THE SEDIMENTATION PROPERTIES OF CHOLINESTERASE FROM A MAYFLY (*HEXAGENIA BILINEATA* (SAY); EPHEMEROPTERA) AND THE HONEY BEE (*APIS MELLIFERA* L.)

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ABSTRACT

1. Solubilized mayfly and honey bee cholinesterase have the same sedimentation coefficient (7·3*S*) at *p*H 8·0.

2. At *p*H 6·8 the mayfly enzyme sediments in 7·3*S*, 10·2*S*, and larger forms; the honey bee enzyme is polydisperse at *p*H 6·8.

3. The mayfly ChE shifts from heavier forms to 7·3*S* following dialysis, dilution, or an increase in *p*H from 6·8 to 8·0.

In spite of the considerable attention that has been accorded cholinesterases (ChE), the enzyme has never been highly purified from insects (Lord, 1961; Dauterman, Talens, and van Asperen, 1962; Kunkee and Zweig, 1963) as compared with the recent success with electric eel acetyl-ChE (AChE) (Leuzinger and Baker, 1967). Two major difficulties which appear to underlie this lack of success are the particulate nature of much of the enzyme (Krysan and Chadwick, 1970) and the presence of multiple ChE forms in soluble preparations (e.g., Menzel, Craig, and Hoskins, 1963). On the one hand, enzymes which cannot be removed from particles are not amenable to purification procedures; on the other hand, the simultaneous presence of several ChE's is likely to make ambiguous the typically dichotomous purification steps. Thus, to face the latter problem, one should have available a knowledge of the molecular properties of the enzyme; however, such knowledge generally stems from successful purification.

The purpose of the work described here was to generate some knowledge about the molecular properties of the enzyme from insects which could serve as a basis for a more rational purification approach. Our results are being communicated at this time because the molecular size and the interconvertibility of sedimentation characteristics of the ChE's observed in this study are of general interest.

MATERIALS AND METHODS

Homogenate Preparation

Samples (25 g.) of whole, frozen, female mayflies (collected near the Mississippi River at Winona, Minnesota, during the adult flight) were homogenized in 100 ml. phosphate buffer (NaCl, 52·6 g.; KH_2PO_4, 3·85 g.; NaOH, 1·00 g.; H_2O to 1 litre; *p*H 6·8: this buffer solution was used throughout) in a Lourdes Waring-type blender for 2½ minutes. The homogenate was centrifuged at 1800 *g* for 10 minutes, the pellet discarded, and the supernatant autolysed under toluene at 25° C. for 80–100 hours with constant shaking. No ChE activity was lost in these steps. The final preparation applied to the gradient was the 105,000 *g* (60-minute) supernatant fluid of autolysed samples. Honey bees (obtained from Robb Brothers, Winona, Minnesota) were frozen and the heads harvested; the heads were homogenized (10 g. per 150 ml. of buffer) as

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described above for mayflies. Dialysed samples were prepared as follows: 20-ml. samples were dialysed with constant stirring at 2° C. against 4 litres of buffer, 2 changes, 24 hours per change.

Ammonium Sulphate Precipitation

Solid ammonium sulphate was added to aliquots of standard homogenate with stirring; *p*H of the system was maintained at 6·8 by the addition of NaOH. The fraction which precipitated between 30 and 40 per cent (weight per starting volume) was dissolved in buffer and used for these studies.

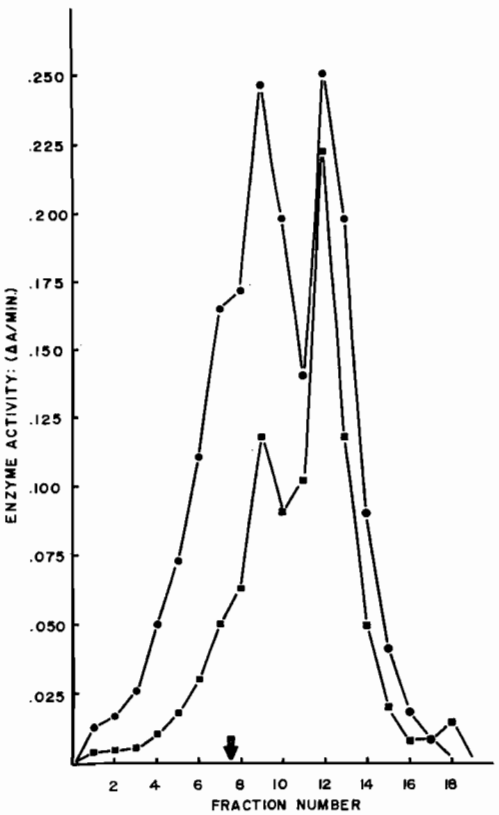

Fig. 1.—Pattern of ChE distribution following density-gradient centrifugation of standard (●) and dilute (■) (diluted 1 : 1 with buffer) mayfly preparations. The arrow indicates the peak location for the catalase standard.

