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INSECT BLOOD-BRAIN BARRIER: A RADIOISOTOPE STUDY OF THE KINETICS OF EXCHANGE OF A LIPOSOLUBLE MOLECULE (*n*-BUTANOL)

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SUMMARY

About 90 % of the butanol uptake by the cockroach abdominal nerve cord washed out with half-times of a few seconds, in good agreement with an electrophysiological estimate, and the temperature sensitivity suggested an activation energy of 3 Kcal mole^{-1}. The remaining activity washed out far more slowly, with a similar time course to that observed in a previous investigation which had not detected the fast fraction. Its size was similar to the non-volatile uptake, and was considerably affected by the butanol concentration and incubation period. It apparently consisted of butanol metabolites, which could be detected by chromatography.

INTRODUCTION

The insect central nervous system possesses an ionic diffusion barrier between the extraneuronal spaces and the haemolymph, as revealed by the insensitivity of the neurones to elevated potassium and low sodium ion concentrations unless the nerve sheath is removed (Twarog & Roeder, 1956). Ultrastructural studies suggest that the barrier is provided by lateral occlusions between the perineurial cells, which form an epithelial layer below the fibrous zone of the sheath (Lane & Treherne, 1970). Knowledge of the permeability of this barrier to organic molecules is of considerable use in insecticidal research, as many insecticides act on the central nervous system (Narahashi, 1971), but little work has been done on the subject. Toxicological data (O'Brien & Fisher, 1958) suggested the presence of a barrier discriminating against charged molecules, but later investigations by Eldefrawi, O'Brien and co-workers, who studied the rates of radioisotope exchange in isolated nerve cords of the cockroach, *Periplaneta americana*, were indicative of a somewhat more complex situation. By this technique they investigated the movements of fatty acids (Eldefrawi & O'Brien, 1966), quaternary ammonium salts (Eldefrawi & O'Brien, 1967*a*), alcohols (Eldefrawi & O'Brien, 1967*b*) and also the effects of polarity (Eldefrawi *et al.* 1968). They reported that both influx and efflux of all compounds studied were comparatively slow, and the efflux was always biphasic, with respective half-times of several minutes and several hours. The effect of desheathing the ganglia was comparatively small,

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although it was greater for positively charged compounds. The data obtained with quaternary ammonium salts suggested that increasing liposolubility tended to increase influx, and increasing size decreased it. However, influx of the fatty acids was faster than that of the analogous alcohols, in spite of their higher liposolubility.

Thomas (1974, 1976) investigated the permeability of the cockroach abdominal nerve cord to aliphatic alcohols by an electrophysiological technique, and obtained very different results to those of Eldefrawi & O'Brien (1967*b*). Using the anaesthetic effects of the alcohols as a measure of their concentrations at the neuronal surfaces, it was concluded that the movements of the lower alcohols were very rapid, with exchange half-times of only a few seconds which were little affected by removal of the nerve sheath. Exchange of the higher alcohols, particularly of octanol, was slower, and was interpreted in terms of a reservoir effect as a result of their higher liposolubility, but even so, the influx half-time for this alcohol was only 2 min.

None of these findings could be related to those of Eldefrawi & O'Brien. There are two possible explanations for the discrepancy. The first arises because the electrophysiological technique measures the alcohol concentrations only at the neuronal surfaces, whereas the tracer technique measures the average concentrations throughout the tissue; clearly, these are not necessarily identical. Such a difference has already been demonstrated for ionic movements, albeit in the converse sense, as it has been shown that radioisotopically-measured cation fluxes in the cockroach nerve cord were considerably faster than those predicted from electrophysiological data (Treherne, 1961*a*, *b*; Tucker & Pichon, 1972). This discrepancy is presumably due at least in part to active transport of these ions within the tissue, so it is most unlikely to be applicable to the alcohols, and in any event it is difficult to envisage why the alcohols should equilibrate with the neuronal surfaces so much more rapidly than with the other compartments of the central nervous system. The second possible explanation is that although rapidly-exchanging components of the alcohol fluxes may be of a significant size, they could have escaped detection by Eldefrawi & O'Brien. The technique used by these authors was not entirely suitable for the identification of such components, since they collected fractions no more often than once per minute in their efflux experiments, and they apparently took no precautions against loss of activity from the nerve cords by volatilization (a potentially important factor as the lower alcohols have boiling points below that of water).

