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Effect of pH on *Blepharisma intermedium*. 5. Studies on Esterases

Synopsis. Esterases of *Blepharisma intermedium* grown in buffered media of different pH values and in unbuffered media have been characterized. Selective inhibition studies indicate six carboxylesterases, two arylesterases and one cholinesterase in all cases. Although the type and number of the enzymes remain constant, there are variations in their inhibitor sensitivities.

Esterases of ciliated protozoa have been studied to a considerable extent. These enzymes are said to have a catabolic role in vacuolar digestion (Parker 1968) and their presence seems to play a role in ciliary movement (Seaman and Houlihan 1951). It is also postulated that they are involved in synthetic reactions in ectoplasmic structures (Parker 1968).

Much of the work that has been carried out on the esterases of protozoa is on *Tetrahymena*. A number of nonspecific esterases have been shown to be associated with cytoplasmic granules and food vacuoles of *Tetrahymena* (Allen 1958). The esterase activity was found to increase with the aging of the culture in *Tetrahymena pyriformis* (Fennell and Pastor 1958, Koehler and Fennell 1964), and the distribution pattern of the aliesterases has been shown to depend on the stage of the growth cycle (Allen 1958, Koehler and Fennell 1964). Koehler and Fennell (1964) have used specific esterase inhibitors to separate and identify esterases of *Tetrahymena* by means of starch gel electrophoretic technique.

There are not many reports on the esterases of *Blepharisma*. Nonspecific esterases have been demonstrated by cytochemical methods in the infraciliary complex and cytoplasm of whole and regenerating cells of *Blepharisma intermedium* by Parker (1968). He has also reported the presence of lipase activity in the organism.

Esterases from various sources have been characterized by using differential inhibitor sensitivities (Bersohn et al. 1966, Forster et al. 1959 and Myers et al. 1957). This technique, which has not so far

been employed in the study of esterases of protozoans is employed to characterize the esterases of *B. intermedium*.

Material and Methods

Organisms grown in hay infusion media, and in citrate (pH 5.0 and 6.0) and acetate buffered media (pH 5.0 and 5.6) were used in the present investigation. Culture conditions were as described in our earlier communications (Shadaksharaswamy and Jyothy 1973, Kasturi Bai et al. 1969). Organisms in unbuffered hay infusion media are referred to as control in the text.

Enzyme Preparation

Organisms grown under the various growth conditions were harvested by low speed centrifugation (275 × g). The pellet obtained was repeatedly washed with distilled water. The packed organisms were homogenized in distilled water in an all glass Potter-Elvehjem type homogenizer surrounded by ice and salt mixture. The homogenate was centrifuged at 18000 × g for 30 min. Protein determinations were made on the supernatant by the method of Lowry et al. (1951) and it was diluted suitably and used for assay. The enzyme preparation was used within 24 h although it was stable for over a week at 0°C.

Substrates and Inhibitors

The substrates used were 1-naphthyl esters of acetate (NA), propionate (NP) and butyrate (NB), obtained from Sigma. The inhibitors dichlorvos (2,2-dichloro--vinyl-dimethyl-phosphate), parathion (diethyl p-nitrophenyl thiophosphate) and carbaryl (1-naphthyl N-methyl carbamate) were purchased from Chemservice Corporation. Eserine sulphate was got from Sigma and PCMB (p-chloro mercuribenzoate) from Fluka.

Assay Procedure

Esterase activity was measured quantitatively by the colorimetric technique of Gomori (1953) as modified by van Asperen (1962). A stock solution of the substrate (3×10^{-2} M) was prepared in acetone. This was buffered with 0.034 M phosphate buffer at pH 7.0 by diluting 1.0 ml of the stock solution to 100 ml with the buffer, to give a substrate concentration of 3×10^{-4} M 0.2 ml of the enzyme was incubated with 1.0 ml of the inhibitor for 30 min at 25°C. To this 5.0 ml of the buffered substrate was added and the tubes were incubated at the same temperature for 30 min. At the end of the incubation period 1.0 ml of the colour reagent (a mixture of 5% sodium lauryl sulphate and 1% diazo blue B in the ratio 2.5 : 1) was then added both to stop the reaction and for the development of colour. The tubes were allowed to stand for 15 min and the optical density was determined at 600 nm.

