Table 1. *The quantitative distribution of the carotenoids in the flowers of Tecoma capensis*

Pigment	Amount (mg./100 g. fresh wt.)
β -Carotene	2.2
γ -Carotene	4.3
Lycopene	5.9
Rubixanthin-like pigment	7.3
Total epiphasic pigments	19.7
Zeaxanthin	8.9
Total pigments	28.6

The hypophasic fraction. Zeaxanthin was the only pigment found.

Quantitative experiment. The amounts of the pigments in *T. capensis* are given in Table 1.

DISCUSSION

Goodwin (1950, 1952b) stated that flowers contain less carotenoids than the corresponding leaves, but that flower carotenoids are more specific to plant species than are leaf carotenoids. This appears to be true in the case of the petals of the two species of *Tecoma* investigated. Although the corresponding amounts of β -carotene and zeaxanthin in the two species do not differ appreciably, *T. capensis* petals contain much more of the red pigments lycopene and γ -carotene and the deep orange rubixanthin-like pigment. This not only results in the total amount of carotenoids in *T. capensis* being 2.3 times that of *T. stans*, but alters the ratio of epiphasic to hypophasic pigments from 1:6 in *T. stans* to 2.2:1 in *T. capensis*.

Epoxy-carotenoids are widely distributed in blossoms (Goodwin, 1952b), but in the case of the two species of *Tecoma* none of these epoxides was detected; zeaxanthin was, however, found in abundance. The difference in colour between the yellow flowers of *T. stans* and the red flowers of *T. capensis* is due to the presence of lycopene, γ -

carotene and the rubixanthin-like pigment in the latter. In *C. officinalis* the red varieties contain much more lycopene than the yellow varieties (Zechmeister & Cholnoky, 1932).

SUMMARY

1. The carotenoids of the flowers of two species of *Tecoma*, *T. stans* and *T. capensis*, have been investigated.

2. Both species contain β -carotene and zeaxanthin, whilst *T. capensis* contains in addition γ -carotene, lycopene, a rubixanthin-like pigment and a trace of the colourless polyene phytofluene.

3. The concentration of carotenoids in the red flowers of *T. capensis* and the yellow flowers of *T. stans* are 28.6 and 12.6 mg./100 g. fresh weight respectively. The corresponding ratios of epiphasic to hypophasic pigments in the two species are 2.2:1 and 1:6.

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The Purification and Properties of Horse Liver Esterase

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When this work was begun in 1948 an extensive literature on the liver esterases existed, but very little purification had been carried out. It was known that esterase activity was not readily extracted from fresh liver, and prolonged autolysis (Bamann, Mukherjee & Vogel, 1934) or drying with

acetone followed by extraction of the powder with dilute alkali (Willstätter & Memmen, 1924) had been used to prepare active solutions. Many recorded observations on the esterases (reviewed by Ammon & Jaarma, 1950) seemed to be most readily explained by the presence of two or more enzymes in the preparations; and more recent work has shown repeatedly that more than one enzyme hydrolysing

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the same ester may occur in a single tissue (Adams & Whittaker, 1949; Whittaker, 1949).

Purification was undertaken, starting with an acetone powder of horse liver, and a method obtained (and repeated several times) which gave a product with 140-fold greater activity per mg. nitrogen than the starting material. This preparation was used by Bournsnel & Webb (1949) in a study of inhibition by diisopropyl phosphorofluoridate.

During the purification work, the crystallization of horse liver esterase was reported by Mohamed (1948); and after the present purification had been completed, a new esterase preparation from horse liver was published by Connors, Pihl, Dounce & Stotz (1950). The relationship between these preparations and the present material will be discussed.

A method was required for following the hydrolysis of esters by the purified enzyme. The manometric technique was unsuitable because of the presence of bicarbonate, found by Murray (1930) to inhibit liver esterase, and because large losses of volatile substrates occur while filling the manometers with gas. In the measurement of initial reaction rates at substrate concentrations of the order of the Michaelis constant, the method must be sensitive to the hydrolysis of very small concentrations of ester. In the Warburg manometer, 1 μ l. carbon dioxide corresponds to at least 10^{-5} M acid produced in the reaction mixture, while the system described below can detect concentration increments of less than 5×10^{-7} M. The liberated acid is titrated continuously, using glass electrodes for pH control. Two principal modifications of previous methods (Glick, 1937; Sanz, 1944; Schümmelfeder, 1947) give substantial improvement in the effective sensitivity to acid concentration, and reduction in the necessary fluctuations of pH. Changes in electrode potential are detected with about 10 times greater sensitivity than is possible with pH meters of ordinary pattern; and carbonic acid gain or loss by the reaction mixture is minimized by using a closed airtight vessel, which is set up empty and flushed out with carbon dioxide-free air before the reaction mixture is introduced. The use of a current of carbon dioxide-free gas to stir the mixture (Schümmelfeder, 1947) gives a protracted loss of carbon dioxide even from boiled solutions (Edwards & Evans, 1937) and a serious loss of volatile substrates.

METHODS

Nitrogen estimations. These were made by the micro-Kjeldahl method, using about 1 mg. N (Chibnall, Rees & Williams, 1943).

DL- β -Butyrolactone. Prepared according to Johansson (1916) from β -bromobutyric acid obtained by the addition of HBr to crotonic acid (Johansson, 1915). The b.p. of the lactone was 60° at 15 mm. (Johansson, 54.5–55° at 10 mm. and 71–73° at 29 mm.). It contained less than 0.2% free

acid (calculated as hydroxybutyric) and its equivalent weight, determined by hydrolysis with NaOH and back titration of the excess alkali, was 86.7 ($C_4H_6O_3 = 86.1$).

Esterase activities of stages in the enzyme purification. Esterase activity was determined by a variant of the much-used continuous drop titration method with internal indicator (Willstätter, Kuhn, Lind & Memmen, 1927). Two boiling tubes, containing respectively reaction mixture and colour standard, were held in a water-bath at $20 \pm 0.5^\circ$. When the colours of the tubes matched the time was noted, and a drop of approximately 0.05 ml. of 0.01 N-NaOH was added to the reaction mixture. Repetition of this process gave the progress curve of ester hydrolysis. Stock substrate solution: 0.4% (v/v) redistilled ethyl butyrate in water. Reaction mixture: 5.0 ml. stock substrate solution, 3 drops 0.05 M phosphate buffer pH 6.8, 10 drops 0.04% bromthymol blue (made up as the sodium salt in water, without ethanol), enzyme and water to a total volume of 10 ml. Colour standard: 1.3 ml. 0.05 M phosphate buffer pH 6.8, 10 drops 0.04% bromthymol blue, 8.7 ml. water.

