with recurrent pregnancy disorders of unknown etiology indicates an altered production rate of DHT, but the biological significance of this phenomenon remains to be clarified.

1 Acknowledgment. The authors thank Prof. A. Dražančić, Mrs Lj. Vukelić and Mrs Z. Tomašković for their collaboration in this study. This study was supported by the Scientific Fund of SR Croatia (Zagreb), SIZ V.

2 K.G. Nygren, E.D.B. Johanson and L. Wide, Am. J. Obstet. Gynec. *116*, 916 (1973).
3 J. Kunz and P.J. Keller, Br. J. Obstet. Gynec. *83*, 640 (1976).
4 S.K. Yip and M.L. Sung, Fert. Steril. *28*, 151 (1977).
5 M.Y. Dawood and B.B. Saxena, Am. J. Obstet. Gynec. *129*, 37 (1977).
6 M. Gerenčer, A. Dražančić, I. Kuvačić, Z. Tomašković and A. Kaštelan, Fert. Steril. *31*, 401 (1979).
7 S. Furujama, D.M. Mayes and C.A. Nugent, Steroids *16*, 415 (1970).
8 M.A. Rivarola, M.G. Forest and C.J. Migeon, J. clin. Endocr. *28*, 34 (1968).
9 G.P. August, M. Tkachuk and M.M. Grumbach, J. clin. Endocr. *29*, 891 (1969).
10 J.M. Saez, M.G. Forest, A.M. Morera and J. Bertrand, J. clin. Invest. *51*, 1226 (1972).
11 T. Ito and R. Horton, J. clin. Endocr. *31*, 362 (1970).

Acetylcholinesterase activity in an *Aedes aegypti* cell line

E. Cohen[1]

Department of Entomology, The Hebrew University of Jerusalem, Faculty of Agriculture, P.O. Box 12, Rehovot (Israel), 8 September 1980

Summary. Considerable acetylcholinesterase (AChE) activity was detected in an *Aedes aegypti* established cell line. The enzyme is blocked by 10^{-6} M eserine sulfate, displays excess substrate inhibition and slowly hydrolyzes butyrylthiocholine. A 2-fold stimulation of AChE activity was shown after 2 days exposure to 3×10^{-7} M β-ecdysone. AChE activity found in the fresh medium is the contribution of the fetal calf serum portion. A direct relationship between levels of serum and the AChE activity in the cultured cells was demonstrated.

The search for an understanding of the mode of action of the molting hormone at the cellular and biochemical level involves studies using various in vitro systems[2-6], including established insect cell lines[7-12]. Depending on the cell system, it has been demonstrated that the hormone causes either an increase in cell multiplication rate[7,9] or an extensive growth arrest[9,12]. In general, the hormonal effect was accompanied by morphological alterations[10]. Decreased RNA and DNA synthesis as well as changes in the electrophoretic profile of proteins were also detected[13,14]. Cherbas et al.[15] were the first to report that physiological levels of β-ecdysone induced acetylcholinesterase (AChE) activity in Kc cell-line of *Drosophila melanogaster*. Subsequently other accounts of the same phenomenon of induction in *Drosophila* lines were published[14,16]. It is of great significance that specific, well known and thoroughly investigated enzymes are induced by an insect hormone. The implications might be far reaching concerning major questions in insect endocrinology. The phenomenon of AChE induction offers an approach to elucidate the mode of action of β-ecdysone at the molecular level with ramifications to hormone receptors and the complex biochemical events leading to expression of AChE gene(s).

This report presents evidence for the existence of AChE in a mosquito cell line and deals with the effect of β-ecdysone on the enzyme activity. The presence of high AChE activity in the medium is discussed with regard to the recent finding of enzymatic induction by ecdysone in *Drosophila* cell lines.

