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RESOLUTION, PURIFICATION AND CHARACTERIZATION OF RABBIT SERUM ATROPINESTERASE AND COCAINESTERASE*

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Abstract—Atropinesterase and cocainesterase from commercially available, pooled rabbit serum have been resolved using conventional methods of enzyme purification. Using a sequence of ammonium sulfate fractionation, Sephadex G-75 gel filtration and QAE-Sephadex ion exchange chromatography, atropinesterase was purified 750-fold and cocainesterase was purified 220-fold. The two enzymes have been characterized with respect to pH optima, Michaelis constants and substrate specificities. Both hyoscyamine and scopolamine appear to function as substrates for atropinesterase; pH optima and K_m determinations are reported for both substrates. Both cocaine and tropacocaine appear to function as substrates for cocainesterase; pH optima and K_m determinations are reported for both substrates with this enzyme.

In 1852, it was reported that rabbits were able to thrive on a diet solely of belladonna leaves, which contain atropine [1]. Atropine (*dl*-hyoscyamine) was incubated with the blood and tissues of various animals, and in certain cases it was found that the drug was inactivated by a thermolabile catalyst [2]. It was recognized by early workers that, since atropine is an ester, it may be susceptible to hydrolysis by an esterase in these animals [3, 4]. However, Oettingen and Marshall [5] claimed that inactivation under these conditions was not due to the hydrolysis of atropine but due to its adsorption on proteins which made it biologically inactive. They found that egg white inactivates atropine in this way. In 1938, Bernheim and Bernheim [6] used manometric techniques to assay atropine inactivation. Their experiments provided evidence that atropine was hydrolyzed and not adsorbed. The enzyme atropinesterase (atropine acylhydrolase, EC 3.1.1.10) hydrolyzes *l*-hyoscyamine into tropic acid and tropine [7].

Glick and Glaubach reported in 1941 [8] that the hydrolysis of cocaine and tropacocaine is catalyzed by rabbit serum. On the basis of genetic studies using crude rabbit serum, they presented evidence that the hydrolysis of these three tropine esters, atropine, cocaine and tropacocaine, is catalyzed by three different enzymes. Subsequently, also in genetic studies, Werner and Brehmer [9] reported similar findings which furthermore indicated that atropinesterase catalyzed the hydrolysis of scopolamine at about the same rate as atropine. Later, Otorii [10], using crude rabbit serum, proposed that scopolaminesterase and atropinesterase are the same enzyme. This conclusion was based on the observation that the relative rates of enzymatic hydrolysis of atropine and scopolamine by rabbit serum, at various concentration ratios of substrate to enzyme, at various pH values of the reaction system, and at various temperatures, are very similar.

Ammon and Savelsberg [11] tested 22 samples of serum and observed that some of the rabbits possessed atropinesterase but not cocainesterase. Contrary to this report, Stormont and Suzuki [12] found that all serums tested (245 samples) which had atropinesterase also exhibited cocainesterase activity. They also found some samples which exhibited neither atropinesterase nor cocainesterase activity and some samples positive for cocainesterase but negative for atropinesterase. Van Zutphen [13] also found only the three phenotypes reported by Stormont and Suzuki.

The first investigators to report purification of atropinesterase were Glick *et al.* [14] who reported from electrophoresis studies, which represented a 17-fold purification of the enzyme, that atropinesterase is found mainly in the α- and β-globulin fractions of rabbit serum. Werner [15], using ammonium sulfate fractionation, purified the enzyme 40-fold from rabbit serum but reported no data on the specific activity of the purified fraction. Margolis and Feigelson [16] purified the enzyme 122-fold from atropinesterase-positive rabbit serum (sp. act., 0·91 units/mg) and reported it to be a nonspecific B esterase. The resolution and purification from rabbit serum of two enzymes, one which catalyzes the hydrolysis of atropine, the other which catalyzes the hydrolysis of cocaine, are described below.