Reactions were followed for about 15 drops, and the gradient of the linear part was taken as the reaction rate. Duplicate determinations agreed within about 5%, and the rate was proportional to the quantity of an enzyme preparation used. Rates between 0.7 and 2.5 drops/min. were employed, and 1 unit of esterase activity was defined as the amount giving a rate of 0.05 ml. 0.01 N-NaOH/min. That the substrate concentration employed gave the maximal rate was verified for stages E_1 , E_2 and E_3 in the purification. Murray (1930) had reported the inhibition of liver esterase by bicarbonate. All water used in the activity tests was therefore boiled to expel CO_2 . It was shown that bicarbonate concentrations which could be introduced into the reaction mixture by absorption of atmospheric CO_2 into enzyme preparations had no effect on the measured rate. Using stage E_1 of the enzyme purification, 1.0×10^{-3} M- $NaHCO_3$ added to the reaction mixture was without effect, while 4.0×10^{-3} M reduced the measured rate by 15%.

Potentiometric titration apparatus (Fig. 1). Three vessels containing 50 ml. respectively of 3.5 M-KCl solution, reaction mixture (A) and a reference buffer (B), are held in a constant temperature bath which is filled with NaCl solution earthed through an AgCl electrode and heated electrically by d.c. This provides adequate electrostatic screening. Salt bridges connect A and B to the KCl solution, which contains an earthed AgCl electrode. There is a glass electrode (Cambridge Instrument Co.) in A and another in B, and a potentiometer is used simply as a null instrument to indicate when the potentials of the two are equal. Standardized buffer of the pH at which the reaction is to occur is first put into vessel A, and the reference buffer in B is titrated to give zero p.d. between the glass electrodes. A is then filled with reaction mixture, to which alkali is added from a micrometer burette at a rate sufficient to maintain zero p.d.

The circuit is shown in Fig. 1. The key K rests centrally, with the valve grid floating; K may be moved to connect the grid either to t_1 or t_2 . The potential divider C is adjusted to give zero microammeter deflexion with floating grid. The grid is connected to t_2 and potential divider D adjusted to give zero microammeter deflexion: the floating grid potential is thus made equal to the potential of the glass electrode in reference buffer, to obtain minimum grid current. On connecting the grid to t_1 , a change in microammeter reading indicates a p.d. between the electrodes.

Fig. 1. Electrical circuit of potentiometric titration apparatus, with details of salt bridges. Resistances shown in ohms. Pentode EBL 31. μA , microammeter, central zero, full scale deflexion $\pm 50 \mu A$; g , glass electrode; j , liquid junction; S , silver chloride electrode.

During the continual readings required in the titration process, C and D need be adjusted only occasionally, since small microammeter deflexions when the grid floats are unimportant, and the maximum maladjustment of D which may occur, before the resulting grid current introduces a detectable p.d. error, is readily determined for any pair of glass electrodes.

The microammeter response to p.d. between t_1 and t_2 is $5 \mu A/mv$; and the continual rapid readings of kinetic work can be made to $0.5 \mu A$, corresponding to $0.1 mv$. The grid current is proportional to the difference between the grid potential and the floating grid potential, and is $10^{-12} A/mv$. Since single glass electrode resistances have been found to be about $3 \times 10^8 \Omega$, the minimum detectable e.m.f. change in the glass electrode is $0.08 mv$, corresponding to $0.002 pH$ unit at 25° . Grid currents occurring during normal use of the apparatus cause no polarization effects in the electrodes. The speed of response of the microammeter is such that a p.d. reading takes about 3 sec.

Adequate insulation of each part of the apparatus is essential. It may be tested directly, by using an ordinary dry battery to apply an e.m.f. of several volts with respect to earth, to each relevant part: e.g. to the bath liquid, the screening of the electrode leads and the metal clamps holding vessels, bridges and microburette. Any leakage pathway is then shown by a change in the potential reading of the terminal t_1 or t_2 . During use, all metal clamps and motor casings are earthed.

The reaction vessel is a Pyrex glass tube (diameter 4 cm., length 12 cm.) closed by a rubber bung, through which glass electrode, salt bridge, stirrer, micrometer burette, and a tube admitting the air current enter by airtight joints. A further tube passing through the bung is closed with a small additional bung, and admits reaction mixture, enzyme, etc. The bridge and burette are removable, passing in through glass tubes to which they are sealed with rubber tubing.

Fig. 2. Mercury-sealed stirrer for reaction vessel.

The stirrer shown in Fig. 2 eliminates contamination of the reaction mixture by drops of water from the space between guide-tube and stirrer.

A small electric pump passes air into the vessel, through a series consisting of a reservoir, a soda-lime tube, a vessel of phosphoric acid, and one of distilled water. A leak in the reservoir is opened or closed with a tap: when closed about 100 ml. air/min. pass into the vessel, and when open about 10 ml./min. The higher rate is used for replacing the air in the empty vessel. Thereafter, when the vessel is opened momentarily for the introduction of solutions, air is passed at the lower rate to protect against atmospheric CO_2 . A small manometer enables the vessel to be tested for air leaks.

The micrometer burette is based on that described by Scholander (1942) and Scholander, Edwards & Irving (1943). Deliveries of NaOH solution (from a fine capillary dipping into the reaction mixture) are accurate to $0.1 \mu\text{l.}$, and the rate of diffusion of alkali from the capillary is negligible.

The rubber bung of the reaction vessel, and the metal parts of the burette, are in electrical connexion with the reaction mixture and must be insulated. Rubber-covered clamp jaws grip the vessel as far from its bung as possible, and rubber tubing insulates the burette from its support and the operator's hand.

