(*4*) into baccharan-3β-ol (*8*a) are known compounds, certain aspects of their synthesis deserve mention. The dehydrogenation of (*4*) into (*5*) is usually [2] effected by a large excess of mercuric acetate (12 g per 1 g of lupenyl acetate) in acetic acid (reflux 5 h). We found that a reasonable yield of (*5*) (60 %) can be obtained with a smaller amount of mercuric acetate (4.5 g per 1 g of lupenyl acetate) in propionic acid (reflux 30 min).

The oxidation of the dihydroacetate (*6*) [3] (in mixture with the tetrahydroacetate) in CCl_4 with ruthenium dioxide and aqueous sodium metaperiodate leads to the diketone (*7*).[3,4] Ozonisation with aqueous work-up,[5] on the other hand, gives the epoxide (*10*), m.p. 238°C, $[\alpha]_D + 40°$, which is probably the same as that obtained by treatment of (*6*) with perbenzoic acid.[3,4] This is a further example of epoxide formation in the ozonolysis of highly hindered olefins.[6-9]

For the Huang-Minlon reduction of the highly hindered C-18 carbonyl in the diketone (*7*), anhydrous hydrazine and prolonged refluxing time is required. Only 10 % of baccharan-3β-ol (*8*a) is produced, the main product being the ketone (*9*a),* m.p. 199–200°C, $[\alpha]_D + 31.9°$ ($c = 0.37$), ν_{max} (KBr pellet) 3450 and 1698 cm^{-1}; acetate (*9*b),* m.p. 184°C, $[\alpha]_D + 31°$ ($c = 1.58$), ν_{max} 1725, 1698 and 1245 cm^{-1}.

Acknowledgement. We are grateful to prof. T. Bruun for a sample of authentic baccharan-3-one, and for the mass spectrum of synthetic (*8*a).

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Received July 5, 1971.

* Both new compounds gave acceptable analyses. Melting points are uncorrected.

Use of Flow Microcalorimetry for the Determination of Cholinesterase Activity and Its Inhibition by Organophosphorus Pesticides

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Recently a general analytical technique was described for the determination of enzyme activities by the use of flow microcalorimetry.[1] The present study is part of a project where the usefulness of this technique in pesticide research and related areas is explored.

The experimental procedure adopted consists simply of pumping an enzyme solution, or suspension, mixed with an excess of a suitable substrate, through the calorimetric cell. In the ideal case the kinetics of the process follows zero order for some time and there will be established a steady state heat effect signal. This is recorded as a voltage-time curve parallel to the instrument baseline. The baseline displacement is directly proportional to the heat effect and will thus provide a measure of the enzymatic activity under the conditions of the experiment.

In the earlier study [1] assay methods were described for several enzymes, including cholinesterase. In the present work the calorimetric technique has been used for a study of the inhibition of cholinesterase by the organophosphorus pesticide Dimefox. From the calorimetric results the inhibition rate constant was calculated.

Experimental. Apparatus. The flow microcalorimeter, a prototype to the LKB 10700-1 microcalorimeter, has been described in detail elsewhere.[2] The flow-through cell used for the present experiments was of the same design as those used in the commercial instruments.

Throughout the work the 10 μV range of a Keithley 150 B Microvolt Ammeter was used. The calorimetric signal was recorded by means of a Servogor RE511 recorder, 100 mV range. A calorimetric baseline shift (Δ) of 1 mm corresponded to 0.05 μV being equivalent to 0.9 μW. The reaction mixtures were pumped through the calorimeter at a flow rate of 20 ml/h. All

calorimetric measurements were performed at 25°C.

Materials. Cholinesterase (Horse serum cholinesterase grade IV) and acetyl choline chloride were obtained from Sigma, St. Louis, Mo. Stock solutions of enzyme (0.1 %) and substrate (10 %) were prepared from 0.1 M tris buffer adjusted to pH 7.4. Tris (trishydroxymethyl aminomethane) was a recrystallized sample of Sigma "pH 7 – 9" or Merck *p.a.*

Dimefox (Terra-sytam; tetramethylphosphorodiamidic fluoride, mol.wt. 154.1) was kindly supplied by Dr. I. Hrdy, Czechoslovak Academy of Sciences, Prague. Stock solutions of Dimefox were prepared in distilled water.

Stock solutions of enzyme, substrate, and inhibitors were stored at 4°C and were used within two days after preparation.

Calorimetric procedure. Enzyme assay. Each series of experiments was started by establishing the instrument baseline. Substrate solution (2 ml, 0.55 M) was diluted with tris buffer (8 ml, 0.1 M, pH 7.4) and the mixture was pumped through the calorimeter until the calorimetric signal reached a steady state. This value was taken as the experimental baseline. A significant difference was found between this value and the baseline found for pure water or buffer without substrate, see Fig. 1. The difference was presumably due to spontaneous hydrolysis of the acetylcholine.

For an enzyme assay, stock solutions of enzyme (0.2 ml) and substrate (2 ml) were mixed with tris buffer (7.8 ml) and the mixture was pumped through the calorimeter until a new steady state was established. The baseline displacement, which is directly proportional to the heat effect evolved in the zero order reaction was taken as the enzyme activity value.