EXPERIMENTAL PROCEDURE

Materials. Pooled, normal rabbit serum was obtained from Baltimore Biological Laboratories, Baltimore, Md. Atropine sulfate and hyoscyamine sulfate were purchased from Mann Research Laboratories, Orangeburg, N.Y. Cocaine hydrochloride was obtained from Merck & Co., Inc., Rahway, N.J. Tropacocaine hydrochloride was supplied by Aldrich

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Chemical Co., Milwaukee, Wis. Scopolamine hydrochloride was purchased from Schwartz–Mann, Orangeburg, N.Y., and hyoscine hydrobromide was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Hyoscyamine sulfate and hyoscine hydrobromide were checked for optical purity immediately before use in a polarimeter. All filtration gels and ion exchange gels were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., and have a particle size of 40–120 μ. All unspecificed reagents were analytical reagent grade and were used without further purification.

Protein determination. Protein was concentrated on an Amican model 52 ultrafiltration cell with a UM-20E Diaflo membrane. The concentration of protein was routinely determined by measuring the ratio of the absorbance at 280 to 260 nm. This method was checked periodically by the method of Lowry *et al.* [17], using crystallized bovine serum albumin for standard curve determinations.

Enzyme assay. Esteratic activity was determined according to the method of Margolis and Feigelson [16] by determining the rate of acid release by ester hydrolysis measured by continuous titration with a Sargent Recording pH Stat model S-30240. The reaction was carried out in a 10-ml reaction vessel containing a combination electrode, a temperature sensor (thermistor type), thermometer and alkali and gas inlet tubes. To exclude possible interference from atmospheric CO_2 a constant current of N_2 (acid, alkali, and water—washed) was introduced through a gas inlet tube and allowed to pass slowly over the surface of the reaction mixture. A magnetic stirrer was employed to ensure thorough mixing during titration. The reaction vessel, ground-glass bottom, was mounted on a thermo-electric hot/cold heat exchanger maintained at 37° ± 0·5°. Unless otherwise noted, all assays were performed at a concentration of 0·1 N KCl in a total volume of 10 ml at 5×10^{-4} M substrate concentration. After attaining thermal equilibrium, the solution was adjusted to the appropriate pH and nonenzymatic hydrolysis was determined. Enzyme was then added and, after the buffers present were titrated, initial reaction rates were obtained. Sodium hydroxide (0·005 M) was introduced through the alkali inlet tube to maintain a constant pH (±0·01). Usually less than 0·3 ml base was required per assay. Each titration was allowed to run at least 20 min. Using this assay, the rate of alkali addition is a linear function of the amount of enzyme added. One enzyme unit is defined as the amount which gives an initial reaction rate equivalent to the uptake of 1 μmole base/min.

Enzyme purification. All purification procedures were done at 0–4°. Selected fractions from each column were assayed for enzyme activity, absorbance was measured at 280 nm, and the fractions containing the peak of enzyme activity were pooled. Column effluents were monitored continuously with an Isco, model UA-2, u.v. liquid-flow analyzer operated at 280 nm in a 2-mm light path. The protein was eluted under a hydrostatic pressure of 20 cm.

Ammonium sulfate fractionation. Saturated ammonium sulfate, pH 7·5, was added in small drops with continuous stirring to 400 ml rabbit serum to reach 30% saturation. The 30–40, 40–45 and 45–57% saturated fractions were obtained in a similar manner. The 0–30, 30–40 and 40–45% precipitates and the 57% supernatant were discarded. The 45–57% precipitate was collected and dissolved in 50–60 ml of 0·002 M potassium phosphate buffer, pH 7·0. It was then dialyzed overnight against 4 l., one change, of buffer. Typically, the volume of protein solution inside the dialysis typing was 90–100 ml and contained approximately 20 mg protein/ml.

Gel filtration. In a typical fractionation sequence (see Table 1), 102 ml dialyzed ammonium sulfate fraction (45–57%) was divided into five equal volumes. Each portion was concentrated, using Amicon ultrafiltration, to a volume of approximately 10 ml. The ultrafiltration cell was then rinsed with about 2 ml of 0·002 M phosphate buffer, pH 7·0. The 12-ml volume (10 ml retentate plus 2 ml rinse) was loaded on the Sephadex G-75 column (2·5 × 34·5 cm).

