude of the EEG, but the consumption of glucose was usually unchanged.

Results

EEG. In the control experiments, the EEG was stable throughout the 40-min perfusion period. The EEG of the brains perfused with morphine (50 μg/ml) showed a slow and continuous decrease in amplitude, with little change in frequencies. The EEG of the brains perfused with methadone (50 μg/ml) was relatively stable, but there were occasional high amplitude slow waves and spikes.

Glucose consumption and lactate production. From the changes in the concentrations of glucose and lactate in the perfusion fluid, the rates of consumption of glucose and production of lactate were calculated. The results (Table 1) show that morphine caused increases in both glucose consumption and lactate production, but methadone caused an increase in glucose consumption without a significant increase in lactate production.

Energy reserves and metabolic intermediates. The results (Table 2) of the analyses of cerebral tissue after 40 min of perfusion with morphine (50 μg/ml) show that the energy reserves were significantly decreased; the levels of creatine phosphate and ATP were decreased, as were the levels of glycogen and glucose. The concentrations of lactate and several of the glycolytic intermediates were increased, as well as the lactate/pyruvate ratio. There was also a significant decrease in the total adenine nucleotide (ATP + ADP + AMP) level.

In contrast to morphine, perfusion with methadone (50 μg/ml) caused almost no significant change in the energy reserves (Table 2). There was a marginally significant decrease in ATP concentration but no significant change in the levels of creatine phosphate, glycogen or glucose. The lactate level and the lactate/pyruvate ratio were increased, but to a much lesser degree than in the brains perfused with morphine.

Discussion

The isolated perfused rat brain is quite similar to the brain of the intact rat with respect to its electrical activity [5], blood–brain barrier function [11] and rate of glucose consumption [12]. However, it is not identical with the normal brain because the spinal cord has been cut and the isolated brain does not receive a neural input. Therefore, in the isolated brain the activity of neurons and the effects of drugs on this activity may be significantly different from that of the intact brain. However, the effects observed in the isolated brain can be ascribed to a direct action of the

Table 2. Effects of morphine and methadone on energy reserves and metabolic intermediates in cerebral tissues of isolated, perfused rat brain*

	Control	Morphine (50 μg/ml)	Methadone (50 μg/ml)
Creatine phosphate	2540 ± 54	1740 ± 84†	2420 ± 78
ATP	1960 ± 39	1250 ± 75†	1855 ± 28‡
ADP	720 ± 31	680 ± 28	750 ± 20
AMP	260 ± 23	425 ± 42†	250 ± 11
ATP + ADP + AMP	2940 ± 34	2340 ± 77†	2860 ± 20
ATP/ADP	2.75 ± 0.14	1.80 ± 0.11†	2.43 ± 0.10
Glycogen (as glucose)	950 ± 38	670 ± 92‡	930 ± 90
Glucose	1770 ± 170	1320 ± 66‡	1890 ± 26
Glucose-6-phosphate	72 ± 5	66 ± 3	94 ± 3†
Fructose-6-phosphate	22 ± 2	20 ± 1	21 ± 2
Fructose-1,6-diphosphate	82 ± 4	87 ± 9	83 ± 4
Triose phosphate	51 ± 4	56 ± 4	56 ± 4
Glycerol-3-phosphate	91 ± 7	132 ± 14‡	82 ± 4
3-Phosphoglycerate	46 ± 3	54 ± 2‡	50 ± 1
2-Phosphoglycerate	14 ± 1	19 ± 1†	19 ± 1†
Phosphoenol pyruvate	10 ± 1	14 ± 1‡	12 ± 1
Pyruvate	63 ± 5	55 ± 4	63 ± 6
Lactate	2330 ± 230	4000 ± 310†	3000 ± 170‡
Lactate/pyruvate	37 ± 3	73 ± 4†	50 ± 3‡

* All concentrations are in nmoles/g (wet wt) of tissue. All values are the mean ± S.E. for ten experiments in each group.

† Significantly different from the control value ($P < 0.01$).

‡ Significantly different from the control value ($P < 0.05$).

drug on the brain; effects observed after injection of a drug into the intact animal may be due to the action of the drug or its metabolites on the brain and/or the action of the drug or its metabolites on other organs, with secondary effects on the brain.

Our results show that there is a marked difference between morphine and methadone in their effects on the metabolism of the isolated brain. Both morphine and methadone increased the rate of utilization of glucose (Table 1), but these increases were different in nature. With morphine, the rate of lactate production was increased, indicating that there was an increase in the rate of glycolysis. With methadone, there was no increase in lactate production, indicating that the extra glucose was being utilized largely in oxidative metabolism.

Our results (Table 2) also show that morphine caused a very significant decrease in the energy reserves of the isolated brain, while methadone had almost no significant effect. The brains perfused with morphine showed marked decreases in creatine phosphate and ATP. There was also a significant decrease in the total adenine nucleotides (ATP + ADP + AMP), which would indicate that the effects of morphine might not be readily reversed when the morphine is removed. The glycogen and glucose concentrations of the cerebral tissue were also considerably reduced, while the levels of lactate and several glycolytic intermediates were increased significantly.