For this reason some of Eldefrawi & O'Brien's experiments have been repeated, using techniques more appropriate for the detection of rapidly-exchanging components. It was considered an unnecessary duplication to investigate all the alcohols in this manner, and one alcohol was instead studied in some detail. The choice was to some extent limited by the availability of suitable isotopes, and the alcohol chosen was *n*-butanol-1-C14. The electrophysiological results (Thomas, 1976) suggested it to be the most rapidly-exchanging alcohol, with respective influx and efflux half-times of 7·5 and 9·0 s in the penultimate connectives of the nerve cord, in contrast to the biphasic efflux observed by Eldefrawi & O'Brien (1967*b*), with half-times of 2·5 and 220 min, comprising 22 % and 78 % of the total uptake.

METHODS

Efflux experiments

To detect efflux components having half-times of only a few seconds, the apparatus shown in Fig. 1 was devised. Either whole cockroach (*P. americana*) abdominal nerve cords or the penultimate connectives were used; they were ligatured at each end with a fine nylon monofilament thread, and several centimetres of thread were left attached to the cercal ligature. The tissue was weighed on a torsion balance and then incubated for 30 min in a stoppered vial holding a small quantity of physiological saline (Yamasaki & Narahashi, 1959) which contained either 10 or 100 mM *n*-butanol-1-C14 (New England Nuclear, Boston, Mass.) at respective specific activities of 2·0 and 0·2 mCi/mM. Only about 1 mm of the thread was immersed in the incubation medium, to reduce the possibility of it taking up any significant activity. As their diameter (*ca.* 15 μ) was less than 10 % of that of the nerve cord, the thread and ligatures were not expected to make any significant contribution to the total efflux.

The free end of the nylon thread was passed through the perfusion hook of the apparatus. At the end of the incubation period, the vial was opened and the thread was pulled through the hook until the tissue was suspended over it as shown in the lower inset of Fig. 1. Perfusion of the tissue with normal saline was immediately started by opening the valve (upper inset), and the shape of the hook was such that it trapped a drop of the saline, totally immersing the nerve. The tissue was thus directly exposed to the air for only a very few seconds, thereby minimizing loss of activity by volatilization.

The perfusate was collected in scintillation counting vials, which were mounted in holes around the edge of the turntable, and were fitted with collecting funnels to prevent perfusate from falling between them when the turntable was rotated. The vials contained dry ice, which immediately froze the perfusate, thereby preventing loss of activity. The perfusion rate was initially about 10 ml min^{-1}, but this was progressively reduced after the first minute, to allow collection of subsequent fractions over longer periods. The first fraction was collected for 2 s, a further six were collected for 5 s each, and the periods were subsequently increased to a maximum of 30 min, the duration of each experiment being 1 h. The possibility of retention of activity by the apparatus was investigated by spotting a small quantity of the radioactive solution onto the perfusion hook, and it was found that essentially all the activity was washed off in the first 2 s of perfusion.

The fractions were counted by the emulsion method of Patterson and Greene (1966), in a 2:1 (by volume) cocktail of toluene and Triton-X-100, using 0·8 % butyl PBD as a scintillant (Scales, 1967). This technique gives a high and replicable efficiency for C14, provided that the aqueous volume is kept within reasonable limits (Turner, 1967). To determine the activity remaining in the tissue at the end of the experiment, it was dissolved in Soluene-100, and counted in toluene using a PPO/DMPOP scintillant mixture (see Scales, 1967, for details), as the Soluene-100 caused appreciable chemiluminescence with the butyl PBD scintillant. The vials were counted in a Packard Tri-Carb scintillation counter, and counting efficiency was determined and corrected for by use of a toluene-C14 internal standard.

