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PARTIAL PURIFICATION AND PROPERTIES OF FLYHEAD CHOLINESTERASE

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Abstract—Housefly head cholinesterase was purified using the following steps: (1) freeze-drying of flyheads, (2) solubilization of the enzyme by butanol extraction, (3) ammonium sulphate precipitation at pH 7, (4) heat denaturation of proteins in the presence of acetylcholine for protection of the cholinesterase, (5) ammonium sulphate fractionation at pH 7 and at pH 6, (6) calcium phosphate gel absorption and elution, and (7) acetone fractionation. The final preparation, a solution in glass-distilled water, hydrolysed acetylcholine at a rate of 1600 μM/hr/mg of organic matter (157 × purification). It proved fairly stable and was used for studying some properties of the enzyme. The substrate specificity did not change much in the course of purification. The purified enzyme differed from purified bovine cholinesterase in that it hydrolysed butyrylcholine, triacetin, and phenylbutyrate at a much higher rate. The evidence strongly points to one single enzyme being responsible for the hydrolysis of all substrates studied, including butyrylcholine. Inhibition experiments with organophosphates indicate a probable turnover number in acetylcholine hydrolysis of about 100,000. Experiments on the influence of organic solvents showed that 2–3% *n*-butanol increases the enzymic activity on choline esters about 60%, and that *n*-butanol, acetone, and ethanol all lower the rate of inhibition by an organophosphorus compound (diazoxon). Agar gel electrophoresis at pH 8·4 showed the cholinesterase to migrate, probably together with other proteins still present in the purified preparation, at a speed which is about 0·9 times the speed of human serum albumin.

INTRODUCTION

PURIFIED cholinesterases have been obtained from different sources. The extremely successful purification of electric eel cholinesterase by ROTHENBERG and NACHMANSOHN (1947) resulted in a preparation that hydrolysed acetylcholine at a rate of 400 mM/hr/mg protein. The specific activities of some other preparations were: 270 μM/hr/mg for ox red cell cholinesterase (COHEN and WARRINGA, 1953a), 440 μM/hr/mg for human red cell cholinesterase (ZITTLE *et al.*, 1954), 360 μM/hr/mg for human serum cholinesterase (SURGENOR *et al.*, 1949), and 12 mM/hr/mg for horse serum cholinesterase (STRELITZ, 1944).

Although insect cholinesterases attract much attention, especially with regard to their probable importance in organophosphate-poisoning and nerve transmission, the only successful attempt at purifying an insect cholinesterase was made by LORD (1961). He obtained a 40-fold purified preparation from the German cockroach (*Blattella germanica*) and studied its properties.

The purpose of the present attempt at purifying housefly head cholinesterase was to obtain a preparation that would allow for a more reliable characterization of this enzyme than that obtained in studies on crude homogenates (VAN ASPEREN, 1959, 1960; VAN ASPEREN and OPPENOORTH, 1960).

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METHODS

Assay of enzyme activity

Two methods for the determination of cholinesterase activity have been used.

(a) *The Warburg-manometric method.* 2 ml of a diluted enzyme solution were introduced into the main compartment of the Warburg flask and 0·4 ml of 1·5% acetylcholine-chloride in 1·26% $NaHCO_3$ was tipped in from the side bulb after gassing the contents of the flask with a 95% N_2–5% CO_2 mixture. Final concentrations: NaCl, 0·5 M; acetylcholine 0·015 M at the beginning of measurement; pH 7·6. Measurements were performed at 37°C. The manometers were closed 5 min after mixing enzyme and substrate and readings taken at 10-min intervals for 1 hr. The activity is expressed in Warburg units (W.u.). A Warburg unit is the amount of enzyme that, under the conditions described, produces 1 μl of CO_2 (N.T.P.) per minute.

(b) *The indophenylacetate-colorimetric method.* Indophenylacetate (N-(4′-acetoxy-phenyl)-*p* quinone imine) was introduced as a suitable substrate for ChE-determinations by KRAMER and GAMSON (1958) and by ARCHER and ZWEIG (1959). Since flyhead ChE proved to be very active against this substrate, the rate of enzymic hydrolysis could easily be estimated by the photometric determination of indophenol at pH 8·0. In order to separate ChE activity from that of other esterases, eserine at 10^{-7} M final concentration was added in one of two parallel experiments. The enzyme solution was diluted with 0·1 M phosphate buffer pH 8·0 in order to obtain a ChE-concentration of 0·5–1·5 units/ml (see below). To 4 ml of this diluted solution were added 0·5 ml H_2O or eserine-solution 10^{-6} M (in water) and 0·15 ml of a $3{\cdot}3 \times 10^{-3}$ M solution of indophenylacetate in ethanol. After 30 min incubation at 27°C optical density was measured by means of a Beckman-DU-spectrophotometer at 625 mμ, using a 1 cm cell. Final concentrations: indophenylacetate, 10^{-4} M; eserine, 10^{-7} M; pH 8·0. An indophenylacetate-unit (Ipha.-u.) is the amount of enzyme that, under the conditions described, produces an optical density of 0·1.

