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COMMUNICATIONS

The action of the local anaesthetic, benzyl alcohol and the monoamine oxidase inhibitor, clorgyline on the β-hydroxybutyrate dehydrogenase activity of adult and weanling rat brain mitochondria

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Both local and general anaesthetics (see e.g. Colley & Metcalfe, 1972; Lee, 1976a; Miller & Pang, 1976; and Shieh, Veda & others, 1976) as well as some tranquillizers and antihypertensives (see Lee, 1977a; Singer, 1977) possess the ability to increase the fluidity of lipid bilayers and it has been suggested that this property is related to the phenomenon of anaesthesia (see Trudell, Payan & others, 1975 and Lee, 1976b). However, the activity of a number of membrane-bound enzymes may be modulated by changes in the fluidity of the lipid bilayer (see e.g. Houslay, Warren & others, 1975; Houslay, Hesketh & others, 1976; Hesketh, Smith & others, 1976). Thus a variety of drugs with the ability in common to increase bilayer fluidity, may be expected to have some apparently non-specific effects *in vivo*.

We have taken two different compounds whose action on increasing bilayer fluidity has been characterized in defined lipid systems, namely, the local anaesthetic benzyl alcohol (Colley & Metcalfe, 1972) and the irreversible monoamine oxidase inhibitor, clorgyline [*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxypropylamine)] (Houslay, 1977) and looked at their effects on the β-hydroxybutyrate dehydrogenase activity of weanling (25 day old) and adult (12 week old) rat brain mitochondria. β-Hydroxybutyrate dehydrogenase is of importance, to the brain in its utilization of ketone bodies both as an energy source and a supply of acetyl CoA for synthetic purposes (see Cremer & Heath, 1974; Patel & Owen, 1977). This enzyme mediates the conversion of β-hydroxybutyrate and acetoacetate in an NAD^+-dependent fashion, and although its activity is very low in the brain of perinatal rats it increases some 5-fold to a maximum just after weaning and then the activity slowly falls to the adult levels of some 30–40% of the maximum activity (Klee & Sokoloff, 1967). The β-hydroxybutyrate dehydrogenase activity in other tissues has been demonstrated to be exclusively localized in the inner mitochondrial membrane, with an absolute requirement for the choline headgroup supplied by a phospholipid, to exhibit activity (Sekuzu, Jurtshuk & Green, 1963; Fleischer, Bock & Gazzotti, 1974; Houslay & others, 1975).

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Brain mitochondria were prepared by standard methods on discontinuous sucrose gradients (Clarke & Nicklas, 1970) and were fragmented by freeze-thawing and treatment with an Ultra-Turrax disrupter, ensuring that no latent enzyme activity was present. All the β-hydroxybutyrate dehydrogenase activity could be sedimented by centrifugation at 100 000 *g* for 1 h, demonstrating that it was all membrane-bound. Mitochondria were stored at −20° with no loss of enzyme activity. β-Hydroxybutyrate dehydrogenase activity was assayed at pH 7·2 either by measurement of NADH production as described previously (Houslay & others, 1975) or by the method of Klee & Sokoloff (1967) which estimates acetoacetate produced during the reaction. Both methods gave similar results. The water insoluble compounds myristoyl alcohol and myristic acid were allowed to equilibrate with mitochondrial fragments by partition from azolectin liposomes. Incubations were carried out at either 4° or 23° with identical results, and the maximal changes in activity were recorded, occurring after either 15 min incubation at 4° or 5 min incubation at 23°.

The effect of benzyl alcohol on the activity of β-hydroxybutyrate dehydrogenase of the mitochondrial fragments of adult and weanling rat brain at 23° is shown in Fig. 1. There is progressive reversible augmentation of the activity of the enzyme with increasing benzyl alcohol concentration up to a maximum at about 70 mM benzyl alcohol. With enzyme preparations from adult rat brain there is an activation of some 8·5 fold, compared with only a two-fold increase in activity with weanling brain preparations (Fig. 1). In a comparison of the activation of the enzyme from animals of the two ages in the presence of maximally stimulating benzyl alcohol concentrations (Table 1), it can be seen that the specific activity of the enzyme preparations from adult brain mitochondria attain 76% of that exhibited by the enzyme from weanling brain mitochondria. This contrasts strongly with a value of only 20% observed in the absence of benzyl alcohol.