All experiments were performed at 25 °C, apart from a small number in which

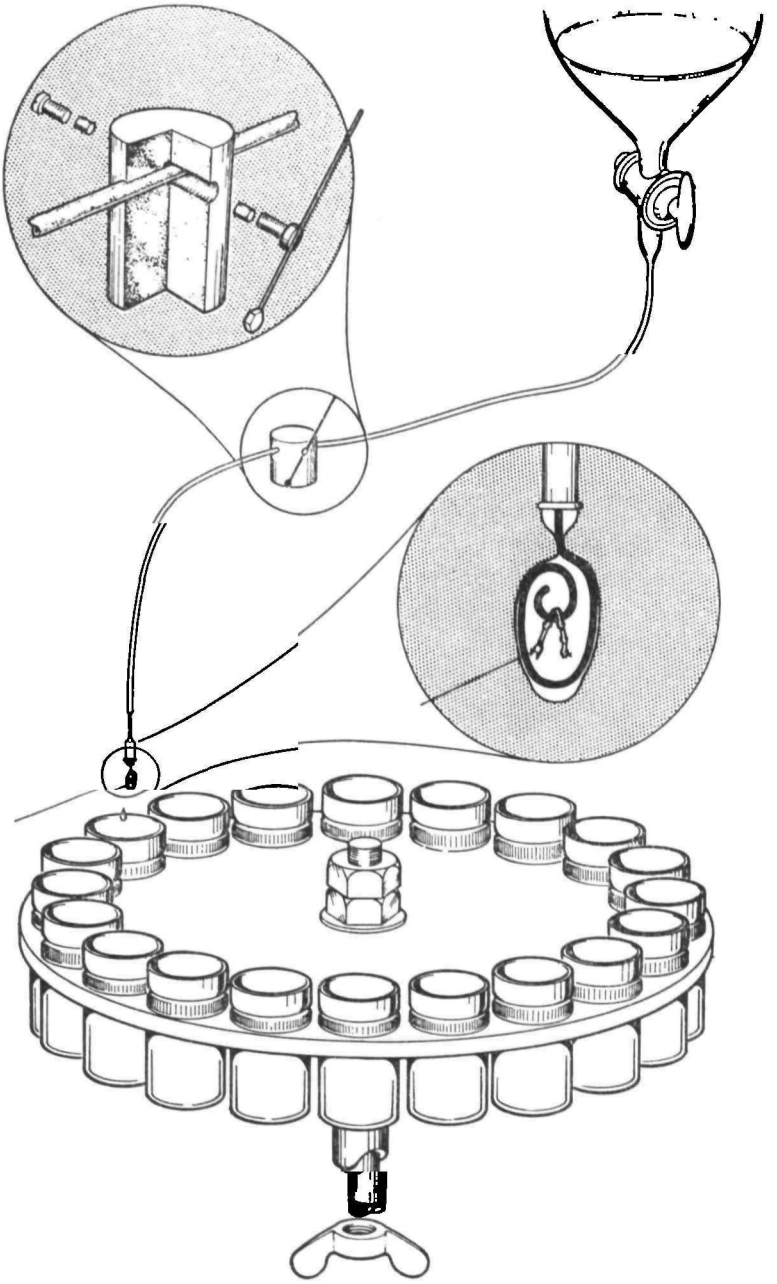

Fig. 1. The design of the perfusion apparatus used in this investigation. (Diagram drawn by Mr J. W. Rodford.)

efflux was measured at 4° in a cold room, after incubation at 25°. In some of the experiments with connectives, the nerve sheath was torn open and rolled back using a pair of electrolytically-sharpened tungsten needles. None of the whole cords were so treated, as it was not practicable to desheath the entire tissue.

Uptake experiments

Eldefrawi & O'Brien (1967*b*) used alcohol concentrations of only 0·1–0·2 mM, whereas those used in the electrophysiological experiments with butanol were 25–125 mM (Thomas, 1976). Therefore, a further series of experiments was performed to investigate whether the movements of this substance were significantly affected by the concentration employed. A much simpler experimental technique was devised, so that a usefully large number of experiments could be performed. Whole nerve cords were incubated for 30 min in closed vials containing butanol at concentrations between 0·1 and 100 mM in normal saline. Each cord was then transferred to 2 ml of saline for 10 min, this period being more than sufficient for washout of any rapid efflux components, but expected to result in only a small loss of the slower components detected by Eldefrawi & O'Brien. The cord was then removed and digested in Soluene-100, and the two fractions were counted under the same conditions as for the efflux experiments.

Another series of experiments was performed to investigate the effect of the incubation period on the uptake. These were carried out in the same manner, using a butanol concentration of 10 mM, the incubation period varying between 5 min and 16 h.

Metabolism

The possibility of metabolism of butanol by the nerve cords, which could cause a serious distortion of the results, was investigated by chromatography. Five nerve cords were incubated for 1 h in saline containing 1 mM labelled butanol, and were then homogenized in 1 N-HCl after washing in normal saline. This was then evaporated under vacuum, and the residue was taken up in methanol, about one-tenth of which was spotted onto a silica gel thin layer chromatography plate. As controls, 5 μl of the 1 mM butanol solution and a small quantity of sodium butyrate solution were also spotted onto the plate. The plate was then subjected to ascending chromatography in 70:30 propanol:water for 3½ h. An autoradiograph of the plate was taken by leaving a sheet of Kodak X-ray film over it for three months.

RESULTS

Efflux experiments

The results of an experiment with a pair of connectives which had been incubated for 30 min in 10 mM butanol are shown in Fig. 2*a*, and those for a whole nerve cord under the same conditions are shown in Fig. 2*b*. It can be seen that in both experiments about 10% of the total uptake washed out very slowly, whereas the other 90% washed out in about 1 min, the efflux being more rapid from the connectives than from the whole cord. The finding is portrayed in more detail by plotting the efflux during the first minute on an expanded scale in Fig. 2 (squares).

