It is tentatively suggested that an effect of the carcinogen may be to cause a deletion of hormone receptor proteins with a subsequent loss of hormonal activity. If these receptor proteins are considered part of the protein synthesis control in the target tissue, then a role of carcinogens may be to cause specific protein deletion as suggested by Miller and Miller.[8]

Department of Zoology,
The University,
Sheffield S10 2TN

T. DALTON*
R. S. SNART

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* Present address: Department of Zoology, Westfield College, London.

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Acceleration by free carbamate of the spontaneous reactivation of carbamylated acetylcholinesterase

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SEVERAL workers have shown that inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) by carbamates is adequately described by the mechanism[1–3]

$$E + I \xrightarrow{k_i} EI' \xrightarrow{k_r} E + \text{products} \qquad (1)$$

where E is the enzyme, I a carbamate and EI' a carbamyl enzyme. *In vitro* the velocity declines to a steady state at which rates of inhibition and spontaneous reactivation are equal. The forward bimolecular rate is first order because the concentration of inhibitor is greatly in excess of that of enzyme. Previous workers have estimated inhibition rates by a method which involves discarding some of the data of progress curves, viz. those beyond the range in which a semilogarithmic plot of velocity against time is sensibly linear. Reactivation rates have usually been estimated by greatly diluting enzyme–inhibitor mixtures and observing the rate of increase of velocity. Reiner and Simeon-Rudolf[2] have also estimated them by multiplying the inhibition rate constant by the "equilibrium constant" obtained by assuming that the steady state represents a true equilibrium. A different method, which uses all the data from progress curves, and which has not previously been used in studies of AChE, is based on the assumption that the approach to a steady state is kinetically equivalent to approach to a true equilibrium, a mathematical treatment for which has been presented.[4] Since both forward and reverse reactions are first order the approach to equilibrium is also first order, and is related to the initial velocity v_o, the equilibrium velocity v_e and the intermediate velocities v at times t thus:

$$k = \frac{2{\cdot}303}{t} \log \left(\frac{v_o - v_e}{v - v_e}\right). \qquad (2)$$

This rate is also the sum of the forward and reverse rates:

$$k = k_i(I) + k_r. \qquad (3)$$

From each progress curve a value of k and its variance can be calculated by methods described by Cleland.[5] When several values of k for different values of (I) are obtained they are used to fit equation (3), using the inverse variances as weighting factors.[5] Thus estimates of k_i, k_r and their standard errors are found.

Rates of spontaneous reactivation of inhibited particulate AChE preparations were also measured by washing off the surplus inhibitor, analogous to the method of great dilution employed for studies on highly purified solubilized preparations. The estimates were found to differ significantly from those calculated by the first method, as will now be described.

Particulate AChE was prepared from diaphragm muscle by the "short method" of Berry and Rutland[6] and the activity was determined by automatic continuous titration, using a twin-syringe assembly to keep the substrate concentration constant. The saline medium was 0·15M KCl, pH 7·42, 38°, and the substrate was 5·5 mM acetylcholine. A typical progress curve of an inhibition experiment is illustrated. The normal velocity was first recorded (AB), the slope of AB giving a measure of v_o. A small volume of carbamate solution was added at B. The trace became curved as inhibition progressed, and it was ultimately recognized that a steady rate had been reached, the straight line CD providing a measure of v_e. Any intermediate velocity v_j is measured by the slope of the tangent at the point J. To avoid the difficulty of drawing tangents, each short portion of the trace IJK, defined by the points of intersection of the time marks on the chart paper, is regarded as an arc of a circle of large radius, the point J being, with sufficient accuracy, the midpoint of the arc IK. The tangent at J is parallel to the chord IK, the slope of which is the required measure of v_j. In control experiments in which no carbamate was added, the traces remained linear during the consumption of more titrants than was consumed during an inhibition run: no corrections for dilution were therefore required.

Direct measurement of spontaneous reactivation was made by incubating a portion of preparation for 30 min at room temperature with enough carbamate to give 95–100 per cent inhibition. The

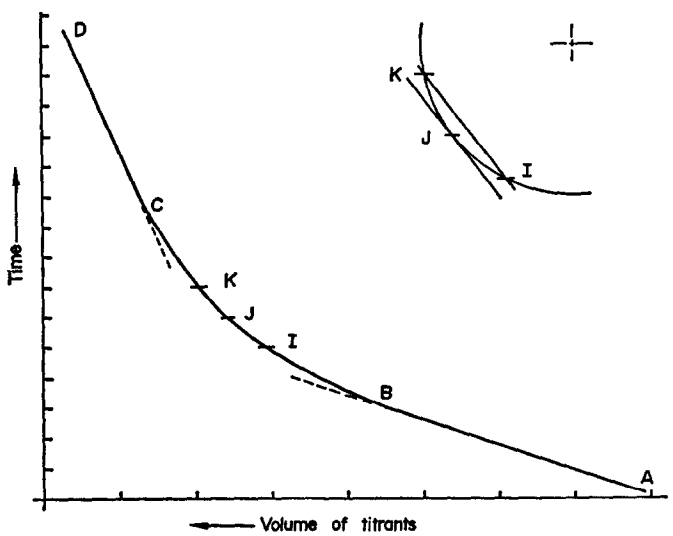

FIG. 1. Copy of trace of representative inhibition experiment. AB, normal velocity; BC diminishing velocity during approach to the steady "equilibrium" velocity CD. Inset, method of drawing tangent at J parallel to the chord IK. The curvature has been exaggerated to show clear separation of chord and tangent. See description in text.