The salt bridges are shown in Fig. 1. Bridge *B* is filled with 3.5M-KCl solution, and bridge *A* contains reaction mixture itself (without enzyme) to prevent contamination by diffused KCl. For this arrangement the reaction must be carried out in solutions of adequate conductivity: 0.1M-KCl has been used. Closed, ungreaed stopcocks prevent siphoning. To form each liquid junction, the greased stopcock *T* is opened, the rubber bulb compressed, *T* closed and the solution stirred. *T* is opened very gently and the junction drawn a short distance into the bridge. *T* is then closed. Mechanical vibration of the junctions is reduced by the rubber insertion in bridge *A*, and by the fact that the clamps holding the bridges are on stands separate from those holding reaction vessel and bath stirrer. This method is reported to be the best practicable for obtaining junction potentials which are stable with time (Guggenheim, 1930; MacLagan, 1929; Dole, 1941). Their constancy is a critical factor, since variations will be measured as errors in the reaction rate. The use of concentrated KCl, while ensuring that the junction potentials are less than 1 mv (Kline, Meacham & Acree, 1932), does not of itself give sufficient stability.

When vessels *A* and *B* are filled with concentrated buffer, to prevent genuine pH changes, the p.d. between the two electrodes usually shows slow drifts, positive or negative, at rates up to about $0.3\text{ mv}/20\text{ min.}$ (or $3 \times 10^{-4}\text{ pH units/min.}$). When the reaction vessel is filled with very dilute buffer, drifts due to residual acid changes, at rates up to about $\pm 1.5 \times 10^{-7}\text{ m/min.}$, are also encountered. Although the drifts would be undetected in pH measurements with a meter of ordinary pattern, they are a limiting factor in the accuracy with which reaction rates can be determined by this apparatus. To minimize the error they introduce, p.d. readings are taken at frequent intervals for 10 min. immediately before the reaction is started by the addition of the last component. Any p.d. drift is converted into acid concentration units, using an approximate value for the buffer power of the solution, and subtracted from the measured reaction rate.

Ester hydrolysis by the purified enzyme. The enzyme was the final product (E_6) of the purification, stored as a freeze-dried powder. A solution in 0.0033M phosphate, pH 7.4,

was prepared at intervals and centrifuged to remove very small amounts of insoluble material. Samples of the solution in small tubes were frozen solid, stored at -10° , and one was used for each day's work. After thawing, the tube was kept in ice between experiments. Under these conditions no change in activity occurred during the day. Different tubes, however, differed in activity by several per cent, due probably to small losses during freezing and storage.

Methyl and ethyl butyrates were obtained commercially, washed with cold dilute Na_2CO_3 solution, CaCl_2 solution and water, dried over anhydrous Na_2SO_4 , and fractionally distilled several times. The final product was collected over a temperature range of less than 0.5° .

Reactions were carried out at $25.0 \pm 0.03^\circ$, at pH 7.4, in 0.1M-KCl and the dilute phosphate concentrations stated. The mixture was made up (with all components except enzyme), from stock solutions prepared with boiled water and protected from CO_2 . The salt bridge was filled with this mixture, and 50 ml. run into the CO_2 -free reaction vessel. After equilibration, titration to the standard pH, and frequent p.d. readings during the 10 min. 'drift' period, the reaction was started by addition of 0.5 ml. enzyme solution. The NaOH was 0.0696M , carbonate free. Reaction velocities were expressed as the molar concentration of alkali added per min. in the 50 ml. volume. Unless otherwise stated, initial velocities only were measured, over periods for which the progress curves remained linear. Spontaneous hydrolysis of either substrate was negligible. The pH increment due to each addition of NaOH was less than 0.05 unit; and on replacing the reaction mixture by standard buffer at the end of the run, the p.d. registered corresponded to less than 0.02 pH . Since the rate of hydrolysis of $3 \times 10^{-3}\text{M}$ methyl butyrate was found to increase by only 15% over the pH range 7.0–7.9, variations in rate due to pH changes were probably very small. Reaction mixtures contained about $3 \times 10^{-3}\text{M}$ bicarbonate. The rate of hydrolysis of $3 \times 10^{-3}\text{M}$ methyl butyrate was unchanged by the addition of $2 \times 10^{-4}\text{M-NaHCO}_3$.

The accuracy of initial velocity measurements varied considerably with the individual conditions (buffer power, rate and duration) of each reaction. An estimate was always made as follows: the uncertainty in drift was taken to be $\pm (1.5 \times 10^{-7} + 3 \times 10^{-4} \beta)\text{ m/min.}$ (where β = buffer power, $\partial[\text{H}^+]/\partial\text{pH}$, of the reaction mixture) and was expressed as a percentage of the measured rate. Time intervals between each pair of consecutive null points in the titration were noted, and the maximum deviation from the mean expressed as a percentage of the total reaction time. The total estimated error in the measured rate was the square root of the sum of the squares of these two errors. It varied from $\pm 1\%$ at high substrate concentrations to $\pm 5\%$ at the lower concentrations used.

Initial hydrolysis rates, at both high and low substrate concentrations ($56.8 \times 10^{-3}\text{M}$ and $2.27 \times 10^{-3}\text{M}$ methyl butyrate), were shown to be proportional to enzyme concentration and independent of phosphate concentration (from $0.5 \times 10^{-3}\text{M}$ to $2.0 \times 10^{-3}\text{M}$).

EXPERIMENTAL

Purification of enzyme

The ratio of the activity in esterase units to the nitrogen content in mg. will be referred to as the A/N ratio.

Preparation of acetone powder. Fresh minced horse liver was added to 400 ml. acetone/100 g. liver, reduced to a fine

suspension in a Waring Blender, and then stirred for 20 min. and filtered by suction. The solid residue was stirred with 300 ml. acetone/100 g. liver and filtered as before. The filter cake was washed with 200 ml. acetone/100 g. liver, dried *in vacuo* over conc. H_2SO_4 and solid NaOH, and ground to a fine powder in a mill. Stirred aqueous suspensions of the powder showed an activity of about 400 units and a nitrogen content of 114 mg./g. powder. A/N ratio = 3.5.

Extraction. The powder was ground in a mortar with a small volume of water until thoroughly wetted. Water was added to a volume of 30 ml./g. powder, and N-NaOH to raise the pH to 9.0. The suspension was stirred for 30 min. and centrifuged. The supernatant (E_1) contained 90% of the activity of the suspension. A/N ratio = 4.9.