Fractions containing atropinesterase activity, which were the same fractions containing cocainesterase activity, were pooled. A typical elution pattern is shown in Fig. 1.

Chromatography on QAE-Sephadex. QAE-Sephadex Q-25 was suspended in equilibrium buffer, 0·002 M phosphate pH 7·0, containing 0·1 N KCl. The ion exchanger was poured into a Pharmacia K 15/30 column (1·5 × 29 cm). The eluate from five Sephadex G-75 columns (total volume approximately 50 ml) was pooled for atropinesterase and cocainesterase activity and exchanged into QAE-Sephadex equilibration buffer and applied to the QAE-Sephadex column. The elution pattern of protein, cocainesterase and atropinesterase is shown in Fig. 2. The protein was eluted with a linear 400-ml KCl gradient (0·1 to 0·4 N KCl) made up in the above buffer.

pH Optima determinations. The activity vs pH curves were determined by a series of constant pH titrations similar to the standard assays. For the determinations reported here, the enzyme preparations were the result of ammonium sulfate fractional precipitation, Sephadex G-75 gel filtration and Sephadex-QAE ion exchange chromatography as described above.

Determination of Michaelis constants. All determinations of reaction velocity were made by constant pH titration with $5{\cdot}2 \times 10^{-3}$ N tetraethylammonium hydroxide, at 37°. The KCl concentration was 0·1 N and the total reaction volume was 10 ml. In each case the pH used was near the pH of maximal activity as determined by separate experiments. Each K_m determination was done without monatonic order in substrate concentration to decrease errors due to enzyme denaturation. For all Michaelis constants reported here, the enzyme preparations used resulted from ammonium sulfate fractional precipitation, Sephadex G-75 gel filtration and Sephadex-QAE ion exchange chromatography. The data are shown in the Lineweaver–Burk presentation. Michaelis constants were determined from abscissa intercepts from an unweighted linear least squares fit of these data.

Chloride determination. Chloride ion concentration was determined by the method of Schales and Schales [18].

RESULTS

Enzyme purification. Difficulties were encountered in attempts to assay rabbit serum for atropinesterase

Table 1. Purification scheme of atropinesterase and cocainesterase from 400 ml pooled rabbit serum

Fraction	Enzyme (units × 10^{-3})	Protein (mg × 10^{-3})	Sp. act. (units × 10^{-3})	Yield (%)
Atropinesterase				
Serum*	29·2	20·7	1·41	100
$(NH_4)_2SO_4$ (45–57%)	22·1	2·00	11·1	76
Sephadex G-75 pool	8·2	0·116	70·7	28
QAE-Sephadex pool	4·68	0·0044	1060	16
Cocainesterase				
Serum*	28·0	20·7	1·35	100
$(NH_4)_2SO_4$ (45–57%)	20·2	2·00	10·0	72
Sephadex G-75 pool	13·0	0·116	112	46
QAE-Sephadex pool	2·28	0·0076	300	8

* Dialyzed against 0·002 M potassium phosphate buffer, pH 7·0.

and cocainesterase before dialysis; however, after dialysis of the serum, measurement of acid released upon ester hydrolysis produced smooth titration curves. A typical assay of dialyzed serum for atropinesterase and cocainesterase (Table 1) indicated a sp. act. of $1·41 \times 10^{-3}$ and $1·35 \times 10^{-3}$ units/mg, respectively; the protein concentration of dialyzed serum was 51·8 mg/ml. Sufficient saturated ammonium sulfate solution was added to the dialyzed serum to produce 30% saturation. As shown in Table 1, atropinesterase in the ammonium sulfate fraction collected (45–57% saturation) is enriched 7·9-fold; similarly, the same fraction contains the bulk of cocainesterase activity; the fraction is enriched 7·4-fold with respect to the latter enzyme activity.