Gradient Preparation

Linear sucrose gradients (5–20 per cent; volume 6·0 ml.) in buffer were prepared in 0·5 × 2 in. nitrocellulose tubes. Gradient *p*H was 6·8 in all cases. Catalase (beef liver, Mann Research Labs.) and ChE samples (0·1 ml.) were layered on the gradients immediately prior to centrifuging at 50,000 r.p.m. for 12 hours at 3° C. using an SW-50 swinging bucket rotor in a Spinco Model L preparative ultracentrifuge. Four-drop fractions (volume 0·3 ml.) were collected from a puncture hole in the bottom of each tube.

Assay Procedures

ChE was assayed in fractions (0·2-ml. aliquot) from density gradients by the method of Ellman, Courtney, Andres, and Featherstone (1961), using as substrate acetylthiocholine iodide (Sigma Chemical Company).

Catalase activity was determined by observing over a period of 1 minute the change in absorbance (240 mμ) of a mixture containing 0·005 ml. of enzyme fraction, 3 ml. buffer, and 0·25 ml. of 3 per cent H_2O_2.

RESULTS

About 90 per cent of the ChE activity in a mayfly homogenate is sedimented by centrifugation at 100,000 *g* for 1 hour. It is only slightly solubilized by the surfactants Tween 80 and Triton X-100. However, some 70 per cent of the particulate enzyme can be made soluble by autolysis under the conditions described in Materials and Methods, above. NaCl at 1 *M* greatly enhances autolytic solubilization yields; addition of NaCl does not solubilize the enzyme from non-autolysed preparations. The soluble enzyme has no activity towards the substrate butyrylthiocholine at 10^{-3} *M* and the enzymatic hydrolysis of acetylthiocholine is inhibited completely by eserine at 1×10^{-5} *M*.

Fig. 1 (●) illustrates the distribution of mayfly ChE from a standard preparation in a sucrose density gradient. The slower sedimenting form (peak at fraction 12) is 7·3*S* relative to catalase (calculated by the method of Martin and Ames, 1961). The other peak is 10·2*S*. For the sake of clarity, the 7·3*S* form will be called 'deaggregated' and the 10·2*S* and larger forms will be called 'aggregated'; it is not known from our data whether the interaction is heterologous or homologous. The proportion of enzyme activity in the 10·2*S* and larger forms, but not the location of the heavier peak (i.e., 10·2*S*), is dependent on several, possibly interrelated, factors. When the above described sample was diluted by half with buffer (*Fig.* 1, ■), the 10·2*S* peak became smaller and the 7·3*S* peak larger, relatively. Indeed, among

some 150 gradients observed, it was clear that as homogenate concentration increased, the 10·2*S* and larger forms came to contain relatively more enzyme activity. However, one heavy peak always appeared in the 10·2*S* location; the presence of forms with $S > 10{\cdot}2$ varied from homogenate to homogenate (e.g., compare *Figs.* 1 and 3 A, which are from different homogenates). Simple serial dilution alone never resulted in total loss of the 10·2*S* form. The total activity recovered from the gradient was always at least 85 per cent of the amount placed on it.

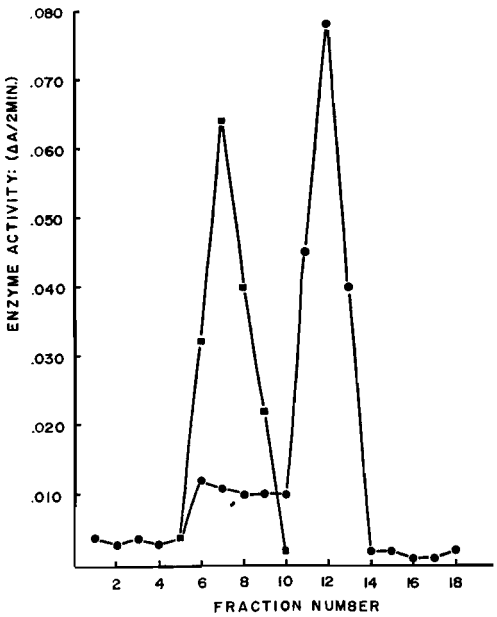

FIG. 2.—Sedimentation pattern of mayfly ChE (●) recovered from the 10·2*S* peak location. Catalase activity (■). Fractions from the 10·2*S* location of three centrifuged gradients were massed, dialysed against buffer to reduce sucrose concentration, and concentrated by adding Sephadex G-25. A 0·25-ml. aliquot was layered on a gradient along with catalase and centrifuged in the usual manner.