Dialysis. (E_1) was dialysed in cellophan tubing against 10 vol. tap water in the refrigerator for 40 hr., the tap water being changed once after 20 hr. About 15% of the nitrogen was lost and the activity was unchanged.

Acidification and heating. On bringing the dialysed extract to pH 5.5 with N-HCl , a heavy precipitate formed. The suspension was heated slowly to 55°, maintained at that temperature for 3 min., cooled to below 30°, and centrifuged. The supernatant (E_2) contained 85% of the activity of (E_1); A/N ratio = 24.

Adsorption on calcium phosphate. The volume V of (E_2) was measured. At room temperature 0.15 V of 2M-NaCl solution was added, and the calculated volume of water (see below). About 0.075 V of calcium phosphate gel (30 mg. dry wt./ml., previously brought to pH 6.0 with N-HCl) was added with stirring, and the mixture was stirred for a further 30 min., centrifuged and the supernatant discarded. Trial experiments were necessary in each preparation to determine the proper amount of gel, which was that leaving between 10 and 15% of the activity in the supernatant. The volume of water added was adjusted to make the total volume of the mixture 1.5 V .

Selective elution. In each elution of the residue, the solid was dispersed, the suspension was stirred for 30 min. and centrifuged. The solutions used were:

- (i) 0.75 V of 1% (w/v) $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ supernatant discarded.
- (ii) 0.15 V of 7% (w/v) $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ } supernatants
- (iii) 0.15 V of 7% (w/v) $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ } (E_3) combined.

(E_3) contained 60% of the activity of (E_2). A/N ratio = 88.

Ammonium sulphate fractionation. 280 g. $(\text{NH}_4)_2\text{SO}_4$ were added per l. of (E_3). The solution was left in the refrigerator overnight, filtered in the refrigerator, and the precipitate discarded. 120 g. $(\text{NH}_4)_2\text{SO}_4$ were added per l. of filtrate. After standing in the refrigerator overnight and filtering as before, the precipitate was dissolved in a small volume of 0.02M-NaCl, to give a solution containing about 400 esterase units/ml. This was dialysed in cellophan tubing against repeated changes of the same NaCl solution, in the refrigerator, until the external liquid gave no opalescence with BaCl_2 solution. Any precipitate formed during dialysis was removed by centrifuging. The product (E_4) contained 80% of the activity of (E_3); A/N ratio = 183. The activity was almost unchanged by the thorough dialysis: losses from 0 to 9% were observed on different occasions. Since the solution before dialysis contained much $(\text{NH}_4)_2\text{SO}_4$, which was therefore present in the activity tests, comparable amounts were added to reaction mixtures in activity tests on stage (E_3), and shown to have no effect.

Adsorption of impurities by calcium phosphate. To the clear preparation (E_4) about 1 ml. calcium phosphate gel (30 mg. dry wt./ml., pH about 7.0) was added per 1000 units of activity present. The suspension was stirred for 30 min., centrifuged, and the supernatant retained. As before, the necessary amount of gel was determined in each case by trial experiments: about 95% of the activity should remain in the supernatant (E_5), when its volume is calculated as the sum of the volumes of (E_4) and gel used. The actual activity yield was about 85% due to the loss of volume in the residue after centrifuging. A/N ratio of (E_5) = 310.

Ethanol precipitation. 0.11 vol. of M-NaCl solution was added to (E_5) and the pH of the mixture adjusted to 6.6 with 0.1N-HCl. Ethanol at -6° was added very slowly with constant stirring to the enzyme solution at +1° to 43% (v/v). The mixture stood at -6° for 30 min., and was spun in a refrigerated centrifuge at that temperature. The precipitate was dissolved immediately in cold 0.033M phosphate buffer, pH 7.5, and the solution centrifuged to give the purified enzyme preparation (E_6). In this precipitation, accurate temperature control during centrifuging was found to be very important, since the solubility of the active material increased greatly with rising temperature. Activity yields were variable (67–95%); A/N ratio = 500. The preparation (E_6) was stable at room temperature for several hours, and no change of activity occurred on dialysis, or on freeze-drying for storage purposes. The whole purification process yielded about 10 000 esterase units/100 g. acetone powder.

RESULTS

Properties of the purified enzyme

Electrophoresis of the purified preparation in 0.0333M phosphate buffer pH 7.5, (0.0280M- K_2HPO_4 , 0.0053M- NaH_2PO_4 ; solute dialysed against buffer) in the Tiselius (1937) apparatus with cylindrical lens (Philpot, 1938) schlieren optical system, gave the diagrams shown in Fig. 3. The protein concentration was 0.77%, temperature 4°, field strength 4.6 v cm.⁻¹.

The major component, whose mobility was approximately 4.2×10^{-5} cm.² sec.⁻¹ v⁻¹ towards the anode, was accompanied by one small minor component. Electrophoresis of an earlier stage in the purification showed that the proportion of the major component increased as the purification proceeded.

Absorption spectrum. Concentrated solutions of the purified preparation were slightly brown in colour. In the Beckman spectrophotometer, the absorption in the visible region was found to rise steadily with decreasing wavelength, showing no maxima. At 405 m μ . a sharp maximum occurred, and after a broad minimum at about 350 m μ . the absorption rose again to the tyrosine-tryptophan maximum at 277–280 m μ . Extinctions in a 1 cm. cell, per mg. N/ml. were as follows: 600 m μ ., 0.039; 500 m μ ., 0.092; 405 m μ ., 0.92; 280 m μ ., 6.8. During electrophoresis, a sharp change in the intensity of brown colour was observed to coincide

with the boundary of the major component. Spectrophotometry of different stages in the enzyme purification showed a steady increase in the ratio of esterase activity to extinction at 405 m μ ., as the purification proceeded. The presence of haematin,

Fig. 3. Electrophoresis schlieren diagrams of ascending boundaries. Protein concn. 0.77% in 0.0333 M phosphate buffer at pH 7.5 (0.0280 M- K_2HPO_4 , 0.0053 M- NaH_2PO_4). Field strength, 4.6 v cm.⁻¹. (a) 35 min., (b) 62 min., (c) 163 min.

Table 1. *Mohamed's activity test*

Esterase activities determined according to Mohamed (1948) were compared with the activity reported for his crystalline material, on the basis of N content. For details of enzyme purification and drop-titration activity test, see text.