Arrhenius plots of the variation with temperature of the activity of the enzyme from adult rat brain in the absence or presence of 50 mM benzyl alcohol showed a decrease in the activation energy of the reaction from 22·3 to 13·4 kcal mol^{-1} (90–54 kJ mol^{-1}) (which is signi-

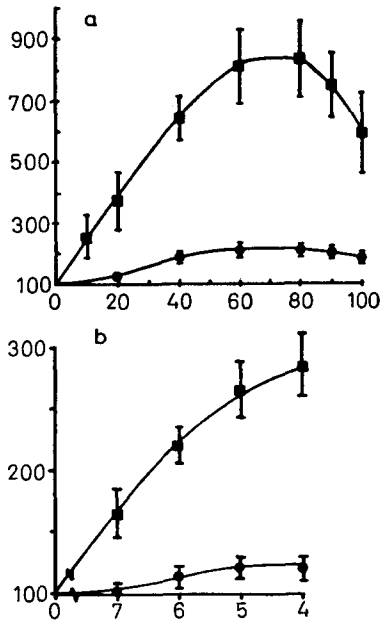

FIG. 1. The effect of benzyl alcohol and clorgyline on 3-hydroxybutyrate dehydrogenase isolated from adult and weanling rat brain. ■ adult rat brain; ● weanling rat brain. The bars represent standard deviations of the enzyme activity in four separate mitochondrial preparations at each age and benzyl alcohol concentration. (a) benzyl alcohol; (b) clorgyline. Ordinate: Activity (%). Abscissa: a, Concn (mM); b Concn (—log M).

Table 1. *The effect of benzyl alcohol on the activity of 3-hydroxybutyrate dehydrogenase in mitochondrial fragments from the brains of weanling and adult rats.*

	Original activity (m units mg^{-1})	Activity with 60 mM benzyl alcohol (m units mg^{-1})	Stimulation*
Weanling rats	5·7 (1·2)	12·3 (2·7)	×2·2
Adult rats	1·1 (0·4)	9·4 (1·4)	×8·5
Weanling rats + azolectin (12·5 mM)	5·2 (0·8)	11·5 (1·0)	×2·2
Weanling rats + azolectin (12·5 mM) + myristic acid (6·25 mM)	2·0 (0·6)	10·2 (1·2)	×5·1
Weanling rats + azolectin (12·5 mM) + myristoyl alcohol (6·25 mM)	2·6 (0·4)	10·5 (0·9)	×4
Adult rats + azolectin (12·5 mM)	0·8 (0·2)	6·4 (0·8)	×8
Adult rats + azolectin (12·5 mM) + myristic acid (6·25 mM)	0·7 (0·3)	6·0 (0·8)	×8·5
Adult rats + azolectin (12·5 mM) + myristoyl alcohol (6·25 mM)	0·7 (0·4)	5·6 (1·0)	×8

The values of the means (with standard deviations) of 4 individual adult and weanling mitochondria preparations.

* Increase in activity relative to original activity.

ficant at the $P = 0{\cdot}001$ level by regression analysis). The so-called pre-exponential term in the Arrhenius expression (related to the entropy of activation of the reaction) is decreased 500-fold by addition of benzyl alcohol. The activation energy of the enzyme from weanling rat brain (16·5 kcal mol^{-1}; 67 kJ mol^{-1}) was not significantly changed by benzyl alcohol, although the pre-exponential term was increased two-fold. The various Arrhenius plots were linear over the temperature range 10°-30° (coefficient of linear correlation >0·96).

At temperatures greater than about 35° the enzyme was rapidly inactivated by benzyl alcohol by a mechanism that was not reversed after cooling the enzyme; the enzyme from ox-heart is also irreversibly inhibited by benzyl alcohol at this temperature (M.D.Houslay unpublished results). This inhibition may be due to the alcohol displacing lipid from the annulus of the protein in such a fashion that it leads to denaturation of the enzyme, or the alcohol may directly denature the enzyme protein. Irreversible inhibition by benzyl alcohol at elevated temperatures is distinct from the reversible inhibition seen at 23° with high alcohol concentrations (Fig. 1), which may be due to the displacement of annular phosphatidylcholine, essential for binding the NAD^+ coenzyme (Fleischer & others, 1974), by benzyl alcohol. Indeed, high concentrations of benzyl alcohol at elevated temperatures have been shown to displace annular lipid from the Ca^{2+}-Mg^{2+}, ATPase from rabbit sarcoplasmic reticulum leading to its reversible inactivation (Hesketh & others, 1976).

