BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 180–185 (1998)
ARTICLE NO. RC988931

Kinetics of Human Erythrocyte Acetylcholinesterase Inhibition by a Novel Derivative of Physostigmine: Phenserine

Abdulaziz A. Al-Jafari,*[,1] Mohammad A. Kamal,* Nigel H. Greig,† Abdullah S. Alhomida,* and Elaine R. Perry‡

**Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia; †Drug Design & Development, National Institute of Aging, National Institute of Health, Baltimore, Maryland 21224; and ‡MRC Neurochemical Pathology Unit, Newcastle General Hospital, Newcastle upon tyne NE4 6BE. United Kingdom*

Received June 5, 1998

The effect of phenserine, a novel cholinesterase inhibitor, was assessed for the first time on kinetic parameters of human erythrocyte acetylcholinesterase (AChE). Phenserine (0.025-0.40 μM) inhibited the activity of human erythrocyte AChE in a concentration-dependent fashion, the IC_{50} was 0.0453 μM. The Michaelis-Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide was found to be 0.124 mM and the V_{max} was 0.980 μmol/min/mg protein. Dixon as well as Lineweaver-Burk plots and their secondary replots indicated that the nature of the inhibition was of the noncompetitive type. The value of K_i was estimated as 0.048 μM by the primary and secondary replots of the Dixon as well as secondary replots of the Lineweaver-Burk plot. A novel relationship between K_i and substrate concentration was also identified which permits more precise prediction of the specific type of noncompetitive inhibition of various enzymes by a wide variety of drugs, chemicals and, in some circumstances, by their own substrates.

As a consequence of the early cholinergic deficit in the Alzheimer brain and the intimate involvement of the cholinergic system in learning and cognitive performance, both reversible and irreversible cholinesterase inhibitors are currently being used in the treatment of Alzheimer's disease (AD). The postulated mechanism to account for the efficacy of this class of drugs is their restoration of activity at cholinergic synaptic sites within the Alzheimer brain by inhibition of acetylcholinesterase (AChE, 3.1.1.7) (1) which attenuates the disease-associated cognitive deficits and memory impairment (2). To this end, several cholinesterase inhibitors have been the subject of both animal and human investigation. The alkaloid physostigmine was the first AChE inhibitor to be tested in AD patients (3) and has since been the subject of various clinical trials (4). Physostigmine, like other first- and second-generation cholinesterase inhibitors, suffers from number of handicaps as a drug. These include a short duration of biological action, low bioavailability, non-selective action between AChE and butyrylcholinesterase (BChE, 3.1.1.8) inhibition and a narrow therapeutic window. A slow-release oral formulation of the drug, Synapton (Forest, USA), is presently in phase III clinical trials for AD treatment and provides the compound and extended action. To overcome the multitude of serious handicaps associated with presently available anticholinesterases, a novel long-acting, brain-directed and AChE selective inhibitor, phenserine, a phenylcarbamate derivative of physostigmine (5), was developed. Phenserine has an highly favorable toxicological profile, interferes with several of the molecular processes involved in AD development and, additionally, possesses an unusually wide therapeutic window to improve cognitive performance in animal models (6, 7). The agent is just entering clinical trials and is predicted to have high activity and minimal toxicity in the treatment of AD and age-associated memory deficit. The rational for investigating anticholinesterases as drugs for AD treatment is based on their ability to reduce the hydrolysis of the neurotransmitter acetylcholine (ACh) released from surviving nerve terminals, and hence prolong and amplify its pharmacological ac-

[1] Corresponding author: Department of Biochemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia. Fax: 966-1-467-5791. E-mail: JAFARI@KSU.EDU.SA.

Abbreviations: AChE, acetylcholinesterase; ASCh, acetylthiocholine iodide; IC_{50}, fifty percent inhibition; K_m, Michaelis-Menten constant.

0006-291X/98 $25.00

tion (8). In the present study, we describe the mode of inhibitory action of phenserine on human erythrocyte AChE. The elucidation of drug-induced erythrocyte AChE inhibition *ex vivo* may predict enzyme action in human brain (9, 10). Although, to date, the precise biological role of AChE present in erythrocyte membrane is unknown, it nevertheless remains a valued source of enzyme for mechanistic studies as erythrocyte AChE possesses many of the properties of enzyme purified from human brain (11).

MATERIALS AND METHODS

Materials. All reagents were of analytical grade. Acetylthiocholine iodide (used as substrate, ASCh) and 5,5′-dithiobis-(2-nitro) benzoic acid (DTNB) were purchased from Sigma Chemical Co. Bovine serum albumin was obtained from Fluka Chemika-BioChemika, (Switzerland). Phenserine, (−)-phenylcarbamoyleseroline, was synthesized as reported earlier (5, 6) and was prepared as its water-soluble L-tartrate salt. It was optically and chemically pure (>99.9%).