Stage of enzyme purification	...	E_4	E_5	E_6
A/N ratio by drop-titration method		150	220	550
Activity by Mohamed's test, divided by activity reported for crystals containing same amount of nitrogen		1.33	1.52	2.05

suggested by the band at 405 m μ ., was confirmed by the pyridine haemochromogen method according to Biörk (1949), which demonstrated 1 g.mol. protohaematin/ 1.5×10^6 g. N in the final enzyme preparation. Published absorption spectra of catalases and methaemoglobins suggest that this quantity of haematin, bound to protein, could account as to order of magnitude for the absorption spectrum of the enzyme preparation at all wavelengths from 405 m μ . upwards.

Mohamed's activity test. Mohamed (1948, 1949) had reported his crystalline horse liver esterase to be electrophoretically homogeneous. However, when intermediate stages of the present purification were assayed by Mohamed's activity test the activity/mg. N was in each case found to be greater than the figure given for the crystals. In this test the enzyme was incubated at 40° with ethyl butyrate in an unbuffered mixture, and the butyric acid titrated after 30 min. With the present preparation the acid liberated was proportional to enzyme concentration up to a certain value, falling off at higher values. The proportional region was used to obtain the results in Table 1.

The activity test of Connors et al. (1950). Dry-weight determinations and manometric esterase-activity measurements according to Connors *et al.* (1950) were carried out on the present preparation. A complication not mentioned by them was encountered in the activity tests: the volatile substrate distilled over from the main chamber of the Warburg manometer into the enzyme in the side bulb, at a speed sufficient to give approximately the full rate of hydrolysis. In order to find whether appreciable loss of activity occurred before the measurement of reaction rate, during the periods (i) while the enzyme was in bicarbonate solution at a high pH before filling the manometers with gas, and (ii) between filling with gas and tipping, both time intervals were first kept as short as possible, and then increased fourfold. This resulted in a 20% fall in measured rates, suggesting that inactivation during the shorter periods could not have been more than a few parts per cent. Rates were proportional to enzyme concentration, and the mean value obtained with short time intervals was 118 000 μ l./hr./mg. dry weight, or 42% of the figure given by Connors *et al.* (1950) for their preparation.

Velocity ratios for three substrates. The drop-titration method was used to measure the esterase activity towards methyl butyrate, ethyl butyrate and ethyl acetate at various stages in the purification of different individual enzyme preparations. The velocity ratios found are shown in Table 2. The substrates were used at concentrations giving maximal velocities, as shown by agreement between the rates at two concentrations of each substrate: methyl butyrate 0.046 M and 0.093 M, ethyl butyrate

0.0158M and 0.0316M, ethyl acetate 0.37M and 0.49M. In testing stage E_3 , the concentration of sodium sulphate in the reaction mixture was necessarily much higher for the ethyl acetate determinations than with the butyrates. However, addition of equivalent concentrations of this salt to reaction mixtures in tests on the butyrates gave no change in hydrolysis rate.

Action on DL- β -butyrolactone. This substance was hydrolysed at a considerable rate by the purified enzyme preparation. By the potentiometric titration method, the initial rate of hydrolysis of 0.05M DL- β -butyrolactone was found to be about 0.042 times that of methyl butyrate at maximal concentration, after correcting for the spontaneous hydrolysis of the lactone. Since well over half of the DL-substrate was hydrolysed enzymically, both D- and L-forms were attacked.

Inhibition by some dyes. Several fluorescent dyes (kindly provided by Dr D. J. R. Laurence) were found to inhibit the enzyme. Activity measurements were made with a preliminary form of the potentiometric system. After incubating enzyme and dye together for 20 min., a small volume of ethyl butyrate solution was added, giving a concentration of 6×10^{-4} M. Fluorescein, eosin, and rhodamine B gave almost complete inhibition at 5×10^{-4} M; pyronine, *o*-5-acridylbenzoic acid, and *N*-methylacridinium chloride gave considerable inhibition at 10^{-3} M. The action of rhodamine B has been studied in detail (Burch, 1954).

Hydrolysis of methyl butyrate and ethyl butyrate

The potentiometric method was used for these experiments.

Substrate concentration. The variation of reaction rate with substrate concentration was found to follow the Michaelis form. Results of three experiments with methyl butyrate are shown in Fig. 4. The straight lines represent the relation

$$1/v = 1/V + K_m/V[S],$$

Table 2. Ratios of maximal rates of hydrolysis of three substrates, determined by drop-titration method

Three separate batches of the esterase were used. For details of their purification, and of the activity measurements, see text.

Preparation	Stage	V ethyl butyrate V ethyl acetate	V methyl butyrate V ethyl butyrate
1	E_3	14.7	2.09
1	E_6	13.0	2.20
2	E_4	14.6	—
2	E_5	16.8	—
3	E_4	15.7	—
Mean		15.0	2.15
Maximum deviation (%)		± 12	± 5

with $K_m = 2.32 \times 10^{-3}$ M. A 360-fold range of substrate concentrations was used, from 0.12 to $43K_m$.

Fig. 5 shows two similar experiments with ethyl butyrate, the straight lines representing the

Fig. 4. Effect of methyl butyrate concn. on the rate of enzymic hydrolysis, determined by the potentiometric method in 0.1M-KCl and 10^{-3} M phosphate at 25°. The vertical line through each point shows the error limits for the velocity measurement, estimated as described in the text. Δ , Expt. 1; $\circ$, Expt. 2; $\bullet$, Expt. 3. Each straight line represents the Michaelis relation with

$$K_m = 2.32 \times 10^{-3} \text{M.}$$

Fig. 5. Effect of ethyl butyrate concn. on the rate of enzymic hydrolysis, determined by the potentiometric method in 0.1M-KCl and 0.5×10^{-3} M phosphate at 25°. The vertical line through each point shows the error limits for the velocity measurement, estimated as described in the text. $\bullet$, Expt. 1; $\circ$, Expt. 2. Each straight line represents the Michaelis relation with $K_m = 6.2 \times 10^{-4}$ M. CD is a continuation of AB.