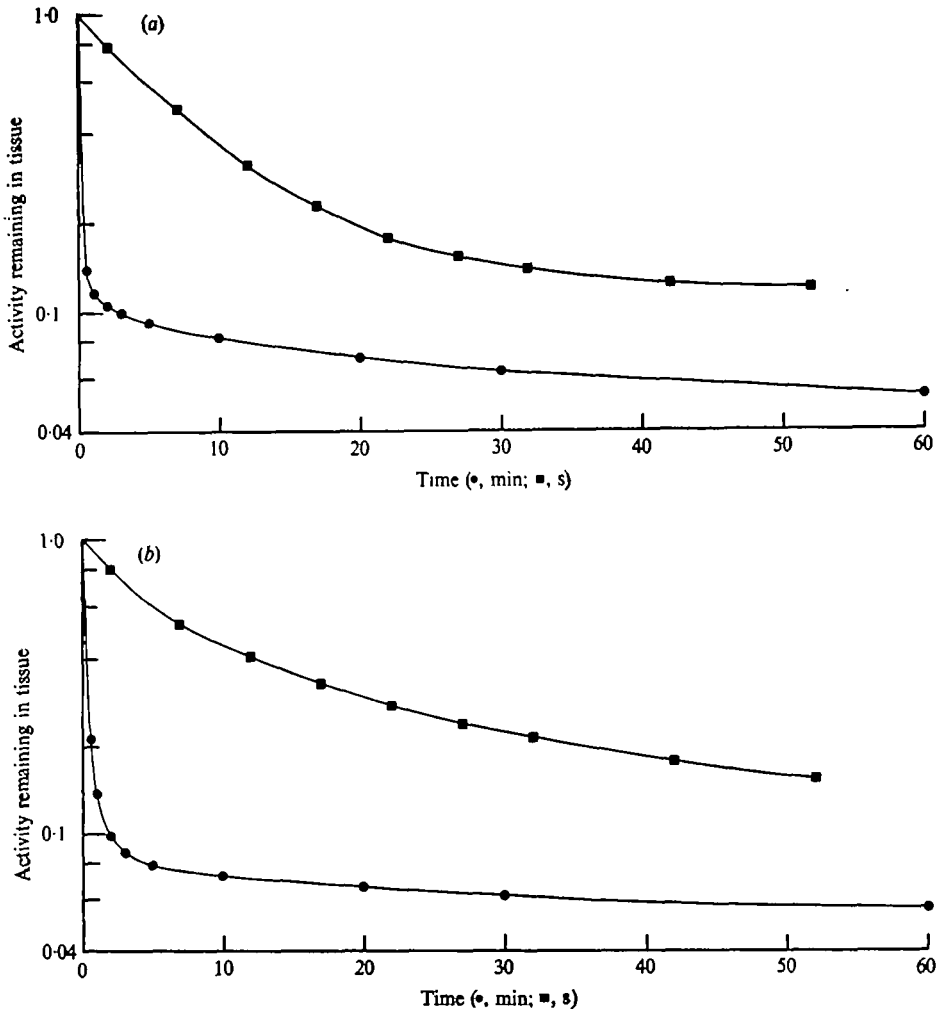

Fig. 2. Efflux of butanol from a pair of penultimate connectives (*a*) and from a whole abdominal nerve cord (*b*).

The results of all the efflux experiments were analysed graphically, which is equivalent to fitting them to an exponential equation of the form $R = A \exp -at + B \exp -bt \ldots + N \exp -nt$, where R is the activity retained by the nerve cord at time t; a, b and n are rate constants, and A, B and N are the initial activities of each component. As pointed out by Solomon (1960), two independent adjustable constants per term provide a tremendous latitude in synthesizing any curve, so the method should be regarded as a convenient way of expressing the results rather than an unequivocal demonstration of the physical existence of such components. This is particularly true for the later portion of the efflux, as the rate fell steadily and never became constant. Over a 1-h period, however, the efflux after the first 2 or 3 min could be described adequately by two components as shown in Fig. 3 and Table 1, although they certainly have no physical existence. They are, nevertheless, of some interest, as their half-times and