The resulting precipitate from ammonium sulfate fractionation was dissolved in 0·002 M potassium phosphate buffer, pH 7·0. Typically, the resulting solution was approximately 100 ml with a protein concentration of 19·4 mg/ml (Table 1). Sephadex G-75 gel filtration of this solution, after concentration (cf. Methods), resulted in a protein solution further enriched in activity with respect to both enzymes (Fig. 1). Both atropinesterase and cocainesterase emerged from the column simultaneously; the fraction containing the peak of atropinesterase activity also contained the peak of cocainesterase activity. Typically, the pool was 48 ml with protein concentration equal to 2·4 mg/ml and enriched, from pooled rabbit serum, 50-fold with respect to atropinesterase and 83-fold with respect to cocainesterase.

Resolution of atropinesterase and cocainesterase. Adsorption and subsequent elution of the pool resulting from Sephadex G-75 gel filtration on a QAE-Sephadex ion exchange column resulted in further purification of the two enzymes and furthermore in the resolution of the two activities (Table 1). A typical elution pattern of the two enzymes from QAE-Sephadex is shown in Fig. 2. Cocainesterase begins to elute from the column at a KCl concentration of 0·15 N, and all esteratic activity with cocaine as substrate was eluted at a KCl concentration equal to

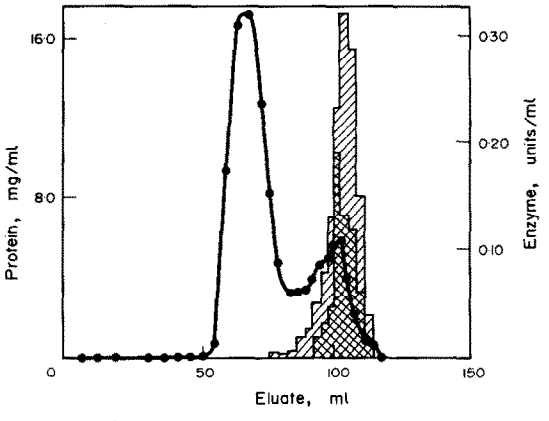

Fig. 1. Gel filtration on Sephadex G-75 of protein precipitated by 45–57% saturated ammonium sulfate. Twelve ml, containing 200 mg protein dissolved in 0·002 M potassium phosphate buffer, pH 7·0, resulting from concentration of 20 ml ammonium sulfate diffusate was applied to a column (2·5 × 34·5 cm) equilibrated and eluted with the same buffer; 64-min fractions containing 5–6 ml each were collected. Key: (—●—) protein, mg/ml; (▨) atropinesterase activity; and (▩) cocainesterase activity.

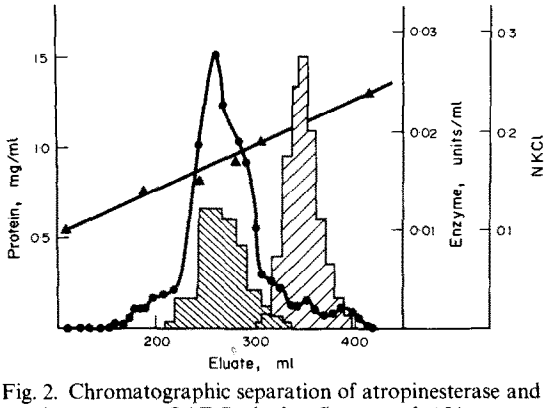

Fig. 2. Chromatographic separation of atropinesterase and cocainesterase on QAE-Sephadex. Seventy ml, 154 mg protein, from Sephadex G-75 eluate pooled for atropinesterase activity in 0·002 M potassium phosphate buffer (pH 7·0) was made 0·09 N KCl and applied to the column (1·5 × 29 cm); the column was initially equilibrated with the same buffer, and elution was initiated with a linear gradient, with 400 ml of the starting buffer in the mixing chamber and 400 ml of 0·4 N KCl in 0·002 potassium phosphate buffer (pH 7·0) in the reservoir; 5–15 ml eluate was collected in 64-min fractions. Key: (—●—) protein, mg/ml; (▨) atropinesterase activity; (■) cocainesterase activity; and (—▲—) Cl^- ion concentration.