To determine the optimum pH, the following buffers of 0.034 M concentration were used: acetate, phosphate and pyrophosphate. The pH range obtained was 5.0 to 9.0. 1-naphthyl acetate was used as the substrate in these experiments.

Results

Substrate Specificity

Activities of the esterases of *B. intermedium* towards the three 1-naphthyl esters used in this study and their K_m values are given in Table 1. The activities are expressed as µg 1-naphthol/100 mg protein/ minute. NB was hydrolyzed at a higher rate than NP and NA.

Table 1

Hydrolysis of 1-naphthyl Esters by *Blepharisma intermedium* Homogenate

Substrate	Activity Units*/100mg protein	K_m (M)
1-naphthyl acetate	13.24	4.0×10^{-4}
1-naphthyl propionate	14.90	3.62×10^{-4}
1-naphthyl butyrate	18.48	2.94×10^{-4}

* 1 unit of activity is 10 µg product/minute.

Inhibitor Specificity

Inhibition patterns of the esterases of *B. intermedium* have been presented in Figs. 1 *1–5*. Plots of pI (negative $\log_{10}$ of M inhibitor concentration) vs. percent inhibition have been used to differentiate esterases (Aldridge 1953, Carino and Montgomery 1968, Montgomery et al. 1968 and Norgaard and Montgomery 1968). These plots enable to differentiate esterases which have different inhibitor sensitivities and are present in the same preparation hydrolyzing the same substrate. Myers et al. (1957) in their work on the esterases of mycobacteria obtained double sigmoid curves suggesting the presence of two esterases, which had different inhibitor sensitivities but hydrolyzing the same substrate. In these curves a plateau was reached where increasing amounts of inhibitor had little effect until sufficient inhibitor was present to start inhibiting the least sensitive esterase. However, enzymes with similar inhibitor and substrate specificities cannot be differentiated by this method.

Figure 1 *1*(a–e) shows the effect of inhibitors between pI values 2 to 8 on the esterase activity of *B. intermedium* grown in unbuffered media (control). With eserine sulphate, concentration corresponding to pI 2 could not be used because of its interference with the colour development. The dichlorvos inhibition curve (Fig. 1 *1* a) shows the presence of at least