Table 1. *The kinetics of butanol efflux from the abdominal nerve cord*

	Connectives (10 mM) (n = 3)		Whole cords (10 mM) (n = 5)		Whole cords (100 mM) (n = 3)	
	T 0·5 (s)	%	T 0·5 (s)	%	T 0·5 (s)	%
Slow	15000 ± 5200	11·4 ± 2·5	9600 ± 1500	8·7 ± 0·9	9700 ± 1500	5·1 ± 0·6
	280 ± 38	3·7 ± 0·4	285 ± 54	2·7 ± 0·4	372 ± 83	2·1 ± 0·7
Fast	7·7 ± 1·1	61·9 ± 4·3	23·6 ± 1·6	29·0 ± 3·6	23·8 ± 0·9	29·3 ± 6·5
			6·1 ± 0·2	44·8 ± 3·8	5·6 ± 0·3	43·3 ± 3·9
'Surface'	—	23·0 ± 1·8	—	14·7 ± 4·6	—	20·1 ± 3·2
Uptake ratio	n.d.		0·73 ± 0·05		0·74 ± 0·05	

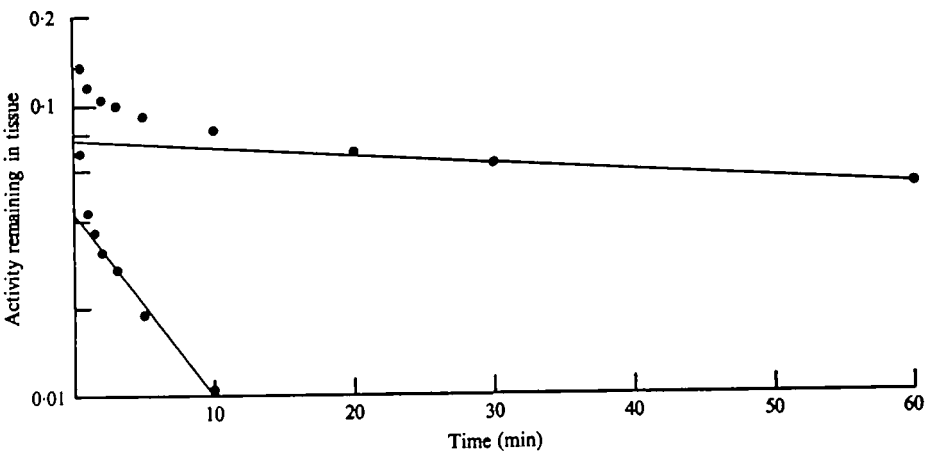

Fig. 3. A figure to show that the efflux after the first 2 min approximately fits a two-component exponential, similar to the ones obtained by Eldefrawi & O'Brien (1967*b*), although these components are *not* expected to have any physical significance. (Same experiment as for Fig. 2*a*.)

relative sizes are very similar to those reported by Eldefrawi & O'Brien (1967*b*), who studied the efflux over a similar period. Their sizes were also somewhat affected by the butanol concentration employed, the difference just being significant for the slower component (P = 0·05) in the 100 mM experiments.

Of considerably more interest, however, is the fast phase of the efflux. The form of this phase in a pair of connectives and in a whole nerve cord is shown in Fig. 4, the results being from the same experiments as for Fig. 2, after subtraction of the slow components. The results with the connectives could be described adequately by a single component having a half-time of 7·7 ± 1·1 s, apart from an initial very rapid component which may have represented activity on the surface of the tissue. The results with whole nerve cords could not be accounted for so easily, and here it was necessary to analyse the fast phase in terms of two major components. They differed in half-time by a factor of only four, and neither the graphical analysis technique nor the data are sufficiently accurate to distinguish precisely between them, so there is likely to be some interaction. It appears, however, that the faster component corresponds fairly closely to the one identified in the connectives, which suggests that

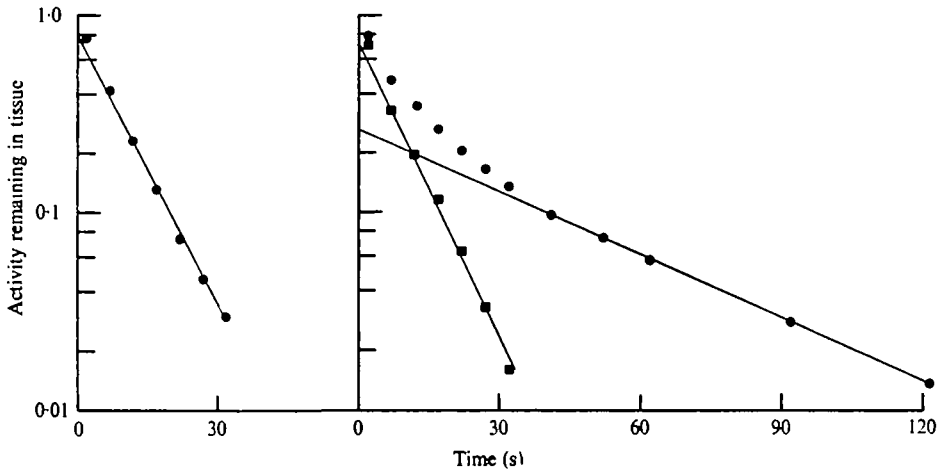

Fig. 4. Fast efflux components from a pair of penultimate connectives (left) and from a whole nerve cord (right).