0·20 N, whereas elution of atropinesterase did not begin until a KCl concentration of 0·19 N was reached; all esteratic activity with atropine as substrate was eluted at a KCl concentration of 0·22 N. The pool containing cocainesterase was, in a typical elution, 19 ml containing 0·40 mg/ml of protein and represented a purification of 220-fold from serum. The atropinesterase pool was 21 ml, 0·21 mg/ml of protein and resulted in a purification of 750-fold from pooled serum. As shown in Fig. 2, some fractions exhibited esteratic activity with both atropine and cocaine as substrates, but the major peaks of these two enzymic activities were completely resolved in this fractionation step.

Effect of pH on the enzymatic activities of atropinesterase. The initial catalytic activity of atropinesterase as a function of pH was determined with 5×10^{-4} M hyoscyamine (added as hyoscyamine sulfate). The rate of hydrolysis of substrate was studied over a pH range of 5·8 to 10·5. The enzyme fraction used had 0·30 mg protein/ml. As shown in Fig. 3, the catalytic efficiency is essentially maximal between pH 7·5 and 8·5. Nonenzymatic acid production is markedly increased above pH 9·0.

The same enzyme preparation was used to study the effect of pH on the enzymatic hydrolysis of scopolamine. Hyoscine hydrogen bromide (final concentration amine ester 1×10^{-3} M) was used as substrate in the reaction mixture (cf. Methods). The reaction velocity was studied over a pH range of 5·2 to 10·0. As shown in Fig. 3, the optimum range of pH for esteratic activity was 6·5 to 7·5. Nonenzymatic activity was not significant below pH 9·0.

Effect of pH on the enzymatic activities of cocainesterase. The initial catalytic activity as a function of pH was determined with 5×10^{-3} M cocaine (added as cocaine hydrogen chloride). The rate of hydrolysis of substrate was studied over a pH range of 6·0 to 10·0. The enzyme fraction used contained 0·39 mg protein/ml. As shown in Fig. 4, the catalytic activity rises smoothly to pH 8·9, at which value it drops precipitously; no appreciable enzymatic activity is observed above pH 9·5. Non-enzymatic hydrolysis of cocaine is significant as low as pH 6·5, and the reaction velocity rises rapidly and exceeds that of the enzymatically catalyzed reaction at pH values above 9·0.

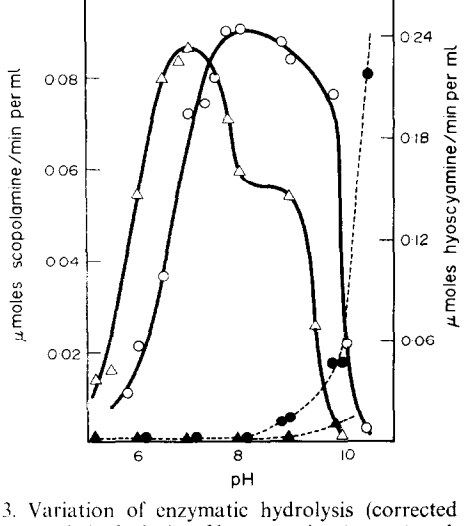

Fig. 3. Variation of enzymatic hydrolysis (corrected for nonenzymatic hydrolysis) of hyoscyamine (—○—) and scopolamine (—△—) and nonenzymatic hydrolysis of hyoscyamine (—●—) and scopolamine (—▲—) with pH.

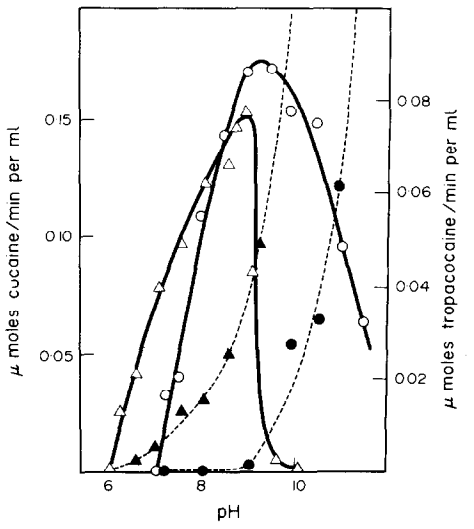

Fig. 4. Variation of enzymatic hydrolysis (corrected for nonenzymatic hydrolysis) of cocaine (—△—) and tropacocaine (—○—) and nonenzymatic hydrolysis of cocaine (—▲—) and tropacocaine (—●—) with pH.