Materials and methods. *Aedes aegypti* cultured cells[17] were grown in a medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, N.Y.) in plastic flasks at 28 °C and subcultured weekly. For experimental purposes the cultured cells were maintained in disposable petri dishes (3.6 cm in diameter) and kept in a humidified 5% CO_2 atmosphere at 28 °C. To harvest the cells, the medium was removed and the culture was washed twice with a salt solution (0.1 M NaCl, 0.007 M KCl, 0.001 M Na HCO_3, 0.007 M KH_2PO_4, 0.0009 M $CaCl_2$). The washed cells were detached from the dish by vigorous pipetting and collected. The cell suspension was spun at $1000 \times g$ for 5 min and washed in the salt solution. After centrifugation the cell pellet was homogenized (Glass-teflon hand homogenizer) in 0.1 M pH-7.0 Na-phosphate buffer containing 0.5 M NaCl, 0.25 M EDTA and 0.5% (v/v) Triton X-100. The homogenate was kept on ice for 30 min and centrifuged at $10{,}000 \times g$ for 20 min. The supernatant served as a source of the enzyme. The assay procedure followed essentially that of Ellman et al.[18]. The reaction mixture in a final volume of 2.0 ml was composed of 1.95 ml 0.1 M, pH 8.0 Na-phosphate buffer, 0.5 mM either acetylthiocholine iodide or butyrylthiocholine iodide as substrates and 0.3 mM dithiobisnitrobenzoic acid (DTNB). After precalibration, the reaction was started by adding 50 µl of the enzyme extract. The absorbance at 412 nm was monitored continuously at 25 °C using Perkin-Elmer double beam spectrophotometer. Protein levels were determined by Lowry's method[19]. Enzyme specific activity is expressed as nmoles/min/mg protein.

β-Ecdysone (Rhoto pharmaceutical, Japan) was dissolved in absolute ethanol and 10 µl of the solution were added to cultures to give a final concentration of 3×10^{-7} M.

Results and discussion. High activity of AChE was detected in *Aedes aegypti* cultured cells (figure 1). It remains con-

Effect of β-ecdysone on AChE activity in *Aedes aegypti* cultured cells

Culture* (days)	β-Ecdysone treatment (h)	AChE activity (% of control)
2	24	134
2	48	194
3	48	199

* Culture age when β-ecdysone (final concentration of 3×10^{-7} M) was added. The cells were thoroughly washed, harvested, extracted and the AChE activity was assayed colorimetrically according to the procedure described by Ellman et al.[18].

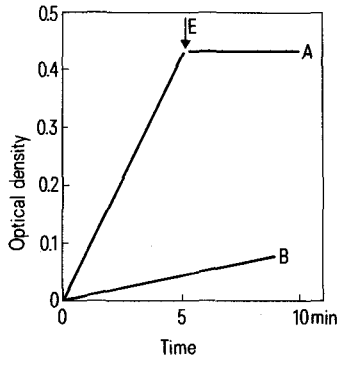

Fig. 1. Cholinesterase activity in *Aedes aegypti* cultured cells. 5-day-old cultures at the monolayer stage were thoroughly washed and the cells harvested, extracted and the AChE activity was assayed colorimetrically according to the procedure described by Ellman et al.[18]. The optical density of the reaction mixture was continuously monitored and recoreded at 412 nm. A, Acetylthiocholine iodide. Arrow indicates time of eserine sulfate (E) introduction. Concentration of the inhibitor was 10^{-6} M. B, Butyrylthiocholine iodide.

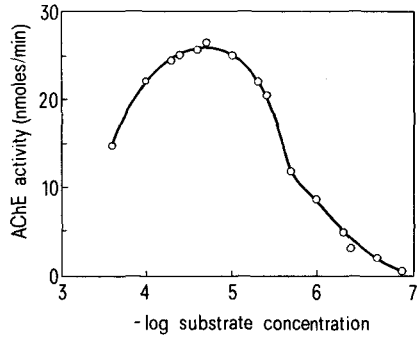

Fig. 2. Effect of substrate concentration on the activity of AChE in *Aedes aegypti* cultured cells. 5-day-old cultures at the monolayer stage were thoroughly washed and the cells harvested, extracted and the AChE activity was assayed colorimetrically according to the procedure described by Ellman et al.[18]. Values were calculated from the slope of the continuously-recorded AChE activity.