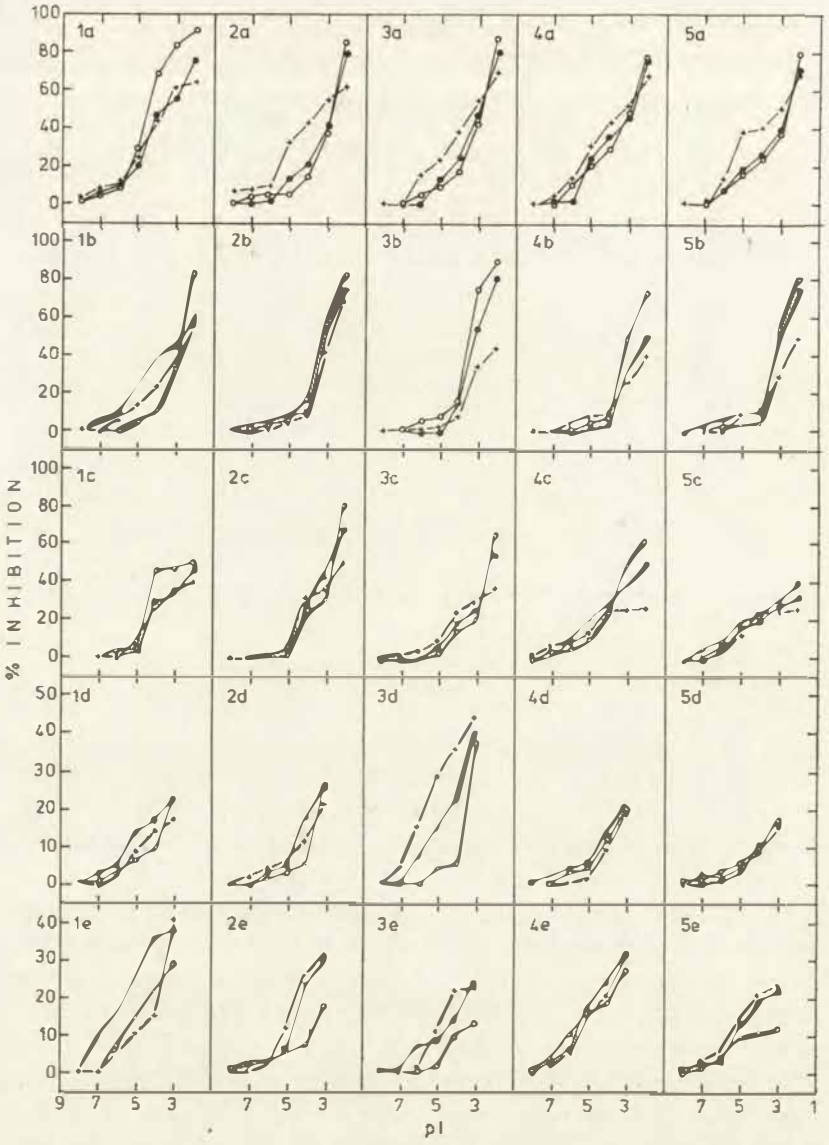

Fig. 1—5. *1* — Inhibition curves for the hydrolysis of 1-naphthyl esters by homogenate of *B. intermedium* (control) *2* — Inhibition curves for the hydrolysis of 1-naphthyl esters by homogenate of *B. intermedium* grown in acetate buffered media, pH 5.0 *3* — Inhibition curves for the hydrolysis of 1-naphthyl esters by homogenate of *B. intermedium* grown in acetate buffered media, pH 5.6. *4* — Inhibition curves for the hydrolysis of 1-naphthyl esters by homogenate of *B. intermedium* grown in citrate buffered media, pH 5.0. *5* — Inhibition curves for the hydrolysis of 1-naphthyl esters by homogenate of *B. intermedium* grown in citrate buffered media, pH 6.0.

Common to Figures 1—5: (a) — Inhibition with dichlorvos, (b) — Inhibition with parathion, (c) — Inhibition with PCMB, (d) — Inhibition with eserine sulphate, (e) — Inhibition with carbaryl. Percent inhibition is plotted against the negative $\log_{10}$ of the molar inhibitor concentration (pI), x- - -x — 1-naphthyl acetate, ●- - -● 1-naphthyl propionate, o- - -o 1-naphthyl butyrate

four enzymes. There is one enzyme between pI 4 and 6 accounting for about 64% NB hydrolyzing activity. The second enzyme is indicated between pI 3 and 4. The difference in inhibition between pI 2 and 3 among the three substrates indicates the presence of the third esterase. This enzyme is more active towards NP. The presence of considerable activity at pI 2 indicates a fourth enzyme which could be classified as a dichlorvos resistant enzyme.

The inhibition pattern with parathion is shown in Fig. 1 *1* b. This also suggests the presence of four enzymes. One between pI 4 and 6, the second between pI 3 and 4 and a third between pI 2 and 3. The second and the third enzymes have greater NB hydrolyzing activity, unlike the first which is more active towards NP. The fourth enzyme indicated at pI 2 is more resistant to parathion than dichlorvos.

The inhibition pattern with PCMB is shown in Fig. 1 *1* c. The enzyme between pI 4 and 6 shows preferential NB hydrolyzing activity and the second enzyme between pI 2 and 4 hydrolyses NP and NA to a greater extent.

Figures 1 *1* d and e show the inhibition pattern with eserine sulphate and carbaryl respectively. Both Figures indicate the presence of two enzymes. One between pI 3 and 4 and the other between pI 4 and 8. The extent of inhibition with both these carbamates is lower when compared with the other two inhibitors. Figure 1 *2*(a–e) show inhibition pattern of the esterases of the organisms grown in acetate buffered media of pH 5.0. Inhibition with different concentrations of dichlorvos (Fig. 1 *2* a) reveals four enzymes. The enzyme between pI 4 and 6 is more active towards NA and the activity decreases with NP and NB. This order of activity changes with the enzyme indicated between pI 3 and 4 (NB > NP > NA). Between pI 2 and 3 the hydrolysis of NB and NP is sensitive to inhibition but that of NA is quite resistant. A resistant enzyme at pI 2 indicates the fourth enzyme. The data presented in Fig. 1 *2* b shows that the major esterase activity is inhibited between pI 2 and 4. Two esterases are indicated in this region, one between pI 2 and 3 another between pI 3 and 4. The third esterase was inhibited between pI 4 and 6. Similar to control there can be a resistant enzyme at pI 2, as nearly 20% activity is not inhibited. The PCMB inhibition pattern in Fig. 1 *2* c shows the presence of two enzymes. The enzyme between pI 4 and 6 is equally active towards all the three naphthyl esters and a second enzyme between pI 2 and 3 has greater NB hydrolyzing activity. Figures 1 *2* d, e show the presence of two enzymes, between pI 3 and 8 of the carbamates.