Michaelis equation with $K_m = 6.2 \times 10^{-4} \text{M}$. In both experiments the velocity at the highest $[S]$ value ($36.6 \times 10^{-3} \text{M}$) was rather lower than that predicted by the equation. It seems possible that excess substrate inhibition was just becoming apparent at this value of $[S]$. This is a common phenomenon with esterases, and was found by Murray (1930) for liver esterases of various mammalian species, the reaction rate obeying the equation

$$v = V[S]/(K_m + [S] + [S]^2/K_2)$$

This relation could apply to the present results with a K_2 of about 0.5M .

Mixed substrates. Table 3 shows the results of duplicate experiments (1) and (2). In each case three velocity determinations were made: (a) with a high concentration of methyl butyrate, (b) with a high concentration of ethyl butyrate, and (c) with a mixed solution of the two esters.

If each of the two substrates were hydrolysed by an independent enzyme in the preparation, or by an independently functioning active centre on the same enzyme, the total rate of hydrolysis in the mixture (c) should have been equal to the sum of the rates of hydrolysis of $2.9 \times 10^{-2} \text{M}$ methyl butyrate and $1.31 \times 10^{-2} \text{M}$ ethyl butyrate measured separately. These two rates were nearly equal to the rates in solutions (a) and (b) respectively, as shown by Figs. 4 and 5. They were calculated from the rates in (a) and (b), using these figures, and the values and their sum are given in Table 4. It is apparent that the found rate of hydrolysis in solution (c) was only 55% of the rate predicted on this additive basis.

If a single type of active centre was responsible for the hydrolysis of both substrates, competition

should have occurred. In this case the total hydrolysis rate v_T may be shown (Haldane, 1930) on the basis of the Michaelis theory to be given by

$$v_T = (V[S]/K_m + V'[S']/K'_m)/(1 + [S]/K_m + [S']/K'_m), \quad (1)$$

where $[S]$ and $[S']$ represent the concentrations of the two substrates, V and V' the maximum velocities and K_m and K'_m the Michaelis constants, observed with each respective substrate alone.

The reaction rate in solution (c) was calculated from eqn. 1, using $K_m = 2.32 \times 10^{-3} \text{M}$ for methyl butyrate and $K'_m = 6.2 \times 10^{-4} \text{M}$ for the ethyl ester, with the results shown in Table 4. In both experiments the rate found was 1.13 times that calculated in this manner. Since the errors for all six rate measurements, estimated as described above, were only 1%, and the results obtained on the two occasions agreed within 1%, this discrepancy must be considered real.

Progress curves. Investigation showed that the progress curves of ester hydrolysis could not be interpreted simply.

(i) Under the reaction conditions, the enzyme was unstable in the absence of substrate. Table 5 shows the results of incubating the enzyme in the stirred mixture for varying times before adding substrate to start the reaction: activity was lost at a rate of about 10%/20 min.

(ii) The presence of substrate in high concentrations protected against this inactivation, since reactions at these concentrations were followed for periods up to 30 min. and showed no decrease in rate.

(iii) At lower substrate concentrations, the reaction rate decreased more rapidly than was

Table 3. *Mixed substrates: experimental results*

Expts. 1 and 2 each consisted of three determinations of initial hydrolysis rate: a, b and c. The potentiometric method (see text) was used, with substrates methyl butyrate (Me) and ethyl butyrate (Et). Estimated errors, approximately $\pm 1\%$ for each run.

	a	b	c	Ratio: a/b
Phosphate concn., 10^{-3}M	1.0	0.5	0.5	—
Substrate concn., 10^{-2}M	Me, 5.8	Et, 2.63	Me, 2.9 + Et, 1.31	—
Reaction rate, $\mu\text{M}/\text{min.}$ (1)	33.5	18.6	27.55	1.80
(2)	35.5	19.5	28.95	1.82

Table 4. *Mixed substrates: comparison of results with theory*

The experimental results of Table 3 are compared with theoretical hydrolysis rates in substrate mixtures, calculated according to two different models as explained in the text.

		(1)	(2)
Found rate for mixture c, $\mu\text{M}/\text{min.}$		27.55	28.95
Calculated rates for mixture c, $\mu\text{M}/\text{min.}$	Two independent centres	Me alone	32.2
		Et alone	18.2
		Sum (Me plus Et)	50.4
	Competition at a single centre	24.2	25.5
Found rate/calc. rate	Two independent centres	0.55	0.55
	Single centre	1.13	1.13

accounted for by the depletion of substrate. Two such reactions were followed, with identical initial substrate concentrations but different enzyme concentrations. By taking tangents to the progress curves at frequent intervals, each curve could be represented by a graph of e/v against $1/[S]$, where e =enzyme concentration in arbitrary units, v =instantaneous velocity and $[S]$ =instantaneous substrate concentration. In Fig. 6 these graphs are shown, together with the straight line representing the relation between e/v and $1/[S]$ found by initial velocity measurements above. In Fig. 7 the instantaneous velocities of Fig. 6 are expressed as fractions of the theoretical velocity at that substrate concentration, i.e. of the velocity given by the straight line of Fig. 6. These fractions (v/v_{th}) are plotted against time. Thus Fig. 7 records the decrease in reaction rate with time, after correcting for the effect of falling substrate concentration.

Table 5. *Inactivation of enzyme without substrate, under the conditions of the potentiometric activity measurements*

The enzyme was incubated in the usual reaction mixture (phosphate concn. $1.0 \times 10^{-3} M$) for time t , before 2 ml. methyl butyrate solution were added (giving a substrate concn. of $4.54 \times 10^{-3} M$) and the resulting hydrolysis rate was measured. Estimated error in each velocity measurement: $\pm 0.3 \mu M/min$.

t (min.)	Velocity ($\mu M/min$.)
2	16.3
22	13.9
38	12.4
2	15.1

Fig. 6. Progress curves of methyl butyrate hydrolysis, represented as graphs of e/v against $1/[S]$. A, experimental curve, enzyme concn. = 1.00 arbitrary units; B, experimental curve, enzyme concn. = 3.95 arbitrary units; C, relation found by the initial velocity measurements of Fig. 4. Phosphate concn. = $1.0 \times 10^{-3} M$ in each experiment.