The fact that the 10·2*S* form is converted into the 7·3*S* form is established by the data in *Fig.* 2. Enzyme from the 10·2*S* location, combined from three gradients, dialysed against buffer for 16 hours to reduce sucrose concentration, and centrifuged through a gradient, yielded mainly 7·3*S* material.

The above observation suggested that dialysis may affect the aggregation state since the 10·2*S* peak is nearly absent. However, before presenting our observations on the effect on aggregation we shall record the effect of *p*H, a parameter which acts in concert with dialysis.

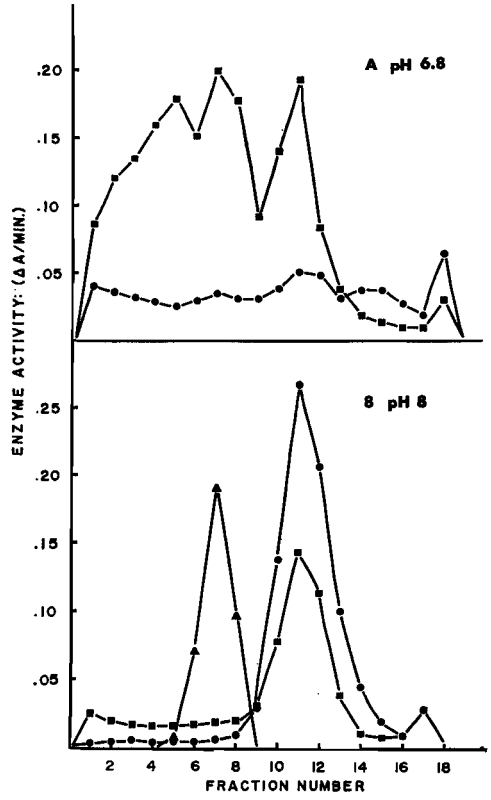

FIG. 3.—A, Distribution of mayfly (■) and honey bee (●) ChE in a density gradient at *p*H 6·8. B, Same preparation as in A in which the samples were adjusted to *p*H 8·0 with NaOH before centrifugation. ▲, Catalase. Gradient *p*H was 6·8 in all cases.

Fig. 3 illustrates the distribution of mayfly and honey bee ChE in the density gradient system at *p*H 6·8 and *p*H 8·0. Note that the gradient itself was *p*H 6·8 in all cases; only the sample *p*H was varied. The shift of all activity to the 7·3*S* form as induced by *p*H change was, in terms of our system, instantaneous; however, 24 hours at *p*H 6·0 and 96 hours at *p*H 6·8 were required to induce

reaggregation of a preparation previously deaggregated by exposure to *p*H 8·0. *Fig.* 4 presents the sedimentation patterns at *p*H 8·0 and 6·0 of a sample purified tenfold (specific activity = 13·3 μm. per mg. protein per minute) by ammonium sulphate precipitation. Note that the purified preparation retained the ability to aggregate at *p*H 6·0 following deaggregation at *p*H 8·0.

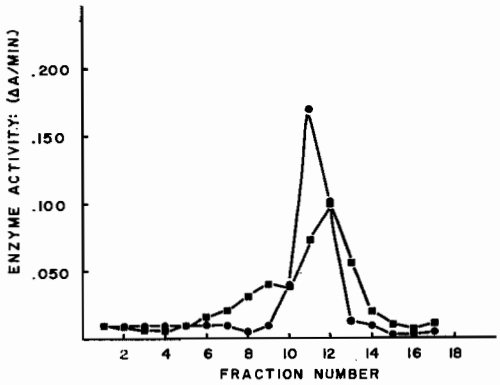

FIG. 4.—Sedimentation behaviour of mayfly ChE purified 10×. Samples were at *p*H 8·0 (●) and 6·0 (■) for 4 and 96 hours, respectively, before centrifugation. The latter sample was at *p*H 8·0 for 4 hours prior to adjusting to *p*H 6·0.

*p*H 6·8 causes some deaggregation. Experiments designed to locate a dialysable aggregation factor were inconclusive.