Total organic matter

This was assayed according to JOHNSON (1949). The method is based on the decrease of optical density of an acid dichromate solution upon heating with organic substances. An aliquot of enzyme solution containing 100–500 μg of organic matter was dried down in an oven at 100°C. To the residue were added 0·4 ml distilled water and 1 ml of oxidizing reagent (5 g $Na_2Cr_2O_7$ $2H_2O$ in 20 ml of water, diluted to 1 litre with 95% H_2SO_4: ca. 0·1 N solution). After reaction for 20 min in a boiling waterbath 10 ml of distilled water were added and optical density read in a Beckman-DU-spectrophotometer against distilled water at 440 mμ, using a 1 cm cell. The O.D. measured was subtracted from that obtained in a blank run (O.D. approx. 0·480) with distilled water instead of the enzyme solution. Preliminary experiments with dried caseine and glucose showed that a change in O.D. of 0·001 corresponds with 1·4 μg of organic matter, in full agreement with JOHNSON's figures.

Heat denaturation

This purification step needs some comment. According to WOLFE and SMALLMAN (1956) flyhead ChE is quickly inactivated at 48°C. However, acetylcholine was found to protect the enzyme against heat-denaturation at 55°C, even if 0·5% *n*-butanol was added as an additional denaturating agent.

PURIFICATION

The following is a typical example of a purification run. It should be emphasized that the reproducibility was rather poor. After every fractionation both enzyme activity and organic matter (O.M.) were checked in all fractions obtained and the purest fraction (or a combination of the purer fractions) was used in further work.

Harvesting flyheads. Flyheads were collected from normal non-resistant houseflies (*Musca domestica* L.) by the method of MOOREFIELD (1957). About 50,000 flies (800 g) were collected over a period of time and stored at −16°C till further treatment; 87·5 g of flyheads were obtained.

Freeze-drying. The frozen flyheads were homogenized in about 450 ml of distilled water, using a small type of Waring-blendor, cooled by ice, and the homogenates were freeze-dried. The total amount of freeze-dried powder was 22·7 g. About 1·5 g was saved for other experiments and 21·19 g for further purification (I).

Solubilization. The method was essentially that of MORTON (1950). The freeze-dried powder was homogenized in cold *n*-butanol (−16°C), using 100 ml for each gram of powder and the homogenate stored at −16°C for 1·5 hr. It was then filtered with suction through a Buchner funnel which was cooled with ice water. The resulting cake of solids on the filter paper was further dried by sucking air through for 15 min and stored under a vacuum above silica-gel at 0°C for 2 hr. The resulting powder was then homogenized by means of a Waring-blendor (cooled by ice), using 100 ml of 0·01 M phosphate buffer, pH 8·0, for each gram of the original freeze-dried material. The homogenate was kept overnight at 9°C and then centrifuged for 30 min at 16,000 *g*. The supernatant was found to contain 2 mg O.M./ml and was (for reason of standardization) diluted twice. The resulting solution (4200 ml) is called the phosphate-extract (P.E., II).

Ammonium sulphate-fractionation at pH 7·0. To 4160 ml of P.E. solid ammonium sulphate (AS, amount calculated by the KUNITZ (1952) formula) was added to make the solution 30% saturated, with adjustment of pH to 7·0. This was left overnight at 9°C and centrifuged (13,000 *g*) the following morning. Further precipitations were performed at 40, 70, and 80% AS-saturation. The fraction precipitated between 40 and 70% saturation was picked up in 500 ml of distilled water. After dilution with 240 ml phosphate buffer (1 M, pH 8·0) and 460 ml distilled water to 1200 ml (III) it was used for further purification.

Heat denaturation. To solution III (cooled in ice) were added 6 ml cold butanol and 29 g acetylcholine-chloride. The concentrations were then: phosphate, 0·2 M (pH 8); butanol, 0·5%; and acetylcholine, 0·133 M. The solution was kept at 54–55°C for 10 min and then left at room temperature for 4 hr. At the end of this period the pH had dropped to 6·4 due to the enzymatic hydrolysis of acetylcholine. Since no further drop of pH occurred the hydrolysis was thought to be complete at the end of this period. The solution was stored overnight at 4°C. After centrifugation at 16,000 *g* and 2°C for 30 min the precipitate was discarded and a second AS-fractionation performed. Three fractions (0–40% sat., 40–60% sat., and 60–70% sat.) were prepared as described above. The fraction precipitated between 40 and 60% saturation was dissolved in 300 ml 0·1 M phosphate buffer pH 7·0 (IV) and used for further purification.