The effect of pH on the enzymatic activity of cocainesterase was also determined using tropacocaine as substrate. Tropacocaine hydrogen chloride (final concentration amine ester $2{\cdot}5 \times 10^{-3}$ M) was added to the reaction mixture and the reaction velocity was observed over a pH range of 7·0 to 11·5. The enzyme preparation used contained 1·0 mg protein/ml. As shown in Fig. 4, the optimum pH range for enzymatic activity with tropacocaine as substrate is 9·0 to 9·5. Nonenzymatic hydrolysis was not significant below pH 9·0.

K_m *determinations, atropinesterase.* The velocity of the atropinesterase-catalyzed reaction was determined as the concentration of hyoscyamine was varied from 3×10^{-5} to 1×10^{-3} M (Fig. 5) using 0·20 ml enzyme solution containing 0·043 mg protein/ml (pH 7·84). The value of the Michaelis constant was $4{\cdot}30 \times 10^{-5}$ M. Atropinesterase activity was also determined as a function of scopolamine concentration (Fig. 5). The protein solution, 0·5 ml (0·21 mg/ml), was assayed at pH 7·5; substrate concentration was varied from 5×10^{-5} to 1×10^{-3} M. The Michaelis constant had a value of $8{\cdot}72 \times 10^{-5}$ M.

K_m *determinations, cocainesterase.* Cocainesterase activity was studied as a function of cocaine concentration (Fig. 5). The enzyme solution, 1 ml (1·00 mg protein/ml), was assayed at pH 7·84 with substrate concentrations varying from 4×10^{-5} to 1×10^{-3} M. The Michaelis constant was determined to be $1{\cdot}73 \times 10^{-4}$ M. Cocainesterase activity was also studied as a function of tropacocaine concentration (Fig. 5). Using 1·0 ml of the same enzyme solution, reaction velocity was determined at pH 8·84 with substrate concentrations varying from 2×10^{-5} to 1×10^{-3} M. The Michaelis constant was $2{\cdot}4 \times 10^{-4}$ M.

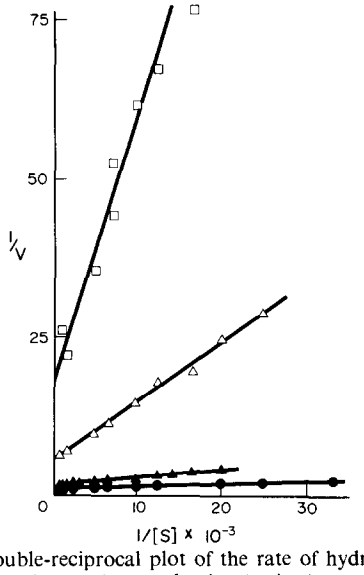

Fig. 5. Double-reciprocal plot of the rate of hydrolysis of hyoscyamine (—●—●—), scopolamine (—▲—), tropacocaine (—□—) and cocaine (—△—) by QAE-Sephadex pool as a function of substrate concentration. The enzyme solution, containing 0·043 mg protein/ml (hyoscyamine), 0·21 mg protein/ml (scopolamine) and 1·00 mg protein/ml (cocaine and tropacocaine), was incubated with 0·1 N KCl at 37° as described (cf. Methods).

DISCUSSION

It is clear from the results reported here that pooled rabbit serum contains two different proteins which catalyze the hydrolysis of tropine esters. These apparently correspond to the *cis*-(α)-tropinesterase and *trans*-(β)-tropinesterase characterized in genetics experiments by Werner [15]. These proteins have been separated and purified; atropinesterase has been found to catalyze the hydrolysis of hyoscyamine and hyoscine but not cocaine or tropacocaine, and cocainesterase catalyzes the hydrolysis of cocaine and tropacocaine but not hyoscyamine or hyoscine. Previously the properties of these enzymes have been characterized in crude rabbit serum or protein fractions in which both enzymes were present.