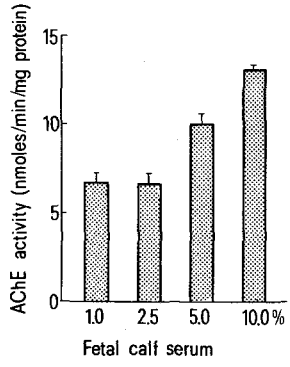

Fig. 3. Effect of levels of fetal calf serum on the AChE activity in *Aedes aegypti* cultured cells. Inoculum was washed 5 times with salt solution and transferred to media with the indicated levels of fetal calf serum. 5-day-old cultures were thoroughly washed and the cells harvested, extracted and the AChE activity was assayed colorimetrically according to the procedure described by Ellman et al.[18]. Values were calculated from the slope of the continuously recorded AChE activity. Numbers indicate percent of fetal calf serum and bars indicate SE. AChE in fetal calf serum hydrolyzes acetylthiocholine and butyrylthiocholine at a rate of 20.3 and 0.7 nmoles/min/mg protein, respectively.

stant throughout the life cycle of normal cultures. In this respect, the mosquito cells resemble 'AChE constitutive' Ca cells of *Drosophila* which display enzyme activity in the absence of β-ecdysone[20]. AChE activity, which is linear up to 45 min, is blocked by 10^{-6} M eserine sulfate. Compared with acetylthiocholine, butyrylthiocholine is hydrolyzed very slowly (about 6-fold less) by the enzyme extract (figure 1). Further indication that the enzyme is a true AChE is based on its inhibition by excess of substrate (figure 2). Unlike the Ca *Drosophila* line, exposure of the mosquito cultured cells to 3×10^{-7} M β-ecdysone for 48 h resulted in a 2-fold increase in AChE activity (table). Stimulatory effects can be observed 24 h after the hormonal treatment. The above time response corresponds with the morphological and biochemical changes induced by the hormone[12,13]. Similar observations have been reported on other insect cell lines exposed to the molting hormone[10,14]. Recently, it has been demonstrated that AChE and β-galactosidase activities are stimulated in several lines of *D. melanogaster* by β-ecdysone[15,16,20]. No clear physiological function can be assigned to both enzymes. Based on morphological changes in hormonally-treated *Drosophila* cultured cells, it was suggested that the corresponding lines had been established from embryonic nervous tissues[15]. It appears that morphological criteria are insufficient. *A. aegypti* and *Drosophila* line 2 cells[14] are not neural-like, although they have considerable AChE activity. This point can be clarified by studies with specific biochemical markers and by applying electrophysiological methods. Nevertheless, the induction of specific and well recognized enzymes is of fundamental importance to elucidate the mode of action of a major insect growth hormone at the cellular level. It was surprising to detect high levels of AChE activity in the fresh medium, where the fetal calf serum is the source of the enzyme. This raises the possibility that in *A. aegypti* and also in *Drosphila* lines the detected AChE activity can be attributed, at least in part, to absorption of the enzyme from serum-containing media. This is apparently not the case with the *Drosophila* Kc0% line which is adapted to develop in serum-free medium, and in which AChE is induced by the molting hormone[20]. The level of AChE activity in *A. aegypti* cells is correlated with the amount of serum present in the medium (figure 3). Medium containing 1.0% serum still supports normal cell growth. To minimize adhering serum proteins, the cells used for the inoculum were washed 5 times with the salt solution. The AChE specific activity measured in cultures maintained at 1.0% and 2.0% serum was about 7 nmoles/min/mg protein. When 5.0% and 10.0% serum were used, the activity increased to 10 and 13 nmoles/min/mg protein respectively. β-Ecdysone seems to affect cell membranes either directly or indirectly[13]. Kambysellis and Williams[5] suggested that spermatogenesis in diapausing silk moth pupae proceeds following alteration in the testicular membranes caused by the molting hormone. This allows the penetration of a stimulatory 'macromolecular factor', presumably a protein. It is conceivable that in the case of insect cell lines, β-ecdysone might stimulate uptake of proteins from the medium including growth factors and presumably ACHhE molecules.