Ammonium sulphate-fractionation at pH 6·0. The AS-concentration was increased stepwise (0–40–45–55–60–70% saturation). After each addition of AS the pH was adjusted to 6·0 and the solutions kept at 2°C for at least 4 hr. The precipitates obtained after each step by centrifugation (13,000 *g*) were dissolved in 50 ml of distilled water. The three fractions covering the range from 40 to 60% saturation were combined (150 ml; V) and subjected to the next purification step.

Calcium phosphate-gel-fractionation. Successive amounts of about 1 ml of calcium phosphate-gel (prepared according to KEILIN and HARTREE (1938), dry weight 30·8 mg/ml) were added to the solution in a centrifuge tube. After each addition the contents of the tube were stirred for 5 min and then centrifuged for 15 min at 16,000 *g* and 2°C. The supernatant was transferred to another centrifuge tube for the next addition of gel. Enzyme activity and O.M. were checked in the supernatants, and it was concluded that most of the

enzyme was present in the three precipitates obtained between the addition of 3·7 and 6·7 ml gel total, which contained less than one-third of the O.M. These precipitates were suspended in distilled water and combined. The gel was then extracted (homogenization followed by centrifugation) with, successively, distilled water and 0·002 M, 0·01 M, 0·02 M, 0·03 M, and 0·04 M phosphate buffer pH 8·0. The last four extracts were combined (252 ml; VI) and purified further.

Ammonium sulphate-precipitation. The enzyme solution was 90% saturated with AS (pH drops to 6·7) and left overnight at 2°C. After centrifugation the supernatant was discarded and the precipitate picked up in 25 ml glass-distilled water (VII).

Acetone-fractionation. The procedure was essentially that described by ASKONAS (1951). The acetone concentration was increased stepwise (0–25–30–50–60% acetone) by adding cold acetone (−16°C) to the enzyme solution while vigorously stirring at ca. −5°C. The precipitate formed in each case after 1 hr standing was removed by centrifugation (30 min, 12,000 *g*, −5°C). The four precipitates were kept overnight at −16°C and each was dissolved in 5 ml glass-distilled water. The third fraction (from 30 to 50% acetone precipitate) contained nearly all the enzyme activity (2430 W.u.) and only 4·1 mg of total organic matter: final product (VIII).

Table 1 shows a schematic picture of the results obtained in this purification run. The final preparation (593 W.u./mg) hydrolysed acetylcholine at a rate of 1600 μM/hr/mg dry wt.

TABLE 1—PURIFICATION OF HOUSEFLY HEAD CHOLINESTERASE

Purification stage	Volume (ml)	Total O.M. (mg)	Total activity		Specific activity		Yield (%)	Purification factor
			(Ipha. u.)	(W.u.)	(Ipha.u./mg O.M.)	(W.u./mg O.M.)		
I. Freeze-dried		16,530		62,500		3·78	100	1
II. Phosphate-extract	4200	4200		47,250		11·3	76	3·0
III. After AS-fractionation pH 7	1200	952		32,000		33·6	51	8·9
IV. After heat-denaturation and AS-fractionation pH 7	300	213	52,950	18,360	249	86·2	29	22·8
V. After AS-fractionation pH 6	150	126	37,100	12,900	294	102·4	21	27·1
VI. After Ca-phosphate absorption and elution	252	16·9	15,000	5000	887	296	8	78·3
VII. After AS-precipitation	25	13·0	9000	3200	692	246	5·1	65
VIII. After acetone-fractionation	5	4·1	6680	2430	1630	593	3·9	157

Some other purification-steps were occasionally used in other purification-runs. In one case treatment with vegetable carbon (Norit) improved the purification from 71 to 106 times, but in other cases, as with ethanol fractionation, it was unsuccessful. No purification was obtained by addition of a protamine sulphate solution (brought to pH 6) in order to remove nucleic acids. Absorption on magnesium carbonate was also unsuccessful.