The following agents, when added to the gradient, had no observable influence on the distribution of ChE between the 7·3*S* and 10·2*S* peaks: acetylcholine bromide, 0·01 and 0·1 *M*; gallamine triethiodide, 2×10^{-3} *M*; pilocarpine hydrochloride, 1×10^{-2} *M*; and nicotine, 1×10^{-4} *M*.

DISCUSSION

The demonstration of alterable sedimentation properties of insect ChE deserves comment from several points of view: approaches to purification; our understanding of the molecular size and multiplicity of form of ChE; and the physiological mechanism of action of the enzyme. Each of these points will now be considered in order.

Preliminary results in this laboratory (Dibella, 1969) with mayfly ChE showed a tenfold purification with ammonium sulphate fractionation at *p*H 6·8 (7·3*S*, 10·2*S*, and greater than 10·2*S* ChE forms present), whereas at *p*H 8·0 (all ChE is 7·3*S*) a 118-fold increase in specific activity resulted. At *p*H 6·8, ChE precipitated between 5 and 45 per cent ammonium sulphate; at *p*H 8·0 it precipitated between 25 and 45 per cent. The rational choice of a purification condition appears to have borne fruit in this one preliminary attempt.

Table I.—EFFECT OF *p*H AND DIALYSIS ON THE AGGREGATION STATE OF MAYFLY ChE

*p*H	NOT DIALYSED	NO. OF EXPERIMENTS	DIALYSED	NO. OF EXPERIMENTS
8·0	95 (95–96)*	3	—	—
7·4	62 (59–65)	2	84 (82–87)	4
6·8	37 (31–50)	7	71 (69–72)	2
5·6	28 (28–28)	2	—	—

* Values given represent percentage of total activity in the 7·3*S* form; remaining activity is in the 10·2*S* or heavier forms. Numbers in parentheses are ranges. With the exception of the data at *p*H 5·6, all data represent density gradient runs on samples from at least two different homogenates.

Data relating the combined influence of *p*H and dialysis on the distribution of mayfly enzyme between the peaks are presented in *Table I.* Briefly, at *p*H 6·8 both aggregated and deaggregated ChE are present; at *p*H 8·0 all ChE is 7·3*S*. At *p*H 7·4 some deaggregation occurs; dialysis at *p*H 7·4 eliminates nearly all aggregated ChE; and, finally, dialysis at

According to the system of Markert and Whitt (1968), our mayfly ChE appears in at least four isozymic forms: in the pellet

(100,000 *g*, 1 hour) of non-autolysed homogenates; in high molecular weight polydisperse forms (greater than 10·2*S*); and as the 10·2*S* and 7·3*S* forms. All of the above are reducible to the 7·3*S* form. Multiple forms of soluble ChE have been demonstrated electrophoretically from insects by several workers (Menzel and others, 1963; Chaudhary, Srivastava, and Lemonde, 1966; Edwards and Gomez, 1966). Hoover (1968) examined our soluble mayfly preparation by acrylamide-gel electrophoresis at *p*H 8; he observed only one band of ChE activity.

Our observations with the insect enzyme, as regards molecular size and diversity of sedimentation behaviour, are quite different from those of workers studying electric eel AChE, the most thoroughly studied ChE. As reviewed by Grafius and Millar (1967) the observations on electric eel AChE have been typified by a great diversity of sedimenting forms, even as observed from preparation to preparation in the same laboratory. The ChE of insects for which sedimentation properties have thus far been described (honey bee: present report and Kunkee and Zweig, 1963; housefly: Krysan and Chadwick, 1966; mayfly: present report) are reducible, with one exception, to a form with the same sedimentation coefficient (7·3*S*) in all three species. The exception is the report of Kunkee and Zweig (1963) for honey bee ChE. They concluded that their ChE preparation had a molecular weight in the millions because 50 per cent of it sedimented when centrifuged at 100,000 *g* for 1 hour; after 2 hours of centrifugation nearly all of the enzyme had sedimented. It is doubtful whether a unit which sediments under such conditions is a molecule. The enzyme appeared somewhat polydispersed upon density-gradient centrifugation. It is significant that Kunkee and Zweig had solubilized the enzyme with n-butanol; when Krysan (1965) solubilized housefly ChE with n-butanol, the enzyme was excluded from Sephadex G-200, indicating a molecular weight of over 200,000. Yet that same housefly enzyme, when solubilized by autolysis, had a molecular weight of 160,000. Perhaps butanol releases the particulate enzyme in some supramolecular structure. Another apparent discrepancy is the ChE fraction in the supernatant of housefly head homogenates which is excluded from Sephadex G-200 when chromatographed at *p*H 7 (Krysan and Chadwick, 1966). However, careful examination of their data shows that when the fly head supernatant was dialysed and then centrifuged in a gradient at *p*H 7·4 only a very minor peak of activity was seen which could have corresponded to the sizeable fraction excluded from Sephadex. We suggest that dialysis and the *p*H of the gradient system account for the loss of heavier ChE from this fly head preparation in the same fashion that these parameters cause deaggregation in mayfly homogenates.

