

# Quantitative Studies of Enzymes and Drug Actions in Cells and Microslices using $^{14}\text{C}$ -labelled Substrates

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## INTRODUCTION

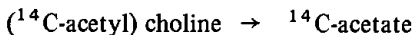
Reed<sup>1</sup> has reviewed the methodology of radiotracer enzyme assays, illustrating the advantages and disadvantages and giving a list of more than 250 enzyme assay methods using radioisotopes. Many of these methods have not been used or adapted for the micro-scale but the application and value of isotopic methods to cytochemistry have been reviewed by McCaman.<sup>2, 3</sup>

During the past few years interest in our laboratory has centred on the relationship between enzyme levels and the organisation and function of the nervous system. Originally we used colorimetric methods with a 'sensitivity limit' of approximately  $10^{-10}$  moles.<sup>4</sup> For this purpose sensitivity limit means the minimum detectable amount of product from the enzyme reaction. Microgasometry, fluorimetry and radiochemistry were the possibilities for increased sensitivity. We chose to use radiochemical methods because of their simplicity and versatility.

## METHODS

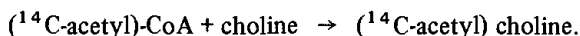
The work has been concerned with the enzymes cholinesterase and cholineacetylase. To estimate these enzymes it is necessary to separate the labelled products, acetate and acetylcholine, from the labelled substrates acetylcholine and acetyl-CoA, respectively. We consider that for a micromethod it is essential to be able to obtain the product of the reaction in one step, e.g. extraction or precipitation.

The method for cholinesterase is illustrated thus:



On addition of acid to pH 1 the  $^{14}\text{C}$  acetate is extracted into toluene/isoamyl alcohol leaving the  $^{14}\text{C}$  acetylcholine in the aqueous phase.

The method for cholineacetylase is illustrated thus:



The  $^{14}\text{C}$ -acetylcholine is extracted using Kalignost dissolved in 3-heptanone, the  $^{14}\text{C}$ -acetylCoA remaining in the aqueous phase.

The volume of the initial reaction mixture is usually about  $1\ \mu\text{l}$ , and the volume of organic solvent containing the labelled product is usually about  $50\ \mu\text{l}$ . An important consideration in assessing the value of these methods is that, because of the small amounts of material used, the costs are low enough to be discounted.

## RESULTS

### Cholinesterase at the Myoneural Junction

Most of the information about cholinesterase at the myoneural junction has come from histochemical staining techniques. Whilst giving good information about the localisation of the enzyme these methods yield little or no quantitative data. Colorimetric methods are not sensitive enough to tackle the problem and microgasometric methods are too slow and cumbersome to give sufficient data for statistical analysis. The radiotracer method has been used with much success to answer several important questions.

Buckley and Heaton<sup>5</sup> showed that the myoneural junctions of extraocular muscles have a wide range of cholinesterase activities. They also showed that myoneural junctions from singly innervated fibres have higher cholinesterase activities than myoneural junctions from multiply innervated fibres. Subsequently we have shown that myoneural junctions from twitch fibres have higher cholinesterase activity than tonic fibres in muscles from frogs, chickens, rats, cats, rabbits and man. The level of cholinesterase does not appear to be simply related to speed of contraction however, since there appears to be little or no difference between the cholinesterase activities of myoneural junctions from slow as opposed to fast twitch muscles.

A further important series of data that the radiotracer method has yielded is the changes of cholinesterase activity at the myoneural junctions during postnatal growth. Histochemical evidence, suggested that the level of cholinesterase in rat myoneural junctions does not change appreciably after the age of three weeks. We have shown, however, that whilst there is a very rapid increase during the first 3 postnatal weeks there is also a large increase after this time. The median cholinesterase activity of myoneural junctions from rat gastrocnemius at one week is  $18\ \text{p.mol/junction/hr}$ , at 3 weeks is  $105\ \text{p.mol/junction/hr}$  and at 52 weeks  $350\ \text{p.mol/junction/hr}$ . There is a similar picture in a twitch muscle of the chick but in a parallel tonic muscle there is only a slight increase in cholinesterase activity at the myoneural junction.

Finally, analysis of the kinetics of hydrolysis of  $^{14}\text{C}$ -acetylcholine by dissected myoneural junctions from rat, frog and chicks has produced evidence suggesting that there are species differences in the organisation of the enzyme at this site. Purified cholinesterase has an optimum substrate concentration of about  $3\ \text{mM}$  acetylcholine, higher concentrations causing substrate inhibition.

This type of kinetics was also found with myoneural junctions from chick muscles.

In the case of rats and frogs, however, the optimum substrate concentration for dissected myoneural junctions was higher than 20 mM but after homogenisation the optimum was 3 to 5 mM.

### **Cholinesterase and Cholineacetylase in single cells and micro-slices of sympathetic ganglia from rats and cats.**

Buckley, *et al.*<sup>6, 7</sup> modified an existing method to enable them to measure the amount of cholineacetylase in single cells of cat sympathetic ganglia. They used the method to show that only about 13% of cells in the L7 ganglion of the cat had cholineacetylase activity higher than the sensitivity limit of the method ( $10^{-12}$ - $10^{-13}$  Moles Ach). They suggested that these cells were cholinergic and the remaining cells were adrenergic.

Since then the method has been used in an investigation of rat sympathetic ganglia. In this study freeze-dried sections of ganglion tissue were weighed (approx. 1  $\mu$ g) and cholinesterase and cholineacetylase measured. The data revealed that the various ganglia studied differ markedly in the absolute and relative contents of the two enzymes.<sup>8</sup> Alternative methods of measuring the amount of acetylcholine produced involve colorimetry or bioassay. The latter is more sensitive than colorimetry but is less sensitive and considerably more time-consuming than the radiometric method.

### **CONCLUSIONS**

Radiotracer methods for the assay of cholinesterase and cholineacetylase have been used to study various physiological and pharmacological problems.

The information so obtained is ample evidence that radiotracer methods of enzyme assay adapted to a microscale provide a powerful analytical tool for the biologist. The techniques are not difficult to acquire and are simpler than many methods of comparable sensitivity. The cost/effectiveness ratio for the methods is comparatively low.

### **REFERENCES**

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### **DISCUSSION**

**P. J. England:** With regard to the precision of your estimates of cholineacetylase activity, is there any chance of micro-reversibility of the enzyme, i.e. are counts being incorporated into acetylcholine without net flow of material? This would have the effect of giving higher estimates of the rate of the reaction than are actually occurring.

**G. A. Buckley:** The equilibrium for the cholineacetylase reaction is very much in favour of acetylcholine production so that, under our conditions, the reverse reaction is negligible.