portion was then washed three times by centrifugation, each with 30–40 vol. of 0·15M KCl. Since in earlier experiments with intact red cells such washing had caused complete reactivation of physostigmine-inhibited AChE[7] it was assumed that washing had effectively removed free carbamate. In the present experiments washing caused only a little reactivation, which could be accounted for as the result of the time taken to wash. The washed inhibited preparation was then put up for assay with acetylcholine and the rate of recovery observed for 1·5–2 hr. Reactivation was assumed to be first order with respect to $(v_o - v)$, using a washed uninhibited portion to estimate v_o.

In all cases the data were graphed to verify that the appropriate first-order equation was obeyed, before computing the results.

TABLE 1. RATE CONSTANTS OF INHIBITION AND SPONTANEOUS REACTIVATION: CARBAMATES AND DIAPHRAGM PARTICULATE ACETYLCHOLINESTERASE IN THE PRESENCE OF 5·5mM ACETYLCHOLINE

Carbamate No.*	Inhibition $l.mole^{-1}sec^{-1} \times 10^{-2}$	Spontaneous reactivation, $sec^{-1} \times 10^{3}$ With carbamate	Washed	Acceleration factor and P that it is unity
1 (Human)	16·6 ± 5·50 (5)	2·50 ± 0·37 (5)	0·59 ± 0·03 (28)	4·2 <0·001
1	8·91 ± 0·18 (5)	1·69 ± 0·08 (5)	0·24 ± 0·028 (17)	7·1 <0·001
2	8·66 ± 0·63 (5)	1·62 ± 0·11 (5)	0·38 ± 0·032 (34)	4·2 <0·001
3	18·2 ± 2·3 (5)	0·99 ± 0·26 (5)	0·90 ± 0·048 (38)	1·1 <0·05
4	85·9 ± 10·8 (4)	1·86 ± 0·26 (4)	0·82 ± 0·077 (35)	2·3 <0·001
5	0·70 ± 0·067 (5)	1·60 ± 0·13 (5)	0·33 ± 0·025 (23)	4·9 <0·001
6	8·54 ± 1·40 (5)	1·77 ± 0·43 (5)	0·225 ± 0·063 (45)	7·9 <0·001

Guinea-pig diaphragm, except where noted. S.E. of estimate. Figures in brackets—Cols. 2 and 3, No. of *k*, (*I*) pairs; Col. 4, total No. of points from two concordant experiments.

* Names of carbamates: 1. Physostigmine sulphate. 2. Miotine, 2-dimethylamino-2-(3′-methylcarbamoyloxyphenyl)ethane dihydrobromide. 3. 2-pyrrolidino-2-(3′-methylcarbamoyloxyphenyl)-ethane dihydrobromide. 4. 3-methylcarbamoyloxy-trimethylaminophenyl bromide hydrobromide. 5. Pyridostigmine, 3-dimethylcarbamoyloxy-*N*-methylpyridinium methylsulphate. 6. Benzpyrinium, 3-dimethylcarbamoyloxy-*N*-phenylmethylpyridinium bromide.

Table 1 shows that rates of reactivation measured on washed preparations were significantly smaller than rates calculated from experiments in which there was an excess of carbamate. Since all measurements were made in the presence of 5·5 mM acetylcholine it may be concluded that decarbamylation was accelerated by the carbamates themselves. An analogous phenomenon has been described by Brestkin and Brik:[8] the hydrolysis of high concentrations of butyrylcholine by a purified cholinesterase (EC 3.1.1.8) was more rapid than would have been predicted by the Michaelis–Menten equation from data at the lowest substrate concentrations. The postulated mechanism involved acceleration of deacylation by high concentrations of substrate.

Kitz, Braswell and Ginsburg[3] used the method of great dilution to measure decarbamylation rates, which is equivalent to the present washing method. They found that decarbamylation was accelerated by certain non-depolarizing neuromuscular blocking drugs, and postulated that these drugs induced an allosteric change in configuration. The acceleration factors, 1·8–4·3, were roughly similar to those reported here, 1·1–7·9. It is therefore tempting to associate the well-known pharmacological antagonism between carbamates and non-depolarizing neuromuscular blocking drugs with such a change in configuration which results in accelerated decarbamylation. However, the present results give grounds for supposing that the acceleration is not specific to nondepolarizing drugs, and is indeed produced by the carbamates themselves, possibly by a similar allosteric mechanism. It is thus very doubtful if the antagonism is associated with the decarbamylation of AChE. Another objection relates to concentration effects. *In vitro*, Kitz *et al.*[3] used 50 μM gallamine in many experiments, and showed that 1 μM had no effect. Since the paralysing dose of the drug, molecular weight 892, is about 1 mg/kg[9] or about 10^{-6} moles/kg assuming even distribution without loss, it is doubtful if a concentration of gallamine could be attained in vital organs *in vivo* sufficiently high to affect decarbamylation.

Chemical Defence Establishment, W. K. BERRY
Porton Down,
Wilts., *U.K.*

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