Under the experimental conditions of the present work 7 min were required from the start of the pumping to the time for reaching a steady state signal.

Inhibition experiments. In the inhibition experiments the enzyme solution was mixed with inhibitor and kept for chosen incubation time. 8 ml of the reaction solution was mixed with 2 ml of substrate solution and the mixture was immediately pumped into the calorimeter. The enzyme activity was established as referred to above. In connection with each series of enzyme inhibition experiments, the activity of the enzyme sample used was established in a control experiment. Between each series of experiments calorimeter cell and tubings were rinsed by pumping through a 5 % NaOH solution or a detergent solution (20 min, 150 ml/h). The rinsing solution was washed out by distilled water.

Results and discussion. In Fig. 1 the calorimetric record from a series of inhibition experiments is shown. Cleaning liquid was rinsed out with distilled water after which the experimental baseline was established by the buffer-substrate solution. The calorimetric value (Δ) for the activity of the enzyme sample used was determined in a control experiment after which five experiments were run with enzyme substrate solutions containing different concentrations of inhibitor. In these experiments the enzyme was incubated with inhibitor 30 min at 25°C before the substrate was added and the calorimetric experiment was started. It is seen from Fig. 1 that good steady state values were obtained both in the control experiment and in the inhibition experiments. This indicates that the enzyme inhibitor process essentially was stopped when the large excess of substrate was added at the start of the calorimetric experiment.

The inhibitor concentration was much larger than the enzyme concentration and the inhibition process can thus be treated as a pseudo first order reaction.[3,4]

$$E_1 = E_0 \exp(-k_i I t) \qquad (1)$$

where E_1 is the enzyme concentration at the time (t) when the inhibition was stopped by addition of substrate. E_0 is the

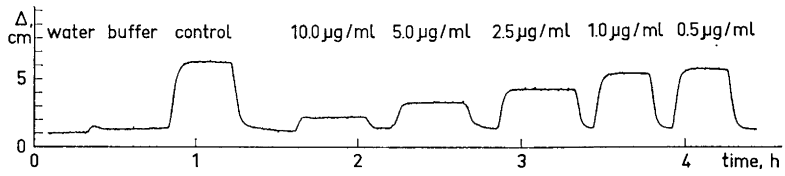

Fig. 1. Calorimetric record from a series of inhibition experiments at 25°C where cholinesterase was inhibited by Dimefox. Inhibitor incubation time was 30 min. Figures denote inhibitor concentrations.

initial concentration and I is the inhibitor concentration which is nearly constant throughout the experiment.

Eqn. (1) can be transformed into

$$\log P = 2 - \left(\frac{k_i I}{2.303}\, t\right) \qquad (2)$$

where P is the % remaining enzyme activity at time t, $(E_1/E_0) \times 100$.

In Fig. 2 results from the Dimefox experiments recorded in Fig. 1 are plotted in the conventional way [5] according to eqn.

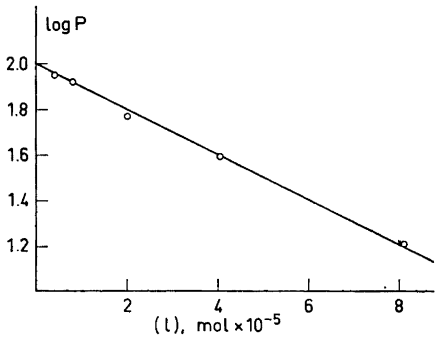

Fig. 2. Inhibition of cholinesterase by Dimefox (*cf.* Fig. 1). log % activity (log P) of the enzyme is plotted *versus* inhibitor concentration, I.

(2). A linear relationship is found with the ordinate intercept equal to 2. The inhibition rate constant, (k_i), calculated from the slope of the line, was found to be 7.5×10^2 M^{-1} min^{-1}.

In other series of experiments the incubation time was varied whereas the inhibitor concentration was kept constant. Results are summarized in Fig. 3. Mean value for the reaction rate constants calculated is 7.8×10^2 M^{-1} min^{-1} in fair agreement with the other series of experiments (Fig. 2).

We conclude that the sensitivity and precision of the present technique is at least comparable with current methods [6] used at determination of cholinesterase activity and its inhibition. Calorimetric analytical methods have a number of valuable features [1] and the present flow technique is very easy to use. It is further judged to be well suited for automatized analytical procedures.

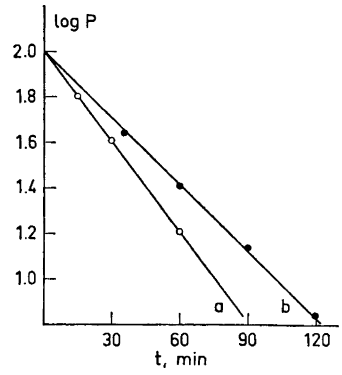

Fig. 3. Inhibition of cholinesterase at 25°C by Dimefox at a constant inhibitor concentration. log % activity (log P) of the enzyme is plotted *versus* incubation time, t. The concentration of Dimefox was 4.05×10^{-5} M (a) and 2.75×10^{-5} M (b).

Acknowledgement. This work has been supported by the *Swedish Board for Technical Development.*

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Received August 21, 1971.