the slower component probably represents efflux from the ganglia of the nerve cord. As with the connectives, a very fast component, possibly representing surface activity, was also tentatively identified. None of the components were significantly dependent on the butanol concentration used, as shown in Table 1.

Table 1 also shows the total uptake ratios for the nerve cords, expressed as the total activity per unit mass of tissue compared with that in an equivalent volume of the incubation medium. No estimates were made for the connectives, as they were too light to be weighed with sufficient accuracy. The values for the whole cords are somewhat below unity, but this may be at least partly because some of the solid matter of the nerve cord, such as connective tissue in the sheath, is unlikely to take up any butanol. To investigate whether there was any significant loss of activity by volatilization during efflux, three experiments were performed at 4°, at which temperature any such loss would be considerably less, after incubation in 10 mM butanol at 25°. The total uptake ratio was $0{\cdot}79 \pm 0{\cdot}04$, which is not significantly different from the mean value observed in all the 25° experiments ($P = 0{\cdot}4$), suggesting any such loss to be only small. It was observed, however, that the mean half-times of the two fast components at the lower temperature were 8·8 and 34·6 s, these being respectively 46 % and 49 % longer than the values observed at 25°. From the temperature dependence of the rate constant, it is possible to calculate the activation energy, *Ea*, for the process, by the Arrhenius equation: $\frac{d \ln K}{dT} = \frac{Ea}{RT^2}$, where K is the rate constant, R the gas constant and T the absolute temperature. Substitution of the appropriate values into the equation gives an apparent activation energy of 3·0 Kcal mole^{-1}, but it must be treated as a tentative estimate, since if the components are from compartments in series, the exponential slopes derived by graphical analysis are not necessarily equal to the rate constants for exchange (Solomon, 1960).

Two experiments were performed on desheathed connectives, using 10 mM butanol, and the results were very similar to those obtained with intact connectives. All the values obtained were within the variation observed for intact connectives, except th

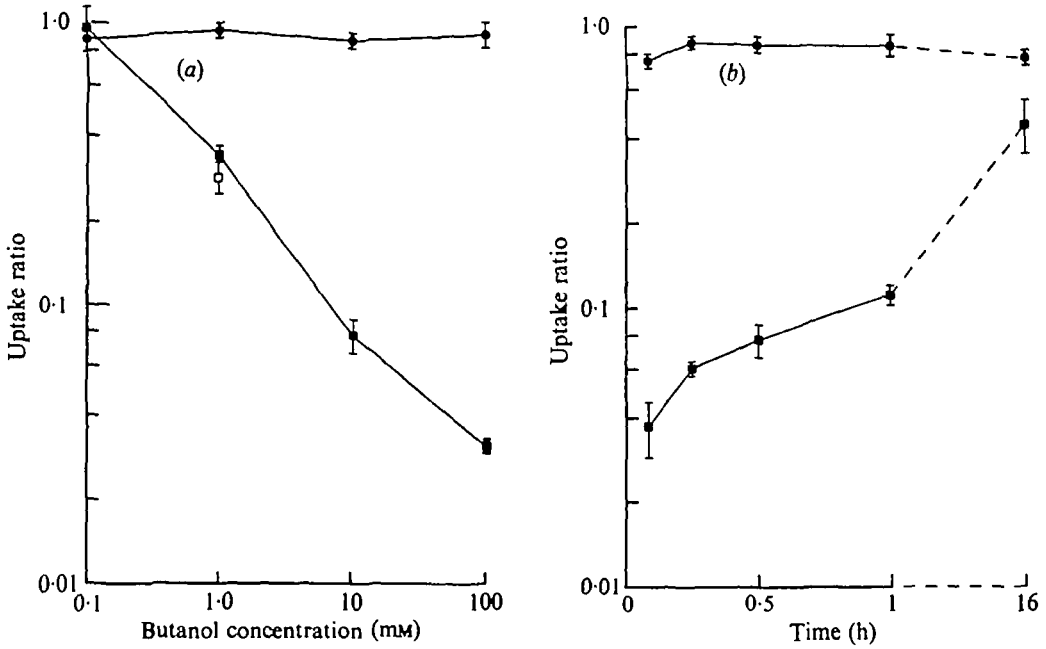

Fig. 5. The effects of butanol concentration (*a*) and incubation period (*b*) on the uptake. Uptake ratios of the fast and slow fractions are respectively indicated by circles and squares. Each point is the mean of six determinations, and the bars indicate ± the standard error.

the half-time of the fast component was apparently a little shorter, values of 5·3 and 6·0 s being obtained, although this difference is not necessarily significant.