Stabilization. In the next section it will be shown that purified enzyme solutions, even if made up in distilled water, are relatively stable. Yet some attempts have been made to obtain stable enzyme powders by freeze-drying small samples of enzyme solution (3·6–5 ml) after the addition of gelatin (1 mg/200 W.u.) and 1 M phosphate buffer pH 7·6 (1 ml/4000 W.u.). This treatment* caused some loss of enzyme activity. Enzyme powder I was obtained from a 79-fold purified enzyme solution with 5% loss of activity, Enzyme powder II from a 93-fold purified preparation with only 4% loss and Enzyme powder III from a 94-fold purified enzyme solution with as much as 50% loss. As pointed out by LESUK (1949) the initial gelatin concentration during the preparation of the powder should be at least 2%. The occasional loss of activity in our experiments could thus be due to the gelatin concentration being too low. However, in two out of three cases the recovery was quite satisfactory.

RESULTS AND DISCUSSION

Stability

The 157× purified enzyme preparation finally obtained in the purification run of Table 1 consisted of an acetone-precipitation fraction (total O.M. 4·1 mg; 2430 W.u.) dissolved in 5 ml of distilled water. This solution was kept in a refrigerator without any addition of salts or gelatin. Its enzymic activity was periodically checked and no loss was observed over a period of 17 days. After 25 days the solution was concentrated 5 times by ultrafiltration through a collodion membrane and 20 days later this concentrated solution was diluted 10 times. After 78 days still 83% of the original activity was present, after 102 days 65% of the activity was retained.

Three other enzyme preparations (79×, 93×, and 94× purified) were converted to enzyme powders with addition of gelatin and phosphate (see above). These powders were kept at −16°C; they had lost about 10% of their activity after 2 months and 50, 40, and 10% respectively after about 5 months.

To a solution of 94× purified enzyme gelatin and phosphate buffer were added. This 'stabilized' solution, kept at 2°C, lost activity at about the same rate as the enzyme powders and the enzyme solution in distilled water as discussed above. The same applied to strongly diluted preparations prepared by dissolving one of the enzyme powders in 3·5% sodium chloride solution.

The present results seem to indicate that the stability of the purified enzyme is fairly high and that none of the methods tried for stabilization gave a clear-cut improvement. It was found, however, that a diluted sample of purified enzyme solution (157×) lost activity at 37°C at a rate of about 3%/min, and that this could be largely prevented by adding 2% gelatin (only 15% loss in 60 min).

* Treatment based on data given on the purified bovine ChE in the N.B.C. catalogue.

Substrate specificity

Special attention was given to the problem of change in substrate specificity during purification.

A change would be expected to appear in the relative activities to acetylcholine and indophenylacetate, that is, in the ratio between the numbers of Warburg-units and Ipha-units. This ratio proved to be nearly constant (2·7–3·0) throughout the last five steps of purification (Table 1) and about equal to the ratio in crude flyhead homogenates if only the eserine-inhibitable part of the activity to indophenylacetate was considered. This was found to be approximately 2·5.

The results obtained in two purification runs with a number of other substrates are compared with those obtained on a crude AD-treated flyhead homogenate and a highly purified preparation of bovine erythrocyte ChE in Table 2. There might be a slight loss of enzyme activity to triacetin, phenylacetate, and possibly

TABLE 2—SUBSTRATE SPECIFICITY OF ENZYME PREPARATIONS

Nature of enzyme preparation	Purification factor	Substrates						
		ACh 0·015 M	BuCh 0·015 M	Triac. 0·036 M	MB 0·048 M	Amylac. emulsion	Phenylac. emulsion	Phenylbu. emulsion
Flyhead homogenate	1	100	57	116	38	65	162	107
AD-treated homogenate	1	100	55	114	2	51	140	73
I. Freeze-dried	1	100	53	109	4	52	129	84
II. Phosphate-extract	3	100	55	110	2	50	130	74
IV. AS-fraction	28	100	52	101	0	54	139	76
VII. Enzyme powder III	94	100	57	84	3	54	90	65
I. Freeze-dried	1	100	59	118	—	65	128	81
II. Phosphate-extract	3	100	53	105	0	53	134	79
IV. AS-fraction	23	100	56	81	0	46	125	75
VIII. Acetone-fraction	157	100	52	99	−3	50	115	62
Bovine erythr. ChE		100	2	32	0	32	104	2

Abbreviations: ACh = Acetylcholine, BuCh = Butyrylcholine, Triac. = Triacetin, MB = Methylbutyrate, Amylac. = Amylacetate, Phenylac. = Phenylacetate, Phenylbu. = Phenylbutyrate.

Technique: Total contents of Warburg-flasks 2·2 or 2·4 ml; substrate (at indicated concentration) and enzyme dissolved in 0·025 M $NaHCO_3$ and 0·5 M NaCl. Gas phase: 95% N_2 + 5% CO_2; pH 7·5; temp. 37°C. Enzyme activities to the different substrates are expressed as percentages of the activity to acetylcholine (taken as 100 per cent). Roman figures refer to the last purification step completed.