1 Acknowledgment. I wish to thank Dr J. Peleg of the Israel Institute for Biological Research for providing the starting culture of *Aedes aegypti* cells.

2 E.P. Marks and R.A. Leopold, Science *167*, 61 (1970).
3 M. Ashburner, Nature New Biol. *230*, 222 (1971).
4 J.W. Fristrom, in: Biology of Imaginal Disks, p. 109. Ed. H. Ursprung and R. Nothiger. Springer, Berlin 1972.

5 M.P. Kambysellis and C.M. Williams, Biol. Bull. *141*, 541 (1971).
6 E. Cohen and L.I. Gilbert, Insect Biochem. *4*, 75 (1974).
7 J. Mitsuhashi and T.D.C. Grace, Appl. ent. Zool. *5*, 182 (1970).
8 E. Cohen and L.I. Gilbert, J. Insect Physiol. *18*, 1061 (1972).
9 A.M. Courgeon, Nature New Biol. *238*, 250 (1972).
10 A.M. Courgeon, Expl Cell Res. *94*, 283 (1975).
11 E.P. Marks and M. Holman, In Vitro *15*, 300 (1979).
12 E. Cohen, N. Lanir and E. Englander, Insect Biochem. *6*, 433 (1976).
13 N. Lanir and E. Cohen, J. Insect Physiol. *24*, 613 (1978).
14 E. Berger, R. Ringler, S. Alahiotis and M. Frank, Devl Biol. *62*, 498 (1978).
15 P. Cherbas, L. Cherbas and C.M. Williams, Science *197*, 275 (1977).
16 M. Best-Belpomme, A.M. Courgeon and A. Rambach, Proc. natl Acad. Sci. USA *75*, 6102 (1978).
17 J. Peleg, J. gen. Virol. *5*, 463 (1969).
18 G.L. Ellman, K.D. Courtney, V. Andres, Jr, and R.M. Fleatherstone, Biochem. Pharmac. *7*, 88 (1961).
19 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
20 M. Best-Belpomme and A.M. Courgeon, FEBS Lett. *82*, 345 (1977).

Insulin effect in vitro on human erythrocyte plasma membrane[1]

P. Luly, P. Baldini, S. Incerpi and E. Tria

Institute of General Physiology, University of Rome, I-00185 Rome (Italy), 15 August 1980

Summary. The effect of porcine insulin has been tested in vitro on human erythrocyte plasma membrane (Na^+-K^+) and Mg^{2+}-ATPase activities as well as on membrane fluidity. The results indicate that the hormonal treatment significantly inhibits (Na^+-K^+)-ATPase activity, and at the same time decreases membrane fluidity.

The presence of a specific insulin receptor on the human erythrocyte plasma membrane has recently been reported[2]. The physiological relevance of this observation is not yet clear, even if altered binding capacities observed in red cells from diabetic patients could be related in some way to metabolic defects[3]. The effect of insulin on isolated mammalian plasma membranes in relation to membrane-bound enzymes has been reported rather extensively[4-9], and very recently a direct action of the hormone on the liver plasma membrane microenvironment has been shown[10,11]. This body of evidences prompted us to study the effect of insulin in vitro on human erythrocyte membrane-bound Mg^{2+}-ATPase (E.C.3.6.1.3) and (Na^+-K^+)-ATPase (E.C.3.6.1.4) activities as well as a possible hormonal action on plasma membrane fluidity.