The pH response curve for the esteratic activity of purified atropinesterase with hyoscine is different from that with hyoscyamine. Furthermore, over a wide range of pH, the rate of hydrolysis of hyoscyamine by atropinesterase is significantly greater than with hyoscine as substrate. As V_{max} and K_m determinations were made at optimum pH values where the activities are different by about 3-fold, the V_{max} is lower and the K_m is higher when hyoscine instead of hyoscyamine is used as a substrate for atropinesterase. These observations do not agree with a report by Otorii [10], who did enzyme–substrate affinity and pH studies with crude rabbit serum containing these activities. He found similar behavior in these properties with both substrates. Possibly because of substrate binding to inert protein, which is removed in purification, maximal velocity is reached at a much lower concentration than reported by Otorii. We have also observed that a higher concentration of substrate is required when crude enzyme rather than purified fractions are used to produce a given specific activity. This behavior may have submerged a significant difference in substrate affinity between the two substrates. In fact, the velocity of reaction of scopolamine over a broad pH range (7 to 9·5) is never close to that of atropine. In any case, although pH response and kinetic parameters were very different, enzymatic activity of these two esters was not resolved using the fractionation procedures reported here. Thus, the conclusion reached by Otorii that the hydrolysis of these two substrates is catalyzed by the same protein is not negated by these experiments using purified atropinesterase.

A number of investigators [8, 12, 13, 15] have reported genetic and electrophoretic evidence that cocainesterase is a protein distinct from atropinesterase. However, Glick and Glaubach [8] found evidence to support the conclusion that there are three azolesterases in rabbit serum, atropinesterase, cocainesterase and tropacocainesterase. We have succeeded in resolving only two thus far.

This is the first report of the purification of cocainesterase. It may be inferred from the behavior of cocainesterase on dextran gel columns, Sephadex G-50, G-75 and G-100, that its molecular weight is similar to that of atropinesterase, which was reported as approximately 65,000 [16]. Since Michaelis constants were determined near the corresponding pH optima, the results may reflect the effect of the pH on the ionic form of the enzyme as well as that of the substrate. Nevertheless, the magnitude of the differences between these kinetic parameters and the comparison of velocities under identical conditions of pH in the high substrate concentration limit indicate that cocaine compared to tropacocaine is a superior substrate for cocain-esterase and that hyoscyamine is superior to hyoscine as a substrate for atropinesterase.

REFERENCES

1. Z. Schroff, *K.K. Ges. Arzte Wien.* **3**, 211 (1852).
2. P. Fleischmann, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **62**, 518 (1910).
3. R. Metzner and E. Hedinger, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **69**, 272 (1912).
4. W. Hesse, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **98**, 238 (1923).
5. W. F. v. Oettingen and I. H. Marshall, *Proc. Soc. exp. biol. med.* **31**, 224 (1933).
6. F. Bernheim and M. L. C. Bernheim, *J. Pharmac. exp. Ther.* **64**, 209 (1938).
7. W. Kalow, *Pharmacogenetics*, p. 55. W. B. Saunders, Philadelphia (1962).
8. D. Glick and S. Glaubach, *J. gen. Physiol.* **25**, 197 (1941).
9. G. Werner and H. Brehmer, *Naturwissenschaften* **46**, 600 (1959).
10. T. Otorii, *Acta med. biol., Nügata* **17** (3), 237 (1969).
11. R. Ammon and W. Savelsberg, *Hoppe-Seyler's Z. physiol. Chem.* **284**, 135 (1949).
12. C. Stormont and Y. Suzuki, *Science, N.Y.* **167**, 200 (1970).
13. L. M. F. Van Zutphen, *Enzymologia* **42**, 201 (1972).
14. D. Glick, S. Glaubach and D. H. Moore, *J. biol. Chem.* **144**, 525 (1942).
15. G. Werner, *Planta med.* **9**, 223 (1961).
16. F. Margolis and P. Feigelson, *J. biol. Chem.* **238**, 2620 (1963).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. O. Schales and S. S. Schales, *J. biol. Chem.* **140**, 879 (1941).