AD-treatment consists of keeping the homogenate for 1 hr at 37°C in a 0·025 M bicarbonate-solution (pH ca. 8·3). This causes denaturation of ali-esterases but leaves ChE-activity unaffected. Not included in the table are the figures for propionylcholine and acetyl-β-methylcholine both at 0·015 M conc. They are 71 and 25 respectively, whereas those for bovine ChE were found to be 79 and 43 respectively.

phenylbutyrate, but the changes are generally small. There can be no doubt that the non-choline esters mentioned are split by a number of esterases (VAN ASPEREN, 1959). The evidence presented in Table 2, however, strongly indicates that both AD-treatment (see legend to Table 2) and freeze-drying remove or destroy

nearly all other esterases leaving only the cholinesterase. The slight changes in the activity to the aromatic esters might be evidence for the initial presence and gradual removal of some 'aromatic' esterase, but this is not very convincing. HOWDEN and SHATOURY (1960) reported on the occurrence of two different cholinesterases in houseflies more or less comparable to the 'true' and 'pseudo-' cholinesterase of vertebrates. No support for this view was obtained from the results of this purification; the ratio of the enzymatic activities to acetylcholine and butyrylcholine was strikingly constant and about equal to that in a crude flyhead homogenate (without AD-treatment). The substrate specificity pattern of the purified flyhead ChE shows marked differences with the bovine erythrocyte preparation in that it reveals much higher activities to butyrylcholine, triacetin, and phenylbutyrate (used at the concentrations mentioned).

One could also object to the use of the name 'cholinesterase' for the enzyme, since most probably the turnover-number to a number of aliphatic and aromatic acetates and possibly even to phenylbutyrate is at least as high as that for acetylcholine. Apart from the physiological argument that acetylcholine is the natural substrate, another point in favour of the term 'cholinesterase' is the extremely high affinity of the enzyme for acetylcholine ($K_m \leqslant 10^{-5}$ M/l.), evidence for which will be presented in another paper.

Inhibition by organophosphates

The inhibition of cholinesterases by organophosphates is a progressive reaction. This especially applies to the housefly cholinesterase, since no indication was ever found for *in vitro* recovery of this enzyme. It follows then that theoretically the enzyme active site concentration can be determined by measuring the inhibition after an 'infinite time of incubation' with some organophosphate. Several difficulties may arise if this technique is used, because of nonspecific phosphorylation, e.g. of enzymically unimportant aminoacids and proteins and because of the duration of an 'infinite' incubation period (for a discussion of these problems see O'BRIEN, 1960).

Previous experiments (VAN ASPEREN and OPPENOORTH, 1960) on crude flyhead homogenates showed the I_{50} (i.e. the concentration that gives 50% inhibition after 'infinite' incubation time) of diazoxon under the conditions described to be about $1{\cdot}2 \times 10^{-9}$ M. It was shown, however, that in these crude homogenates other esterases (ali-esterases) are present that also utilize diazoxon. Elimination of at least part of these esterases thus led to the much lower I_{50} value for the cholinesterase inhibition by diazoxon of 5×10^{-10} M. If it is assumed in the latter experiment that the organophosphate reacts exclusively with the ChE active sites, the concentration of these sites would be 10^{-9} M; and since 2 ml of this enzyme solution produced 124 μl CO_2 per 30 min in a Warburg experiment, the t.o.n. of the enzymic reaction would be 93,000. Several experiments with diazoxon under similar conditions all yielded an 'apparent t.o.n.' of about 100,000. It was clear, however, that these figures could be completely wrong if the homogenates still contained other compounds that utilize organophosphate and thus compete with the

cholinesterase. It was decided, therefore, to measure the inhibition of the preparations that were obtained in the course of purification.

Two organophosphate inhibitors were used throughout: diazoxon [= O,O-diethyl O-(2 isopropyl-4-methyl-6 pyrimidinyl) phosphate] and QAT (= O-ethyl-S-2 triethyl-ammoniumethyl ethylphosphonothiolate iodide). They were selected because of their very rapid inhibitory action; the bimolecular rate constants under the experimental conditions were found to be 2×10^8 for diazoxon and 7×10^8 for QAT (l/M/min). These high figures (approximately 100 times as high as those for bovine erythrocyte ChE) made it possible to obtain the final inhibition values after 1 hr incubation, i.e. 1 hr was considered as an 'infinite incubation time'. This was confirmed in experiments where practically no change in inhibition was observed in a second hour of incubation. QAT, moreover, takes a special position in that it contains a quaternary nitrogen group, which may improve its affinity to acetylcholinesterases and other anionic compounds.

