Renwick & Williams (1972) showed that the gut flora of rats chronically fed with cyclamate acquire the ability to convert cyclamate into cyclohexylamine by splitting the N–S bond, although this did not occur in all rat colonies (Drasar et al., 1970). The splitting of the N–S bond in saccharin could occur similarly to form 2-sulphobenzamide. To investigate the possibility of similar variations in saccharin metabolism, faecal samples (1 g) from control rats, from rats kept on a 1 or 5% sodium saccharin diet in this department and from rats kept on a 5% saccharin diet at The Boots Company Ltd., Nottingham, U.K., in a saccharin-toxicity study, were incubated at 37°C for 24 h with [14C] saccharin (0.5 mg; 0.5 µCi) under both aerobic and anaerobic conditions. The incubation mixtures were examined chromatographically but no hydrolysis of the saccharin could be detected.

It appears that saccharin does not undergo any significant metabolic change in the rat when administered either as a single dose or chronically at 1 or 5% doses in the diet for up to 12 months.

The work was supported by the Calorie Control Council, Atlanta, Ga., U.S.A. We are grateful to Dr. J. W. Shaw of The Boots Company Ltd. for faecal material from rats on a saccharin diet.

Saccharin (1973) Food Cosmet. Toxicol. 11, 1126–1129

---

**Occlusion of Acetylcholinesterase Activity in Nerve Tissue Homogenates from the American Cockroach (Periplaneta americana), and its Release by Detergents**

PHILIP W. BEESLEY* and G. A. KERKUT

Department of Physiology and Biochemistry, Medical and Biological Sciences Building, Bassett Crescent East, Southampton SO9 3TU, U.K.

Insect nerve tissue contains high activities of acetylcholinesterase and in many cases the specific activity of the enzyme is higher than in mammalian brain tissue (Colhoun, 1959, 1963). This activity may be even higher than previously reported as we have observed that measurable acetylcholinesterase activity in nerve tissue samples from *Periplaneta americana* decreases rapidly after homogenization. This has also been reported by Willner & Mellanby (1974). We have investigated the nature of this decrease and the stabilization of the enzyme activity by treatment of samples with detergents. Two methods for accurately determining the acetylcholinesterase activity in such samples are suggested.

**Methods**

Nerve cord tissue was dissected from the animal and cleaned free of surrounding fatty tissue and tracheoles. The dissected tissue was homogenized in 0.1 m-NaH₂PO₄–

---

* Present address: Department of Biochemistry, Royal Holloway College, Egham Hill, Egham, Surrey TW20 0EX, U.K.
\( \text{Na}_2\text{HPO}_4 \) buffer, pH 8.0, in a Voss homogenizer (speed 2000 rev./min) for 1 min. Portions were immediately assayed for acetylcholinesterase activity by the method of Ellman et al. (1961). The concentration of the substrate, acetylthiocholine, used was 1 mM, which is optimal for the enzyme from \emph{Periplaneta americana}. Protein was determined by the method of Lowry et al. (1951).

The detergents used in this study were 1\% (v/v) Triton X-100, 1\% (v/v) Lubrol PX and 0.1\% (w/v) sodium deoxycholate. Higher concentrations of deoxycholate slowly denatured the enzyme. Samples were always rehomogenized after the addition of detergent.

---

**Fig. 1.** Time-course of the loss of acetylcholinesterase activity in nerve tissue homogenates from \emph{Periplaneta americana}

Acetylcholinesterase activity is expressed as nmol of acetylthiocholine hydrolysed/min.

\( \square \), Untreated sample; \( \triangle \), sample treated with 1\% Triton X-100; \( \circ \), sample treated with 0.1\% sodium deoxycholate; \( \blacktriangle \), untreated sample, results plotted on log-log scale.
Results and discussion

A rapid decrease in acetylcholinesterase activity occurred after homogenization of nerve tissue samples. Up to 50% of the initially measured activity was lost during the first 20 h after homogenization of samples. Subsequent decrease in activity was much slower. If the results from such an experiment were replotted as log of acetylcholinesterase activity against log of time after homogenization at which the sample was assayed, a linear relationship between the two parameters was obtained (correlation coefficient = −0.96). This relationship only applied for the time during which there was a rapid decrease in measurable acetylcholinesterase activity.

The non-ionic detergents Triton X-100 and Lubrol PX and the anionic detergent sodium deoxycholate prevented this loss of acetylcholinesterase activity and over a period of days a slow increase in enzyme activity was observed (Fig. 1). These detergents were also tested for their ability to reactivate samples in which loss of acetylcholinesterase activity had already occurred. Triton X-100 had a minimal reactivating effect but deoxycholate increased the enzyme activity to 124% of that measured immediately after homogenization of samples. Lubrol PX was slightly more effective in reactivating samples than Triton X-100 (Table 1).

The rate of enzymic hydrolysis of acetylthiocholine increased markedly during the first 3 min of each assay. Thus, in freshly homogenized samples, the rate of hydrolysis increased by 22% during the first 3 min of assay, whereas in samples aged for 2 h after homogenization the rate of hydrolysis increased by 46% during the first 3 min of assay. Treatment of samples with deoxycholate not only increased measurable acetylcholinesterase activity, but also resulted in the assay becoming linear. Triton X-100 did not have this effect.