Whether the high molecular weight forms are functionally significant is unknown; but the clearly discernible fact with respect to the rapidly sedimenting forms (*S* greater than 50) is variation from preparation to preparation and laboratory to laboratory in the distribution and value of sedimentation coefficients (Grafius and Millar, 1967). This variation suggests random, non-specific interaction. We see no reason to consider the high molecular weight forms reported elsewhere for insects (Dauterman and others, 1962; Kunkee and Zweig, 1963), and the aggregates observed in this report, as being of any real structural or functional significance. We suggest that the 7·3*S* form described here from a mayfly and the honey bee, and as reported by Krysan and Chadwick (1966) from the housefly, is a fundamental unit of insect ChE. The insect enzyme is of lower molecular weight than the 260,000 molecular weight AChE recently described by Leuzinger, Goldberg, and Cauvin (1969) from *Electrophorus electricus*.

The basis for the effect of dialysis and *p*H on the aggregation state is not known. However, one can conclude that some dialysable feature affects the aggregation state directly and in concert with *p*H. That the 10·2*S* form is highly sensitive to *p*H change is shown in the observation that a change in *p*H of six-tenths of a unit will rapidly cause total deaggregation of the ChE in a dialysed preparation. This effect of *p*H is quite

different from that observed by Grafius and Millar (1967) with eel AChE; they saw a slight dissociation of fast (77*S* average) to slow (12*S* average) components as *p*H decreased from 10 to 5. Below *p*H 5 considerable AChE appeared in the pellet. Krysan and Chadwick (1970) observed that soluble housefly ChE (molecular weight 160,000) precipitated at *p*H 5. The rapid deaggregation and slow reaggregation, as induced here by *p*H change, is reminiscent of the kinetics for the same process on electric eel AChE as induced by ionic strength changes (Grafius and Millar, 1965).

While enzyme aggregation itself has been demonstrated to relate to function in several instances (reviewed by Markert and Whitt, 1968), the shift in sedimentation behaviour which we observed may be an artifact; however, it could reflect a *p*H-dependent conformational change in the enzyme, which change may relate to function. In this respect the observations, and speculations therefrom, by Podleski and Changeux (1967) are pertinent. They observed that isolated electroplax cells made insensitive to 'receptor activators' (e.g., decamethonium) are depolarized by the AChE (membrane-bound) caused hydrolysis of acetylcholine. They suggest that local *p*H changes resulting from acetylcholine hydrolysis influence 'diffusion barriers surrounding the AChE molecules integrated into the membrane structures'. Possibly a *p*H-induced perturbation of the enzyme molecule itself is a step in the event and the *p*H-dependent aggregation affect we observe is a consequence of that perturbation.

Also in this vein, it has been suggested (e.g., Zupancic, 1967) that AChE itself is the receptor molecule of the cholinergic system and Changeux (1966) has proposed that the enzyme acts as receptor via an allosteric change. On the assumption that an allosteric shift induced by an effector might be reflected in our system as a change in sedimentation behaviour, we tested four pharmacologic agents which would be expected to influence a receptor protein; of acetylcholine, pilocarpine, gallamine triethiodide (Flaxedil), and nicotine none affected the distribution of enzyme activity between the 10·2*S* and 7·3*S* isozymes in our standard preparation. These hypothetical schemes for the action of AChE in electrogenic membranes have been discussed elsewhere (Grafius, Friess, and Millar, 1968) and little can be added by further speculation on our part. Suffice it to say that we have observed a more discrete and less drastically induced deaggregation of ChE than has been previously reported.

With respect to these comparisons of insect ChE with vertebrate AChE, the following should be kept in mind. The choline-ester-hydrolysing activity of insects is generally confined to the nervous tissue (reviewed by Chadwick, 1963). Although homogenates and partially purified enzymes from insects, including the honey bee (Kunkee and Zweig, 1963) and a mayfly (Blackburn, 1967), have choline ester substrate specificity patterns somewhat different from those of mammalian nervous tissue ChE's, the activity here observed is probably the one associated with nervous function. Therefore, it quite likely has a fundamental functional role comparable to that of the more extensively studied AChE's of the electric eel and the mammalian brain.

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