Morphine might have caused these changes by an effect on autonomic regulation which changed regional blood flow or by an effect on neuronal excitability. Whatever the mechanism of action may be, the decrease in energy reserve of the tissue suggests that there was a decrease in oxidative metabolism, the major generator of the energy reserve.

All the changes observed in the brains perfused with morphine could be accounted for by an inhibition of mitochondrial oxidative activity by morphine. Such inhibition would result in a decrease in oxidative phosphorylation, with resulting decreases in ATP and creatine phosphate. As a result of these decreases, glycolysis would be stimulated, with resulting increases in rates of glucose consumption and lactate production, and also decreases in concentrations of glycogen and glucose in the tissue. The observed increases in the lactate/pyruvate ratio and in the concentration of glycerol-3-phosphate point to a reduced redox state, which would be expected if oxidation were inhibited. The changes in the brain tissue indicate that, in the brain perfused with morphine, the energy supply is not adequate in spite of the increase in glycolysis. This inadequate energy reserve is the most likely cause of the continued deterioration of the EEG during perfusion with morphine.

In contrast, the brains perfused with methadone showed almost no significant changes in energy reserve or metabolites. The comparison of the effects of morphine and methadone is limited because we have studied only one concentration of each drug. Comparison of our results with effects in intact animals is also limited by the possible differences in susceptibility to drug action between the isolated and intact brains. The significant increase in glucose-6-phosphate concentration suggests the possibility that methadone may have some effect on the pentose phosphate pathway. Takemori [13] has reported that injection of methadone or morphine caused an increase in glucose-6-phosphate dehydrogenase activity in brain tissue of rats.

Our results with morphine and the perfused brain are different from those obtained by Dodge and Takemori [14] and Miller *et al.* [3] in rats injected with morphine. Miller *et al.* [3] noted that the results of earlier studies, including that of Dodge and Takemori [14], may have been in error because their method of freezing the brain was too slow to prevent post-mortem changes in the brain metabolites. Miller *et al.* [3], using special apparatus for the rapid removal and freezing of rat brain, found that a single dose of morphine (20 mg/kg) caused an increase in brain glucose, a decrease in creatine phosphate and some other metabolites, but no changes in adenine nucleotide concentrations. In our experiments, the perfused brains were very rapidly frozen, with no prior interruption of perfusion. This makes it unlikely that a technical error would account for the differences in our results. There are several differences

between the perfused brain and the brain of the intact rat, which may account for the differences in results. The results from the perfused brain experiments show only the direct effects of a known constant concentration of morphine on the cerebral tissue. In rats injected with morphine, the concentrations of morphine and its metabolites in the circulation were not known and not constant, and the effects on other organs may have caused secondary effects in the brain. For example, it is known [15] that injected morphine caused a marked hyperglycemia, which probably increased the brain glucose concentration and perhaps significantly altered the metabolism of the brain. In fact, the increase in brain glucose concentration observed by Miller *et al.* [3] may be accounted for in large part by the high concentration of glucose in the blood within the brain at the time of sampling. Another difference was that in our experiments only cerebral tissue was sampled and analyzed, while the samples obtained with the apparatus used by Miller *et al.* [3] were mixtures of unknown proportions of several regions of the brain.

Sablé-Amplis *et al.* [16] found, in the brain of the intact rat, as we did in the isolated rat brain, that morphine caused an increase in lactate concentration and in the lactate/pyruvate ratio. This suggests that oxidative metabolism was inhibited by morphine. Bachelard and Lindsay [17] found that the incorporation of radioactivity from [^{14}C]glucose into citric acid cycle intermediates of the brain was diminished after injection of morphine in rats. This is more direct evidence that morphine inhibited mitochondrial oxidative activity, and it is less likely to be subject to technical error than are measurements of redox state. There is good evidence [18, 19] that respiratory exchange and oxygen consumption are decreased in rats after the administration of morphine. This would suggest that there was an inhibition of mitochondrial activity by morphine and/or its metabolites in some tissues of the animals, perhaps including brain tissue.

We found that methadone significantly increased glucose consumption without a significant increase in lactate production. Some stimulation of metabolism is suggested by our observation of the activation of the EEG. Similar effects were observed in intact animals by Jhamandas and Dickinson [20], who reported that methadone, unlike morphine, produced seizures in animals. Another important difference in the effects of morphine and methadone was reported by Goodlet and Sugrue [21], who found that acutely administered morphine, but not methadone, increased the turnover of 5-hydroxytryptamine in the rat brain.

In summary, perfusion of an isolated rat brain preparation with fluid containing morphine (50 μg/ml) caused increases in both glucose consumption and lactate production; perfusion with methadone (50 μg/ml) caused an increase in glucose consumption but no significant increase in lactate production. After perfusion with morphine for 40 min, the concentrations in cerebral tissue of creatine phosphate, ATP, glucose and glycogen were decreased and those of lactate and several glycolytic intermediates and the lactate/pyruvate ratio were increased. After perfusion with methadone for 40 min, there was only a small decrease in ATP concentration in cerebral tissue and the lactate/pyruvate ratio was increased but to a much lesser degree than in the brains perfused with morphine. The results suggest that morphine may inhibit mitochondrial oxidative activity.

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