Dichlorvos inhibition pattern of the esterases of the organism grown in acetate buffered media, pH 5.6, is given in Fig. 1 *3* a, indicating the

presence of four enzymes between the same pI values. Figure 1 *3* b also reveals four enzymes: Three between pI 2 and 6 and another at pI 2. PCMB inhibition pattern (Fig. 1 *3* c) shows one enzyme between pI 4 and 6 and another between pI 2 and 3. The pattern with carbamates (Fig. 1 *3* d, e) show two enzymes each, between pI values 4 and 8 and 3 and 4.

The number of enzymes indicated in the inhibition pattern of the organisms grown in citrate buffered media pH 5.0 and 6.0 (Figs. 1 *4* a–e and *5* a–e) are the same as those obtained with organisms grown in acetate buffers, except in the case of eserine sulphate.

Discussion

Esterases consist of a heterogeneous family of enzymes. Several workers (Augustinsson 1961, Guss and Krysan 1972, Mounter and Whittaker 1953 and Sinanuvong et al. 1971) have used selective inhibition by organophosphates and carbamates as a criteria to classify these hydrolases as: carboxylesterases, arylesterases and cholinesterases. Carboxylesterases (carboxylester hydrolases EC 3.1.1.1), formerly known as aliesterases or B-esterases, hydrolyze both aliphatic and aryl esters but not choline esters. They are inhibited by most organophosphorus inhibitors but not by carbamates. Arylesterases (arylester hydrolases EC 3.1.1.2) formerly known as A-esterases, are not inhibited by organophosphates or carbamates but are inhibited by PCMB. These enzymes hydrolyze aromatic esters, but normally not aliphatic esters. Cholinesterases (acetylcholine acetyl hydrolases EC 3.1.1.7 and acylcholine acyl hydrolases EC 3.1.1.8) are inhibited by both organophosphates and carbamate inhibitors.

Animal (Augustinsson 1961 and Forster et al. 1959), plant (Carino and Montgomery 1968, Norgaard and Montgomery 1968), insect (Sinanuvong et al. 1971) and protozoal (Koehler and Fennell 1964) esterases have been studied with regard to substrate and inhibitor specificities. Employing the same techniques, the present studies have revealed the existence of carboxyl, aryl and cholinesterases in *B. intermedium* grown under different pH conditions.

Comparison of the inhibition pattern obtained with the organophosphorus inhibitors (dichlorvos and parathion) reveals the presence of six enzymes in the organisms grown in unbuffered media (Fig. 1 *1* a, b). Three enzymes are shown to be present between the pI values 2 and 3, 3 and 4 and 4 and 6 in the dichlorvos inhibition pattern. These three

enzymes are different from those that are inhibited by parathion at the same concentrations. If the same enzyme were inhibited, then the percent inhibition at the plateau would be expected to be the same. At pI 4 it can be seen that there is greater inhibition (68%) of the butyryl ester with dichlorvos than with parathion (10%). This shows the presence of two enzymes in the region pI 4 and 6 which have different inhibitor sensitivities. The inhibition between pI values 3 and 4 is also not due to the same enzyme. Dichlorvos inhibits the hydrolysis of the three substrates to the same extent, while this is not so with parathion. Between pI 2 and 3, there is little inhibition with dichlorvos whereas there is nearly 50% inhibition with parathion of N_B hydrolysis indicating that the two enzymes are different. These six enzymes inhibited by the two organophosphates can be classified as carboxylesterases.

The same number of carboxylesterases are shown to be present in the organisms grown under different buffered conditions. However, they differ in their inhibitor sensitivities. For example, the inhibition of the NB hydrolyzing activity with dichlorvos between pI 2 and 3 is more than 30% under the buffered conditions while it is only 7% in the control. On the other hand, with parathion the extent of inhibition is less in buffered media compared to control. Such differences are also noticed between pI 3 and 4 and more distinctly between pI values 4 and 6. Further, it is observed that between pI 4 and 6, the enzymes of the organisms grown in buffered media are quite resistant to parathion unlike what is observed in the control.