Uptake experiments

The results of the uptake experiments are shown in Fig. 5. They clearly show that the size of the fast fraction is little affected by either the concentration or the incubation period, as expected if it equilibrates rapidly and is not further accumulated by the tissue. In contrast, the behaviour of the slow fraction is far more complex, its relative uptake after 30 min incubation being reduced approximately threefold for every tenfold increase in butanol concentration (Fig. 5*a*), and the uptake in a 10 mM solution increasing steadily with time, showing little sign of saturation (Fig. 5*b*). This non-ideal behaviour suggests that the slow fraction may represent butanol metabolites or possibly very firm binding of this substance.

The open square in Fig. 5*a* indicates the activity remaining in the nerve cord after 30 min incubation in a 1 mM butanol solution followed by evaporation to dryness, and thus represents the non-volatile uptake. This was considerably greater than could be accounted for by non-volatile impurities in the butanol solution, as these were found to account for only about 1 % of the total activity. The size of the non-volatile uptake is not significantly different from that of the slow fraction under the same conditions ($P = 0{\cdot}15$), suggesting a close relation between the two parameters.

Metabolism

Fig. 6 shows the autoradiograph obtained from a chromatography experiment to investigate the possibility of metabolism of butanol by the nerve cord. This shows that there was only one major radioactive impurity in the butanol solution, which ran very close to the sodium butyrate marker on the parent chromatogram. The same impurity could be detected in the nerve cord extract, but a variety of others could also be detected, suggesting fairly extensive metabolism by the nerve cord under these conditions (1 h incubation in a 1 mM solution). No attempt was made to identify any of these products.

DISCUSSION

The present results confirm the existence of both a rapidly-exchanging fraction as detected in the electrophysiological experiments (Thomas, 1975), and of a far more slowly-exchanging one as detected by Eldefrawi & O'Brien (1967*b*). The profound effects of the butanol concentration and the incubation period on the size of the slow fraction, and the demonstration that it is comparable in size to the non-volatile uptake, strongly suggest that it represents butanol metabolites. The results of the chromatography indicate that butanol can be metabolized by the nerve cord, further supporting the hypothesis.

Eldefrawi & O'Brien (1967*b*) observed a total uptake ratio of just over unity after 30 min incubation in approximately 0·1 mM butanol (their Fig. 1), which is in good agreement with the size of the slow fraction observed in the present experiments under similar conditions (Fig. 5*a*). The agreement suggests that these authors were totally unable to detect the fast fraction, as this made a similar contribution to the total uptake ratio in the present experiments under these conditions, which was nearly two. A possible reason, apart from volatility, is that their experimental technique (Eldefrawi & O'Brien, 1966) included rinsing the nerve cords for approximately 10 s on removal from the incubation medium, a procedure expected to wash out over half of this fraction.

The results of the metabolism experiments are rather more at variance with those of Eldefrawi & O'Brien. They could detect no metabolism of octanol, and also reported that no extractable, non-volatile metabolites of ethanol or butanol could be detected after incubation of nerve cords in these alcohols. There appears to be no simple reason for this discrepancy, as the present results provide very strong evidence for metabolism, and the butanol was identically labelled (-1-C14) in their investigation. Further support for metabolism of butanol by the nerve cord is provided by Fig. 6 of Eldefrawi *et al.* (1968), which shows that influx of this alcohol was significantly slowed by the metabolic inhibitor DNP.