The results obtained with diazoxon and four preparations in the purification run of Table 1 are shown in Fig. 1. These and other results lead to the following considerations:

(1) The relation between inhibitor concentration and enzymic activity is strikingly linear, indicating that there occurs a simple stoichometric reaction between diazoxon and ChE in which the inhibitor is completely utilized.

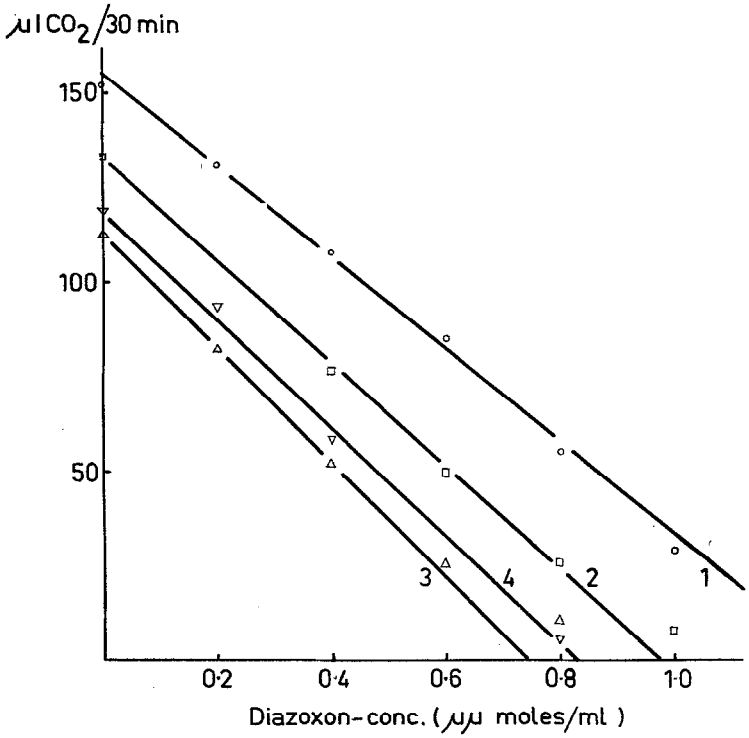
(2) If it is assumed that diazoxon reacts exclusively with the active sites of the ChE, the turnover numbers can easily be calculated from the slopes of the lines. These apparent t.o.n.-values are shown in Fig. 1.

If any other phosphorylatible compounds capable of competing with the ChE for diazoxon were present in the freeze-dried preparation ($1\times$ purified), these are likely to be removed in the course of the $157\times$ purification with a resultant increase of the apparent turnover number. No such increase, or only a very minor one, was actually observed. Phosphorylatible groups other than the active sites could be present in the cholinesterase molecules. It would be rather peculiar, however, if such groups, that have nothing or little to do with enzymic activity, were present only in the cholinesterase molecule. It is very likely, therefore, that diazoxon specifically reacts with the active sites of the cholinesterase and that the apparent turnover number, calculated from our experimental data, equals the true turnover number. This true turnover number would thus be approximately 110,000.

(3) The above conclusions were completely confirmed by the data obtained with QAT in the same purification run and by those with diazoxon and QAT in a number of other preparations, ranging from 3 to $157\times$ purification. Plots of enzyme activity to inhibitor concentration were essentially similar to those of Fig. 1; the calculated turnover numbers ranged from 79,000 to 132,000 with a mean value of $101{,}000 \pm 15{,}000$. No increase of 'apparent' t.o.n. was observed with increasing purity.

(4) For comparison, the turnover number of highly purified bovine erythrocyte ChE (Winthrop) was determined in a similar way, using a rather concentrated

enzyme solution and paraoxon. The apparent t.o.n. was found to be approx. 350,000, which agreed quite well with the true turnover number of 295,000 given by COHEN and WARRINGA (1953b) and COHEN *et al.* (1955). Paraoxon was used in



	Degree of purification	Apparent turnover number
1	1×	92,000
2	3×	103,000
3	23×	114,000
4	157×	108,000

FIG. 1. Inhibition of enzyme activity by diazoxon. 1 ml of enzyme solution and 1 ml of diazoxon solution, both in NaCl 3·5%, were mixed in the main compartment of the Warburg flask. After 1 hr incubation at 37°C and gassing the contents of the flask with 95% N_2–5% CO_2, 0·4 ml of 1·5% acetylcholinechloride in 1·26% $NaHCO_3$ was tipped in from the side bulb. Five minutes later the manometers were closed and readings taken at 10-min intervals for 1 hr.

this experiment, since the bimolecular rate constant for the inhibition of bovine AChE is much higher for this compound ($2{\cdot}5 \times 10^6$ l/M/min) than for diazoxon

(5×10^5 l/M/min). The reverse was found to be true for the inhibition of housefly head ChE: k diazoxon $= 2 \times 10^8$ and k paraoxon $= 6 \times 10^6$ (l/M/min).