Inhibition sensitivities of the esterases of the organisms grown at pH 5.0 differs from those grown at higher pH's in acetate and citrate buffered media. In the case of organisms grown in acetate buffered media, pH 5.6, the enzymes are inhibited to a greater extent by parathion (pI 3 and 4) than in those grown at pH 5.0. Similar effects are also noticed with the organisms grown in citrate buffered media of pH 5.0 and 6.0.

The absence of complete inhibition of esterases of organisms grown under different conditions at pI 2 with both organophosphates indicates the presence of esterolytic activity. Augustinsson (1961) has reported the presence of arylesterases which are inhibited by PCMB but not by organophosphates. The esterolytic activity noticed at pI 2 may be due to the presence of arylesterases. In the case of pea esterases, though there is inhibition by PCMB, Montgomery et al. (1968) have stated that arylesterases are absent because of the complete inhibition of the enzymes by organophosphates. They attribute the action of PCMB to non-specific reaction of this inhibitor with essential -SH groups. However, in the present investigation since the enzymes are not

completely inhibited by organophosphates and there is considerable inhibition with PCMB (80% in acetate buffer) the presence of arylesterases is apparent. The inhibition pattern with PCMB indicates the presence of two arylesterases in all cases, though there are some differences in the extent of inhibition between the buffered and unbuffered conditions.

A comparison of the inhibition pattern obtained with the carbamates (eserine sulphate and carbaryl) reveals the presence of two enzymes, one between pI 4 and 8 and the other between 3 and 4, under the various conditions of investigation. As cholinesterases are generally inhibited by eserine suphate at concentrations of 10^{-5} M and lower (Krisch 1971), the inhibition noticed between pI 3 and 4 may not be that of cholinesterase. Sudderuddin (1972, 1973) in his work on the esterases of the green peach aphid has shown that 10^{-7} M eserine sulphate blocks cholinesterase activity completely and therefore higher concentrations of the carbamates must be inhibiting carboxylesterases. Therefore the enzyme inhibited between pI 3 and 4 may be a carboxylesterase and the other inhibited between pI 4 and 8 is a cholinesterase although its activity with the three substrates is low.

Blepharisma intermedium therefore contains a complex system of ester hydrolases. Under all chosen conditions six carboxylesterases, two arylesterases and one cholinesterase are indicated. Although the type and number of esterases remain constant, there are variations in the inhibitor sensitivities of these enzymes. Substrate specificity studies have also shown that NB is preferentially hydrolyzed. Investigations on insect esterases carried out in our laboratory and with pea esterases (Norgaard and Montgomery 1968), have shown that propionyl esters are hydrolyzed at a greater rate than acetyl and butyryl esters.

In our studies on the effect of pH on *B. intermedium* (Shadaksharaswamy and Jyothy 1973 a, b, 1976) it has been shown that pH of the environment has considerable influence on some macromolecular constituents and activity of some enzymes. It has been pointed out that the changes noticed have been due to the differences in the rate and extent of transport of various buffer components of the medium across the cell membrane. However, the existence of the same number of different types of esterases in the organisms grown under varying conditions indicates their metabolic importance.

ZUSAMMENFASSUNG

Es wurden Esterasen von *Blepharisma intermedium*, die in Puffermedien mit verschiedenen pH-Werten sowie in nichtgepufferten Medien gezuchtet wurden, charakterisiert. Aus Untersuchungen der selektiven Inhibition ergaben sich stets

sechs Karboxylesterasen, zwei Arylesterasen und eine Cholinesterase. Obwohl Art und Zahl der Enzyme konstant bleiben, sind die Inhibitor-Empfindlichkeiten veranderlich.

REFERENCES

Allen S. L. 1958: Cytochemical localization of enzymes in sexual strains of the Protozoan, *Tetrahymena pyriformis*. Anat. Rec., 131, 526–527.

Aldridge W. N. 1953: Serum esterases. 1. Two types of esterases (A and B) hydrolyzing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. Biochem. J., 53, 110–117.

van Asperen K. 1962: A study of housefly esterases by means of a sensitive colourimetric method. J. Insect Physiol., 8, 401–416.