The mean efflux half-time of the fast phase in the experiments with connectives was 7·7 s, which is in remarkably good agreement with the electrophysiological estimates for the connectives of 7·5 and 9·0 s respectively for influx and efflux (Thomas, 1976). The observation that removal of the nerve sheath does not greatly reduce the half-time was also as expected from the electrophysiological results, and thus supports the earlier conclusion that the ionic diffusion barrier does not significantly retard the movements of this substance. By analogy, the tracer technique

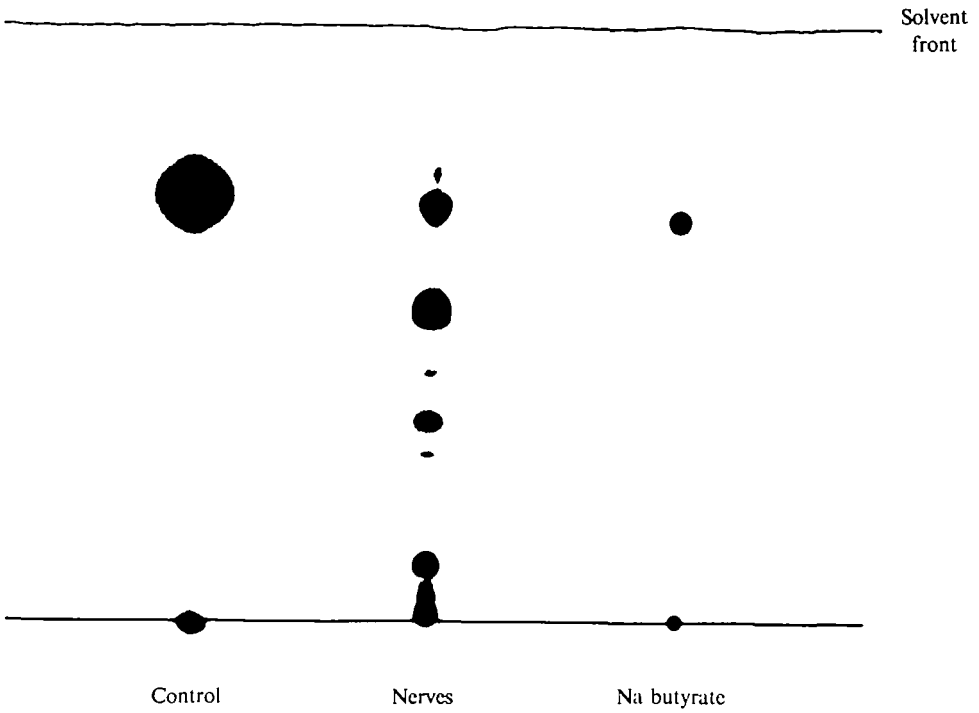

Fig. 6. An autoradiograph to show metabolism of butanol by the abdominal nerve cord.

should be able to detect rapidly-exchanging components for the other alcohols, and they presumably escaped detection by Eldefrawi & O'Brien for the same reasons as for butanol.

The apparently biphasic nature of the fast fraction in the efflux experiments with whole nerve cords (Fig. 4) has been attributed to components from the connectives and ganglia. Efflux from the ganglia is expected to be approximately four times slower, as their surface-to-volume ratio is correspondingly lower than that of the connectives, but the two components are expected to be of similar size. Within the limitations of the graphical analysis technique, the results are in reasonable agreement with this prediction. Further support for the essentially similar nature of the two components is provided by the observation that their half-times have an identical temperature dependence.

The magnitude of the temperature dependence suggests an apparent activation energy of approximately 3·0 kcal $mole^{-1}$, and this estimate is in good agreement with the expected activation energy for the passage of butanol across biological membranes. To permeate a membrane, butanol must presumably first dissolve in it, a process which is exergonic by 3·3 kcal $mole^{-1}$, as calculated from the figure of 815 cal per methylene group observed for the alcohols by Schneider (1968). This also represents the energy required to leave the membrane again and thus, unless the two processes are significantly coupled, the activation energy for crossing it.

The very fast component detected in the efflux experiments has been tentatively ascribed to activity on the surface of the nerve cord. The magnitude of this component is difficult to estimate accurately, but the observed value of about 20 % of the total uptake is of approximately the expected size. Its apparent half-time was of the order of 1 s, which is also reasonable in view of the high initial perfusion rate and the very rapid removal of any butanol solution which was placed on the perfusion hook. It is considered highly unlikely that surface activity could have made any significant contribution to the efflux after the first 2 s.

The present results thus confirm the conclusions drawn from the electrophysiological investigation (Thomas, 1976), that the insect central nervous system is highly permeant to the alcohols, as expected from their comparatively high liposolubility. They are also broadly in agreement with those obtained on the mammalian central nervous system, which also possesses a blood-brain barrier. For example, Davson (1967, p. 267) observed an apparent rate constant for the movement of ethanol from the blood to the cerebrospinal fluid of the rabbit of 0·225 min^{-1}. Oldendorf (1972) compared the uptake of C14 ethanol with that of tritiated water into the rat brain 15 s after injection into the carotid artery, and found them to be very similar, suggesting the permeability to ethanol to be as high as that to water.

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