Treatment of tissue homogenates with detergents is known to disrupt lipid and membrane structures. It is concluded that treatment of nerve tissue homogenates from Periplaneta americana with deoxycholate disrupts such a barrier, which gradually forms after homogenization of the sample and occludes a fraction of the acetylcholinesterase from assay. Treatment of samples with Triton X-100 prevents formation of this barrier, but does not disrupt it once it has formed. This suggests that the Triton X-100-solubilized enzyme is still attached to other membrane components.

Curvature of the assay is attributed to slow penetration of the occlusion barrier by the assay reagents, particularly 5,5′-dithiobis-(2-nitrobenzoic acid). This is shown by the fact that the curvature is decreased from 46% to 11% during the first 3 min of assay if the sample is preincubated with 5,5′-dithiobis-(2-nitrobenzoic acid) before assay. This suggestion is in agreement with the expected lipid solubility of the compound.

Table 1. Effect of Triton X-100, Lubrol PX and sodium deoxycholate on release of occluded acetylcholinesterase activity and on the curvature of the assay

Acetylcholinesterase activity is expressed as a percentage of the value determined immediately after homogenization of samples. The increase in the rate of hydrolysis of acetylthiocholine during the first three min of each assay is expressed as the percentage increase above the initial rate of hydrolysis. All samples were aged for 2 h. Each result is the mean of five experiments.

<table>
<thead>
<tr>
<th>Detergent added</th>
<th>Percentage acetylcholinesterase activity</th>
<th>Percentage increase in rate of hydrolysis of substrate during first 3 min of assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>55</td>
<td>46</td>
</tr>
<tr>
<td>1 % Triton X-100</td>
<td>68</td>
<td>41</td>
</tr>
<tr>
<td>1 % Lubrol PX</td>
<td>77</td>
<td>8.5</td>
</tr>
<tr>
<td>0.1 % Deoxycholate</td>
<td>124</td>
<td>0</td>
</tr>
</tbody>
</table>
These results indicate two procedures for accurately assaying acetylcholinesterase activity in such samples, either by treatment of samples with 0.1% deoxycholate before assay or by assaying the enzyme activity at several accurately known times after homogenization, plotting the results on a log-log graph and estimating the acetylcholinesterase activity at the time of homogenization. The specific activity of acetylcholinesterase present in the metathoracic ganglion of *Periplaneta americana* was determined by both of these methods. The value as measured after treatment of samples with 0.1% deoxycholate was 0.00147 ± 0.000108 μmol of acetylthiocholine hydrolysed/min per μg of protein. The value as measured by the time-based assay was 0.00149 ± 0.000081 μmol of acetylthiocholine hydrolysed/min per μg of protein. Agreement between the two sets of values is good. These values are considerably higher than those previously reported, e.g. Kerkut et al. (1970) report a value of 0.000476 ± 0.0000165 μmol of acetylthiocholine hydrolysed/min per μg of protein. We propose that either of the procedures suggested will give an accurate determination of acetylcholinesterase activity in nerve tissue from *Periplaneta americana*.


---

**t-3-Hexadecenoic Acid in Broad Bean**

JOHN L. HARWOOD

*Department of Biochemistry, University College, Cardiff CF1 1XL, U.K.*

t-Δ3-Hexadecenoic acid appears to be always associated with higher plant chloroplasts, where it is only found in phosphatidylglycerol (Bartels et al., 1967). It has been noted by a number of workers that it is absent from etiolated tissue (e.g. Nichols et al., 1965a) and increases on exposure of the plant to light (Nichols et al., 1965b). Although direct evidence has not been obtained, it appears that, in *Chlorella vulgaris*, t-Δ3-hexadecenoic acid is formed by desaturation of hexadecanoic acid on phosphatidylglycerol (Bartels, 1969).

The broad bean (*Vicia faba* var. *Aquadulce*) was chosen as an experimental plant with which to examine the synthesis of t-Δ3-hexadecenoic acid because it has been reported to contain rather high concentrations of the acid (Crombie, 1958). The fatty acid composition of leaves obtained from broad-bean plants treated with various light/dark periods was determined by g.l.c. and AgNO₃-t.l.c. The double-bond positions in unsaturated acids were obtained after permanganate oxidation. Cis- and trans-bonds were measured by i.r. spectroscopy. These results showed that leaves from mature broad-bean plants contained about 4% of the total fatty acids as t-Δ3-hexadecenoic acid. This is higher than comparable values for *Chlorophytum elatum variegatum* (1%), lettuce (1%) or spinach (2%). It is only found in phosphatidylglycerol and then only esterified to the 2-position. The concentration of the acid increases with the age of the leaf (Table 1) in keeping with the observation that t-Δ3-hexadecenoic acid is associated with the fully mature chloroplast in maize (Leech et al., 1973). On subjecting plants to dark periods, the concentration of the acid can be decreased to negligible amounts after 5–7 days. No other fatty acid is significantly affected. On exposing such plants to normal daylight, synthesis takes place resulting in an over-response and higher than normal concentrations of t-Δ3-hexadecenoic acid after a week. These changes are depicted in Fig. 1(a).

Chloroplasts isolated from broad-bean leaves are capable of high rates of fatty acid synthesis from [1-14C]acetate or [1,3-14C]malonyl-CoA. ATP, NADH, NADPH and CoA are required but exogenous acyl carrier protein gave little stimulation in contrast...