As demonstrated in Fig. 1b clorgyline also increased the activity of the enzyme, and like benzyl alcohol the adult enzyme was more dramatically affected than that from weanling animals.

We would like to suggest that both benzyl alcohol and clorgyline, which are known to increase bilayer fluidity (Colley & Metcalfe, 1972; Houslay, 1977) increase the fluidity of the lipid annulus around the enzyme, hence relieving a constraint on the enzyme with concomitant activation. Similar results have been obtained using these compounds with other enzyme systems (see e.g. Hesketh & others, 1976; Houslay, 1977; Dipple & Houslay, 1978). The decreased activation energy for the reaction observed in the presence of benzyl alcohol would add further support to this (see Dipple & Houslay, 1978). The more pronounced activation caused by benzyl alcohol may be related to its

greater ability to perturb lipid bilayers, e.g. it can depress the lipid phase transition of dimyristoyl lecithin some 8° (Colley & Metcalfe, 1972) whereas clorgyline only achieved a 2–3° depression (Houslay, 1977).

Both benzyl alcohol and clorgyline achieved a far greater augmentation of the enzyme activity from adult rats compared with weanling rats (Table 1, Fig. 1). One explanation of this phenomenon could be that the enzyme protein changes during maturation, accounting for the altered enzyme activities. We would like to suggest that the decrease seen in enzyme activity occurring upon maturation is due, in part, to an increase in rigidity of the ring of lipids surrounding the protein, the phospholipid annulus. Thus a more rigid lipid annulus surrounding the enzyme in the adult mitochondria would give a decrease in activity, and an increased sensitivity to activation by agents increasing bilayer fluidity such as benzyl alcohol and clorgyline. A prediction of such a model would be that agents increasing bilayer rigidity by promoting lipid packing, such as myristoyl alcohol and myristic acid (see e.g. Lee, 1977b), should have little effect on the activity of the adult enzyme, with a rigid lipid annulus, but should have an inhibitory effect on the weanling enzyme if it has a more fluid lipid annulus. Furthermore the sensitivity of the weanling enzyme to activation by benzyl alcohol should increase if it is inhibited by agents increasing the rigidity of its lipid annulus. Experiments demonstrating the effect on β-hydroxybutyrate dehydrogenase activity of partition of myristic acid and myristoyl alcohol into adult and weanling mitochondria is shown in Table 1. As predicted on the above model bilayer rigidifying agents have little effect on the adult enzyme and its stimulation by benzyl alcohol. However the weanling enzyme was markedly inhibited, and this inhibition could be reversed by addition of benzyl alcohol, which activated the enzyme some 4–5 fold compared with only 2-fold in the native state. Such an experiment would be consistent then with the lipid annulus of the weanling enzyme being in a relatively fluid state in native mitochondria and exhibiting little sensitivity to further increases in fluidity achieved by benzyl alcohol or clorgyline, but very sensitive to agents rigidifying the bilayer. The converse would hold for the adult enzyme, its insensitivity to agents increasing bilayer rigidity presumably occurs because its lipid annulus is already in a highly ordered, rigid state constraining the activity of the enzyme and hence making it highly susceptible to activation by agents increasing its fluidity.

To test the hypothesis further, attempts were made to manipulate the lipid annulus of these proteins using various lipid species as described previously (Houslay & others, 1975). Unfortunately, the enzyme activity from brain mitochondria was peculiarly sensitive to inactivation by the detergent, cholate, used to achieve this manipulation, giving $<15\%$ recovery of activity. In experiments where the lipids of mitochondria from both adult and weanling animals were substituted with egg lecithin (95% total), then the response to benzyl alcohol (60 mM) exhibited by the enzyme from both weanling and adult mitochondrial preparations was similar ($\times 1{\cdot}4$ fold activation). This may indicate that when enzyme from both sources is in an identical environment then the response to increasing bilayer fluidity is identical, however, it is not unlikely that exposure to detergent may have impaired the response.