DISCUSSION

No evidence has been obtained for the existence of any dissociable coenzyme for horse liver esterase. The activity is almost or entirely unchanged by thorough dialysis, and proportionality has always been found between the rate of ester hydrolysis and the concentration of enzyme preparation present. With the purified enzyme preparation this has been shown at both high and low substrate concentrations. These results contrast with those of Kraut & Patschenko-Jurewicz (1935) and of Ruffo (1943), who found that separable material of low molecular weight was concerned in the activity of pig liver esterase. Ruffo obtained inactivation on dialysis, and reactivation by the addition of concentrated dialysate. In the purification of horse liver esterase by Connors *et al.* (1950), exhaustive dialysis resulted in only a small loss of activity. Apparently a species difference exists.

The haematin contained in the enzyme preparation, although apparently bound to the major electrophoretic component, seems unlikely to be concerned in the esterase activity. Bournsnel & Webb (1949) found that an esterase preparation obtained by the present method, when completely inhibited by diisopropyl phosphorofluoridate, bound 1 g. atom of phosphorus per 10^5 g. protein (while previously heat-inactivated enzyme bound none). The haematin content is only 0.1 g.mol. per 10^5 g. protein, and perhaps represents material adsorbed during the purification.

The esterase is inhibited by fluorescein, eosin, rhodamine B, *o*-5-acridylbenzoic acid, pyronine and *N*-methylacridinium chloride. The first four compounds possess carboxyl groups, and are thereby structurally related to the ester substrates; but

Fig. 7. Progress curves of methyl butyrate hydrolysis, plotted to show decrease of reaction rate with time, after allowing for substrate depletion. Data from Fig. 6. v_{th} =theoretical velocity, i.e. velocity given by initial rate measurement at the substrate concn. concerned. A, enzyme concn. = 1.00 arbitrary units; B, enzyme concn. = 3.95 arbitrary units.

neither pyronine nor *N*-methylacridinium chloride bears any obviously relevant group. Neither the net charge on the molecule at the working pH (negative, positive or zero) nor the charge on the xanthene or acridine ring appears to play a determining role.

That β -butyrolactone is hydrolysed at least as fast as ethyl acetate by the preparation is of interest, since so far as is known to the author no lactone has previously been shown to be hydrolysed by an esterase. Bamann & Schmeller (1931), Bamann, Schweizer & Schmeller (1933) and Steenshold (1943) tested a considerable number of lactones and similar compounds, including γ -butyrolactone, and found them completely resistant to enzymic hydrolysis. Some relation to the enzyme was shown, however, since they inhibited the hydrolysis of ordinary esters. It may be noted that the lactone rings of all these compounds were five-membered or greater, while β -butyrolactone has a four-membered ring, and is spontaneously hydrolysed in aqueous solution much more rapidly.

The simple experiment represented in Figs. 6 and 7 demonstrated that the progress curves of ester hydrolysis were complex: at least two processes other than substrate depletion were involved in the fall in reaction rate. Fig. 6 shows that no mechanism of rapidly established inhibition by products could explain the two curves fully. The initial substrate concentrations were identical. Thus at points of equal $[S]$ the two mixtures were of identical composition in substrate and products; but reaction rates were not proportional to enzyme concentration, except initially. The rate per enzyme unit was always less in the reaction with lower enzyme concentration. Thus the velocity was not determined by the composition of the mixture alone, but depended also on the time taken to reach that composition: progressive inactivation of the enzyme must therefore have been involved. But if such inactivation had been the sole cause of the fall in rate, the two curves of Fig. 7 would have been expected to coincide. Since the reaction with greater enzyme concentration showed a more rapid decrease in v/v_{th} , amounting to about 25% in 20 min., which is faster than the inactivation in the absence of substrate (Table 5), an inhibition by reaction products was probably also involved.

The degree of homogeneity of the enzyme preparation and its relation to the horse liver esterases of Mohamed (1948) and of Connors *et al.* (1950) remain in doubt. When assayed by Mohamed's procedure, the last three stages of the present purification gave greater activities/mg. nitrogen than had been reported for this author's crystalline material. Connors *et al.* (1950) also observed that an early stage in their preparative method gave nearly twice the activity reported for the crystals.

Electrophoresis of the present preparation and that of Connors *et al.* (1950), each at a single pH only, suggested near-homogeneity for the former material and about 70% purity for the latter; but the present preparation, tested by the manometric method according to Connors *et al.* gave only 42% of the activity/mg. dry weight reported for their material. If a single esterase is responsible for the activity of both preparations, electrophoretic resolution must have been inadequate. The unity of the enzymic activity of a preparation may be investigated by two other general methods: kinetic studies should show whether the presence of a single type of active centre can account for the characteristics of the enzymic activity; and a combination of activity tests with protein separation techniques should show whether fractions of different enzymic properties can be distinguished in the material. Experiments in each class have been carried out on the present preparation.

The kinetic characteristics of the enzyme examined here differ in several respects from those of the preparation of Connors *et al.* (1950), but differences in the experimental conditions used may be responsible. The largest discrepancy is in the Michaelis constant for methyl butyrate, which is given as $2.2 \times 10^{-2} M$, or about 10 times greater than the value found in the present work; but it may be noted that competitive inhibition by bicarbonate is possible (Murray, 1930) and that the manometric technique may involve very considerable losses of this volatile substrate during gassing of manometers. Either effect would raise the apparent value of the Michaelis constant.

Kinetic studies on the present preparation showed that the Michaelis relation between reaction rate and substrate concentration was obeyed with methyl and ethyl butyrates separately. This is itself evidence against the presence of more than one type of active centre for each substrate, since if two or more types of centre with different K_m values act simultaneously on one substrate, the graph of $1/v$ against $1/[S]$ should be not linear, but a curve concave towards the $1/[S]$ axis. This may be shown by taking the expression

$$v = V_1[S]/(K_{m_1} + [S]) + V_2[S]/(K_{m_2} + [S])$$

and differentiating $1/v$ with respect to $1/[S]$: $d\left(\frac{1}{v}\right)/d\left(\frac{1}{[S]}\right)$ is always positive but $d^2\left(\frac{1}{v}\right)/d\left(\frac{1}{[S]}\right)^2$ is necessarily negative.