Augustinsson K. B. 1961: Multiple forms of esterase in vertebrate blood plasma. Ann. N. Y. Acad. Sci., 94, 844.

Bersohn J., Barron K. D., Doolin P. E., Hess A. R. and Hedrick M. J. 1966: Sub-cellular localization of rat brain esterases. J. Histochem. Cytochem., 14, 455.

Carino L. A. and Montgomery M. W. 1968: Identification of some soluble esterases of the carrot (*Daucus carota* L.). Phytochem., 7, 1483-1490.

Fennell R. A. and Pastor E. P. 1958: Some observations on the esterases of *Tetrahymena pyriformis* W. I. Evidence for the existence of aliesterases. J. Morph., 103, 187–202.

Forster T. L., Bendixen H. A. and Montgomery M. W. 1959: Some esterases in cow milk. J. Dairy Sci., 42. 1903-1912.

Gomori G. 1953: Human esterases. J. Lab. Clin. Med., 42, 445–453.

Guss P. L. and Krysan J. L. 1972: Esterases and the identification of Lipases from eggs of *Diabrotica undecimpuntata* Howardi and *D. virgifera*. J. Insect. Physiol., 18, 1181–1195.

Kasturi Bai A. R., Srihari K., Shadaksharaswamy M. and Jyothy P. S. 1969: The effects of temperature on *Blepharisma intermedium*. J. Protozool., 16, 738–743.

Koehler L. D. and Fennell R. A. 1964: Histochemistry and Biochemistry of the polysaccharides, esterases and dehydrogenases of *Tetrahymena pyriformis* (Strain W.) J. Morph., 114, 209-223.

Krisch K. 1971: The Enzymes. (ed. Boyer P. D.) Academic Press Inc., New York and London, 5, 43.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. 1951: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265–275.

Montgomery M. W., Norgaard M. J. and Veerabhadrappa P. S. 1968: Purification and substrate and inhibitor specificities of carboxylesterases of the pea (*Pisum sativum* L). Biochim. Biophys. Acta 167, 567–574.

Mounter L. A. and Whittaker V. P. 1953: Hydrolysis of esters of phenol by cholinesterases and other esterases. Biochem. J., 54, 551–559.

Myers D. K., Tol T. N. and De Jongc M. H. T. 1957: Aliesterases VI. Selective inhibitors of the esterases of brain and saprophytic mycobacteria. Biochem. J., 65, 232–241.

Norgaard M. J. and Montgomery M. W. 1968: Some esterases of the pea (*Pisum sativum* L). Biochim. Biophys. Acta 151, 587–596.

Parker J. W. 1968: A cytochemical investigation of the nuclear and enzymatic activities. Ph. D. Thesis, Stanford University, U.S.A.

Shadaksharaswamy M. and Jyothy P. S. 1973 a: Effect of pH on *Blepharisma intermedium* 1. Changes in fission rate and oxygen consumption. Acta Protozool., 12, 117–124.

Shadaksharaswamy M. and Jyothy P. S. 1973 a: Effect of pH on *Blepharisma intermedium* 2. Cytochemical changes. Ann. Histochim. 18, 149–158.

Shadaksharaswamy M. and Jyothy P. S. 1976: Effect of pH on *Blep-*

harisma intermedium 3. Changes in the levels of some macromolecules, free amino acid and protein pattern. Acta Protozool., 15, 57–65.

Seaman G. R. and Houlihan R. K. 1951: Enzyme systems in *Tetrahymena geleii* S: II. Acetylcholinesterase activity. Its relation to motility of the organism and the coordinated ciliary action in general. J. Cell Comp. Physiol., 37, 309.

Sinanuvong C., Knowles C. O. and Kearby W. H. 1971: Electrophoretic studies of certain hydrolases from the smaller European elm bark beetle *Scolytus multistriatus*. J. Kans. Entomol. Soc., 44, 408–413.

Sudderuddin K. I. 1972: Some biochemical and toxicological studies of organophosphate resistance in *Myzus persicae* Sulz. Ph. D. Thesis, University of London.

Sudderuddin K. I. 1973: An in vitro study of esterases, hydrolyzing nonspecific substrates, of an organophosphate resistant strain of the green peach aphid. *Myzus persicae* (Sulz). Comp. Biochem. Physiol., 44 B, 1067.

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