No attempts were made to assess changes in fluidity of mitochondrial lipids upon maturation or to make any detailed study of them because it has been shown that β-hydroxybutyrate dehydrogenase (Houslay & others, 1975) and other enzymes (Warren, Bennett & others, 1975; Dipple & Houslay, 1978) can segregate specific lipids from the bulk lipid pool to constitute their lipid annulus. Thus, a knowledge of the constituents and properties of the bulk lipid pool is unlikely to give information about the lipid annulus. There is evidence, though, that the bulk lipid pool does change upon maturation, hence altering the 'choice' of lipids available to the enzyme. Marshall, Fumagalli & others (1966) have demonstrated that marked changes in the fatty acids associated with total rat brain phospholipids occur during maturation and this is also true for fatty acids derived from the mitochondrial phospholipids of weanling and adult rat brains (M. D. Houslay unpublished observations). Such changes in the availability, during maturation, of lipids with different physical properties may provide a basis for our model.

Thus, drugs influencing bilayer fluidity may have different effects on ketone body utilization by the brain, dependent on the degree of maturation of the animals. Our work also has the implication that so-called specific drugs, e.g. clorgyline may have far-reaching consequences *in vivo* by modulating the activities of membrane-bound enzymes and transport processes that are sensitive to change in membrane fluidity.

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Circling induced by dopamine uptake inhibitors*

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Analysis of the circling behaviour induced in the rat with a unilateral 6-hydroxydopamine lesion of the substantia nigra has proved to be of great interest in the study of drugs acting on catecholamine neural systems. This animal at rest tends to assume an asymmetric posture with the head, body and tail describing a gentle curve concave on the lesioned side. Administration of amphetamine accentuates the postural asymmetry and induces vigorous circling towards the side of the lesion; that is, ipsiversive circling. In contrast, apomorphine and L-dopa induce circling in the opposite direction (contraversive).

Evidence has been presented by Ungerstedt, Butcher & others (1969), Arbuthnott & Crow (1971), and Ungerstedt (1971), that the response to amphetamine reflects the asymmetric release of dopamine from nigrostriatal nerve endings in the striatum. Since these nerve endings have almost completely degenerated on the side of the nigral lesion, little or no dopamine release can occur on that side, whereas the release occurs normally on the unlesioned side. The animal turns away from the side of greater striatal dopamine activity and consequently, after amphetamine, it turns ipsiversively towards the side of the lesion.

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This interpretation of the mechanism of action of amphetamine in the circling rat suggests that drugs which inhibit the uptake of dopamine but do not cause its release should also cause ipsiversive turning in this animal model. Several drugs have recently become available which make it possible to test this hypothesis. Nomifensine, introduced recently as an antidepressant, is a potent inhibitor of dopamine as well as of noradrenaline uptake (Kruse, Hoffman & others, 1977). The new anorexic and stimulant drugs mazindol (5-hydroxy-5-*p*-chlorophenyl-2,3-dihydro-5H-imidazo-(2,1-α)isoindole and dita (3′,4′-dichloro-2-(2-imidazolin-2yl-thio) acetephenone hydrobromide) were recently shown in our laboratories to be extremely weak releasers but very potent inhibitors of both dopamine and noradrenaline uptake (Heikkila, Cabbat & Mytilineou, 1977). In the present study, we compared the behavioural effect of these three dopamine uptake inhibitors with that of amphetamine in rats with unilateral nigral lesions.

Sprague-Dawley rats, 150–175 g, were subjected to a unilateral chemical nigrotomy performed essentially as described by Ungerstedt (1971) with minor modifications. A solution of 6-hydroxydopamine HBr containing 8 μg/4 μl was injected into the rostro-medial portion of the left substantia nigra under Brevital (Rx) anaesthesia in a David Kopf model 900 stereotactic apparatus. Lesion coordinates derived from the König & Klippel atlas (1963) were A 3·0, L 1·8 and V −2·6. The animals