The effect of organic solvents on activity and inhibition

In the course of our purification work it was found that in one case the total enzymic activity had been increased by the butanol-treatment of step II. This was thought to be possibly due to the presence of small amounts of butanol, since COLHOUN (1961) has found that cockroach ChE is activated by this alcohol. This phenomenon has been extensively studied in the housefly and the results of this study will be presented in a separate paper. Some remarks on the effect of butanol and other organic solvents, referring to purified enzyme preparations, will be made here.

(1) The results of a typical experiment on the influence of n-butanol on the activity of a 157× purified preparation and on the inhibition of this activity by diazoxon are shown in Table 3. They clearly show that n-butanol activates the

TABLE 3—THE INFLUENCE OF n-BUTANOL ON FLYHEAD ChE

	Butanol-conc. (%)					
	0	0·1	0·3	1·0	2·0	3·0
Activation*	0	27	30	50	60	73
Diazoxon-inhibition†	71	78	68	62	46	19

* Activation is expressed as a percentage of the normal activity (without butanol) of 92 μl CO_2 per 30 min.

† Diazoxon-conc. $= 8 \times 10^{-10}$ M. Incubation time (enzyme-inhibitor) = 15 min. Inhibition is expressed as a percentage of the activity in the absence of diazoxon.

flyhead ChE. Optimal activation was obtained with n-butanol concentrations of 2–3%. Higher concentrations gave less activation or even inhibition. The results also show that butanol decreases the inhibition caused by diazoxon. It could be proven that this decrease is due to a lowered rate of inhibition.

(2) The activation of enzymic hydrolysis by n-butanol was only observed if choline esters were used as substrates. With other substrates, such as triacetin, amylacetate, phenylacetate, and phenylbutyrate butanol (2%) caused inhibition, amounting to 17, 72, 28, and 23% respectively.

(3) Some activation (9–20%) was caused by *iso*butanol, tertiary butanol, propanol, and n-amylalcohol. Ethanol and acetone in low concentrations did not affect the cholinesterase activity. At higher concentrations (8 and 16%) these compounds gave slight inhibition.

(4) Like n-butanol, ethanol and acetone strongly lowered the rate of inhibition by diazoxon.

(5) The above data were all obtained with a 157× purified preparation of flyhead ChE. Essentially similar results, however, were obtained with crude flyhead

homogenates and also with a highly purified preparation of bovine erythrocyte ChE (Winthrop). Further details will be published in due course.

Electrophoresis of purified cholinesterases

Some agar-electrophoresis experiments were performed using the method described by WIEME (1959).

Techniques. Microscope slides (76 × 26 mm) were covered with a thin layer (ca. 1·5 mm thick) of agar gel (Difco agar noble 0·9%), made up in veronal buffer (pH 8·6; $\mu = 0{\cdot}05$). The pH of the resulting agar gel was about 8·4. Transverse slits were then made and filled with approximately 1 μl of the solution to be studied. After the plates had been covered with petroleum ether (boiling range 28–40°C) for the purpose of cooling, a potential gradient of circa 30 V/cm was applied. After 4–8 min the slides were removed and treated in one of the following ways:

(a) *Demonstration of proteins.* The slides were transferred into 5% acetic acid–70% ethanol solution in order to precipitate the proteins. After about 1 hr the slides were dried at 37°C between filter paper and coloured with naphthalene black. After differentiation in 5% acetic acid the proteins show up as bluish-black bands.

(b) *Demonstration of esterases.* The agar gel on the slides was covered with a few drops of indophenylacetate solution ($3{\cdot}3 \times 10^{-3}$ M) in ethanol. The presence of esterases, capable of hydrolysing this ester, resulted in the appearance of blue spots after a few minutes due to the production of indophenol which at pH 8·4 has a very strong blue colour.

Results and conclusions

The results that should be considered as preliminary are summarized in Fig. 2. The following conclusions can be drawn.