Materials and methods. Human erythrocyte plasma membranes were prepared from fresh blood, obtained from healthy adult male volunteers, according to Hanahan[12].

Mg^{2+}-ATPase and (Na^+-K^+)-ATPase activities were measured as previously reported[7] in a final volume of 2.2 ml containing the following; about 100 µg membrane proteins, 92 mM Tris-HCl buffer (pH 7.5), 5 mM $MgSO_4$, 5 mM KCl, 60 mM NaCl, 0.1 mM EDTA and 0.1 mM ouabain when employed; ATP concentration was 4 mM.

Fluorescence labeling of human erythrocyte plasma membranes was carried out as follows; $2 \cdot 10^{-3}$M 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran was diluted 1:1000 just before use with the hypotonic Tris buffer used during membrane isolation, then mixed in a 1:1 ratio with the membrane suspension to give a final protein concentration of 50 µg/ml. After 15 min of incubation at 37 °C fluorescence polarization measurements were carried out as recently reported[13] with an Aminco Bowman spectrophotofluorometer equipped with 2 Glan prism polarizers; excitation was set at 366 nm and emission was recorded at 430 nm. The temperature of the sample was checked within ±0.1 °C with a thermistor thermometer.

The degree of fluorescence polarization, P, was calculated from the equation:

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} = \frac{(I_\parallel / I_\perp) - 1}{(I_\parallel / I_\perp) + 1}$$

where $I_\parallel$ and $I_\perp$ are the fluorescence intensities recorded with the analyzing polarizer oriented, respectively, parallel and perpendicular to the direction of the polarized excitation beam[14].

Porcine insulin was denatured according to Loten and Sneyd[4]. Proteins were estimated by the method of Lowry et al.[15] using bovine serum albumin as a standard. Porcine insulin was from Sigma, St.Louis, Mo., USA; porcine proinsulin was obtained from Lilly Research Laboratories, Indianapolis, Ind. USA; ATP was from Boehringer, Mannheim, FRG; 1,6-diphenyl-1,3,5-hexatriene was from Fluka AG, Buchs, Switzerland; all other chemicals were analytical reagent grade from Merck-Darmstadt, FRG.

Table 1. Insulin effect on (Na^+-K^+)-ATPase, Mg^{2+}-ATPase and fluorescence polarization (P at 37 °C) of human erythrocyte plasma membrane

Preincubation time (min)	(Na^+-K^+)-ATPase		Mg^{2+}-ATPase		Fluorescence polarization (P)	
	− Insulin	+ Insulin	− Insulin	+ Insulin	− Insulin	+ Insulin
0	0.62±0.15	0.60±0.16	3.20±0.30	3.40±0.40	0.268±0.008	0.270±0.006
30	0.58±0.12	0.40±0.07[a,b]	3.25±0.28	3.25±0.24	0.269±0.007	0.286±0.004[a,b]
60	0.55±0.14	0.35±0.05[a,b]	3.15±0.23	3.30±0.25	0.272±0.006	0.289±0.005[a,b]
120	0.50±0.10	0.27±0.06[a,b]	3.00±0.19	3.19±0.18	0.270±0.009	0.290±0.007[a,b]
180	0.57±0.11	0.25±0.04[a,b]	2.95±0.16	3.14±0.20	0.274±0.005	0.294±0.006[a,b]

Erythrocyte membranes were preincubated at 37 °C for the time indicated in the presence or absence of $1 \cdot 10^{-9}$ M porcine insulin, before starting the reaction by adding the substrate or measuring the P value. Enzyme activity is reported as µmoles P_i/mg protein per 5 min; results are means ±SD of 6 different membrane preparations. $p < 0.05$, at least, [a] with respect to untreated controls, or [b] with respect to zero time, as assessed by Student's t-test.