When a mixed solution of the two esters was used, considerable competition was shown, and the total hydrolysis rate lay between the rates for the two separate substrates. Thus the simple criterion which is often applied for the action of only a single enzyme on both substrates was satisfied. However, on comparing more closely the observed reaction

rate with that calculated from the Michaelis theory (eqn. 1) assuming competition for a single type of active centre and using the constants already determined for the separate substrates, the rate observed was found to be 1.13 times that calculated. Examination of eqn. 1 shows that errors in the values used for K_m and K'_m change the value of the calculated rate very little. Thus in order to obtain a rate equal to that observed, either the value of K'_m must be raised by a factor of 3, or K_m must be decreased by a factor of 3, or both must be changed by factors of about 1.8. Such values for the Michaelis constants are not consistent with the experimental results of Figs. 4 and 5. Excess substrate inhibition of the Murray-Haldane type (Murray, 1930; Haldane, 1930) cannot account for the discrepancy. If an inactive complex ES'_2 was formed with ethyl butyrate only, and with dissociation constant $K_2 = 0.5M$, the calculated rates would be only 1% greater than those given in Table 4. If mixed inactive complexes ESS' were also formed, calculated rates would necessarily be lower than those in the table.

This unresolved discrepancy may indicate the presence of two or more types of esterase centre in the preparation, since the reaction rate given by such a system, acting on a substrate mixture, may have any value from zero (when each substrate inhibits the hydrolysis of the other) to the additive value obtained when each substrate is attacked by an independent centre. Alternatively, the action of a single type of centre could be more complicated than the simple Michaelis mechanism. For instance, if the presence of high concentrations of ester caused a change in the Michaelis constant due to a medium effect, no deviation from the ordinary relationship would be observed in experiments with single substrates, since at high values of $[S]$ the reaction rate is very insensitive to changes in K_m . But in mixed substrate solutions the rate depends largely on the ratio of the two Michaelis constants, and a differential medium effect would produce a discrepancy between theory and observation.

Activity measurements on the last four stages of the enzyme purification, in which the ratios of the maximal rates of hydrolysis of three different substrates were determined, showed no marked or consistent change in these ratios, although the activity per mg. nitrogen of the preparations increased by a factor of 5.5, indicating considerable protein fractionation. These experiments thus provide evidence against the presence of separable esterases in the preparation.

SUMMARY

1. An apparatus is described for continuous potentiometric titration of the acid produced in the enzymic hydrolysis of esters. It is designed to

measure low reaction rates, with the consumption of very small concentrations of substrate.

2. A purification of horse liver esterase is described, giving a product with 140-fold greater activity/mg. nitrogen than the acetone powder used as starting material.

3. Electrophoresis of the preparation shows a major component constituting about 95% of the refractive material.

4. The rates of hydrolysis of three separate esters remain in constant ratios over the last four stages of the enzyme purification.

5. The progress curves of ester hydrolysis are complex in form; a simple comparison of reactions with equal initial substrate concentrations, but different enzyme concentrations, shows that at least two processes other than substrate depletion are involved in the decrease of reaction rate.

6. The Michaelis relation between initial velocity and substrate concentration holds for each ester separately. Mixed substrate solutions are hydrolysed at a rate which is much less than additive, but is significantly greater than the rate predicted on the basis of simple competition at a single type of active centre.

7. The esterase is inhibited by fluorescein, eosin, rhodamine B, *o*-5-acridylbenzoic acid, pyronine, and *N*-methylacridinium chloride. It hydrolyses DL- β -butyrolactone, attacking both optical isomers.

8. The homogeneity of the preparation, and its relation to two recently published horse liver esterase preparations are discussed.

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The Determination of Creatinine in Plasma or Serum, and in Urine; a Critical Examination

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Many different methods have been proposed for the determination of creatinine in plasma, serum and urine, and most of these have been based on the colour reaction which takes place between creatinine and sodium picrate in an alkaline medium (Jaffé, 1886). On comparison, however, these different analytical procedures have been found to give different results, particularly when applied to plasma or serum.

The Jaffé reaction is not specific for creatinine and it is generally acknowledged that there are present in plasma and serum, and, to a smaller extent, in urine, one or more unknown substances which also give an orange colour with alkaline picrate. Methods which have been claimed to be more specific towards creatinine include a nephelometric method (Barclay & Kenney, 1947) and various procedures employing 3:5-dinitrobenzoic acid in place of picric acid (Langley & Evans, 1936; Benedict & Behre, 1936; Bolliger, 1936). In addition, other methods have been devised to increase the specificity of the Jaffé reaction, and these involve the use of creatinine-destroying bacteria (Dubos & Miller, 1937), adsorption techniques (Brod & Kotátko, 1949; Hare, 1950; Haugen & Blegen, 1953) and destruction of interfering substances with ceric sulphate (Kostir & Rabek, 1950; Kostir & Sonka, 1952).

We have attempted to explain some of the differences in the results of creatinine determinations obtained with different methods by examining

the factors which influence the determination of creatinine in plasma, serum and urine. Attention was confined to the methods based on the Jaffé reaction and to the procedures which have been claimed to increase the specificity of the reaction since methods based on other reactions did not appear to offer any definite advantages.

MATERIALS

Creatinine. Standard solutions were prepared daily by dilution of a stock solution containing 400 mg. creatinine/100 ml. of 0.1 N-HCl. The stock solution was stored at 0° and was never kept for longer than 4 weeks.

Picric acid. An aqueous solution, saturated at room temperature (18-20°), was prepared from picric acid (A.R.) which had been recrystallized twice from water and satisfied the criteria of purity proposed by Folin & Doisy (1917). The solution was kept in dark bottles and was made up at intervals of a few days.

Other reagents. Sodium hydroxide, (A.R.) 2.5 N. Sodium tungstate, (A.R.) a 10% (w/v) solution of Na₂WO₄ · 2H₂O. Sulphuric acid, (A.R.) 0.66 N. Phosphate buffer, 1 M, pH 7.0 (Green, 1933). Oxalic acid, (A.R.) saturated aqueous solution. Lloyd's reagent (hydrated aluminium silicate), Hartmann-Leddon Co., U.S.A.

NC-bacteria. A suspension of these bacteria (sometimes called *Corynebacterium ureafaciens*) was prepared according to the method of Miller, Allinson & Baker (1939).

Plasma, serum and urine were obtained from normal subjects; the plasma was heparinized.

A Unicam SP. 600 Spectrophotometer was used to measure optical densities.