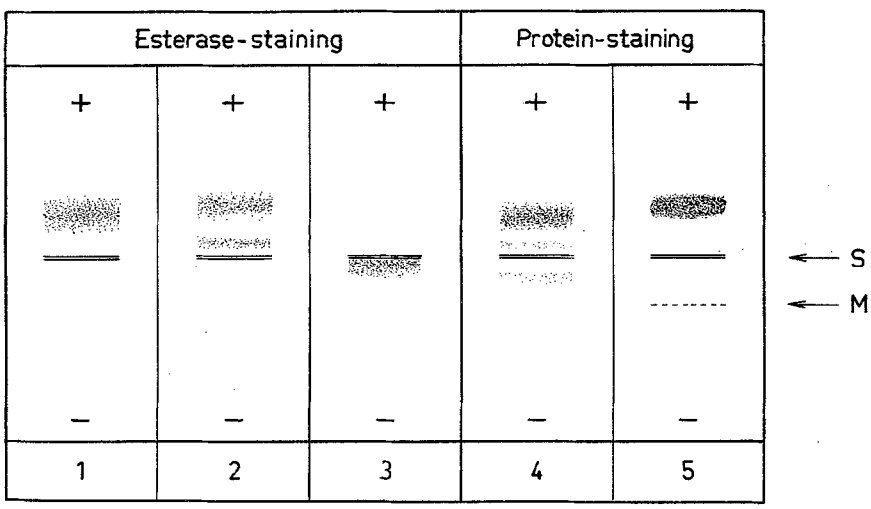


FIG. 2. Results of agar gel electrophoresis. Techniques and conditions are described in the text. The samples are introduced into the slit S. 1. Flyhead cholinesterase, 157× purified. 2. Human serum. 3. Purified erythrocyte cholinesterase (Winthrop). 4. Flyhead cholinesterase, 157× purified. 5. A mixture of human albumin and macrodex. The latter is an electrophoretically inert polysaccharide, which is moved to the negative side by endosmosis and thus marks the real starting point for electrophoresis (M).

(a) The 157× purified preparation of housefly head ChE contains probably three electrophoretically different proteins (column 4). At pH 8·4 these are all negatively charged (N.B. See note under Fig. 2, No. 5). The most important of these proteins moves at about 0·9 times the speed of human albumin (compare 4 and 5).

(b) Comparison of columns 1 and 4 shows that this rapid protein band contains (or is identical with) the cholinesterase. In the presence of eserine this cholinesterase spot did not appear.

(c) The flyhead ChE moves much faster than the bovine erythrocyte ChE (compare 1 and 3). Serum contains two indophenylacetate splitting esterases, one of which is somewhat faster, the other somewhat slower than the flyhead ChE (compare 1 and 2).

Some experiments were performed using an agar gel–phosphate buffer system of pH 7. After electrophoresis and incubation with indophenylacetate the esterases show up as reddish spots. The results obtained with flyhead ChE, purified bovine erythrocyte ChE, and human serum were in agreement with the above conclusions.

GENERAL DISCUSSION

Some speculative remarks can be made with respect to the purity of our final preparation (600 W.u./mg of organic matter) and the molecular weight of the flyhead ChE. If it is assumed: (1) that the preparation is fairly pure, (2) that the turnover number is 110,000, and (3) that the enzyme contains only one active site per molecule, it can be calculated that the M.W. of the cholinesterase is in the order of 3–4 million. Unfortunately, no ultracentrifuge experiments could be done so that no sedimentation data are available for our preparation. In our view, the preparation is probably by no means pure and the cholinesterase has a lower molecular weight and represents only a small proportion of the protein. That other proteins are still present is evident from the two slower-moving protein bands in the electrophoresis experiment (Fig. 2, column 4). These bands are extremely weak and do not comprise more than 10 per cent of the total protein. It seems reasonable to assume that the cholinesterase migrates together with other proteins in the fast-moving protein band. At present it is impossible to decide which of these two views is correct.

The purification was undertaken to obtain a reasonably pure preparation in order to study the properties of the enzyme. The results that were obtained did not appreciably alter the opinions on substrate specificity, the inhibitive properties of organophosphates and the influence of organic solvents, that have been based on experiments with crude flyhead homogenates. With respect to the inhibition by diazoxon, the results confirm the view that diazoxon and probably other organophosphates as well react very specifically with the esterases and that reaction with other proteins does not occur to any measurable extent.

The results obtained in this and other studies clearly indicate that the flyhead ChE is distinctly different from the bovine erythrocyte ChE in many respects, e.g. in that it shows high hydrolytic activity to butyrylcholine. Yet, it should be

classified as a 'true' cholinesterase (if classification is thought necessary). It seems very unlikely that two different cholinesterases were present in our final purification product and the evidence presented makes it doubtful whether a second cholinesterase of the 'pseudo'-type is present in the crude homogenates.

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