Enzyme preparation. Human blood was taken from healthy males by vein puncture, and was collected into tubes containing citrate phosphate dextrose adenine solution as an anticoagulant. Erythrocytes were separated from plasma by centrifugation (1000 × g, 25 min, 4°C), the plasma and the buffy coat removed by aspiration, and the cells were then washed twice with 10 vol. of 172 mM, Tris/HCl buffer, pH 7.6, by recentrifugation. Washed cells were resuspended in the same, 172 mM isotonic Tris buffer, to a haematocrit of 50% and mixed well by inversion for 1 min. The 1 vol. of 50% haematocrit was added drop by drop, with constant stirring, to 7.5 vol. of lysis buffer (11 mM Tris/HCl buffer, pH 7.8) (12). After lysis, the erythrocyte membranes (ghosts) were harvested by centrifugation (100,000 × g, 50 min, 4°C) and the supernatant was removed by suction. The ghosts were washed six times with lysis buffer. Colorless "ghosts" then were resuspended in 25 mM, Tris/HCl buffer, pH 7.2, for determination of AChE activity and protein concentration. The ghosts (100 μg/ml) then were solubilized with 1% Triton X-100. After centrifugation (100,000 × g, 50 min, 4°C; 45 Ti rotor, Beckman ultracentrifuge L8-80), and AChE activity was quantitatively (72%) recovered in the supernatant.

Assay of AChE activity. AChE activity was determined at 22°C by the colorimetric method of Ellman *et al* (13). The assay mixture contained 0.20 mM ASCh and 0.25 mM DTNB. The remaining assay conditions have previously been reported (14). A 5 min. incubation time was selected for the enzyme assay after preliminary experiments were performed to ensure that enzyme activity was linear with respect to reaction time for the enzyme concentration employed. To study the effect of phenserine, the enzyme was preincubated with phenserine for 5 min. prior to the addition of substrate.

Estimation of IC_{50} and kinetic parameters. The transformed data ln % a / % i (where a = activity and i = inhibition) versus log [Phenserine] was plotted for the determination of IC_{50} (15).

Michaelis constants (K_m) were determined by means of Lineweaver-Burk plots (16), using initial velocities obtained over a substrate concentration range of between 0.034 and 0.20 mM. The assay conditions for determining the residual activities in the presence of phenserine were identical to the above assay procedure, except that a fixed concentration (0.01-0.08 μM) of phenserine was used in the assay medium.

Estimation of protein and statistical analysis. The protein content of the enzyme preparation was estimated according to the method of Lowry *et al.* (17), using bovine serum albumin as a standard. The detergent, Triton X-100 interfered with the estimation, but this problem was resolved as described previously (18, 19).

Graphs were plotted by using GraFit program (20). The values of the correlation coefficient, slope, intercept and their standard errors were obtained by the linear regression analysis using this program.

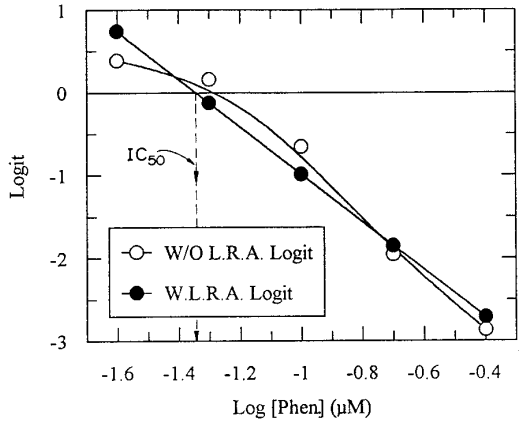


FIG. 1. Inhibition of human erythrocyte AChE by phenserine. Transformed data are presented in the form of a plot, where Logit = ln [% activity / % inhibition]. The correlation coefficient is 0.9773. Each point represents the mean of triplicate experiments.

RESULTS

Phenserine inhibited human erythrocyte AChE in a concentration-dependent manner (Fig. 1). The IC_{50} was calculated from this plot as 0.0453 μM. The regression equation was as follows:

$$\text{Logit} = (-2.8655 \pm 0.3584) \log [\text{Phenserine}] - 3.8519 \pm 0.3895.$$

The pattern of nature of inhibition of AChE activity by phenserine is shown in Figure 2. Inhibition was found to be noncompetitive type. Specifically, phenserine decreased V_{max} (13.51-63.46 %) without producing an appreciable change in the value of K_m (Table 1). The K_m value for human erythrocyte AChE was calculated by three methods: from (i) intersection at x-axis (Fig. 2), (ii) the intersection of $1/V_{maxiapp}$ and (iii), slope plots at x-axis (Fig. 4). The mean value was found to be 0.124 ± 0.0125 mM. Apparent values of V_{max} were determined by linear regression analysis of $1/\nu$ versus 1/[ASCh] data from the Lineweaver-Burk plot over a substrate concentration range of between 0.034 mM and 0.20 mM and at a phenserine concentration of between 0.01 to 0.08 μM phenserine.

The value of the K_i (the dissociation constant of the AChE-phenserine complex into free AChE and phenserine) was determined directly from the intersection of the line for each substrate concentration on the x-axis

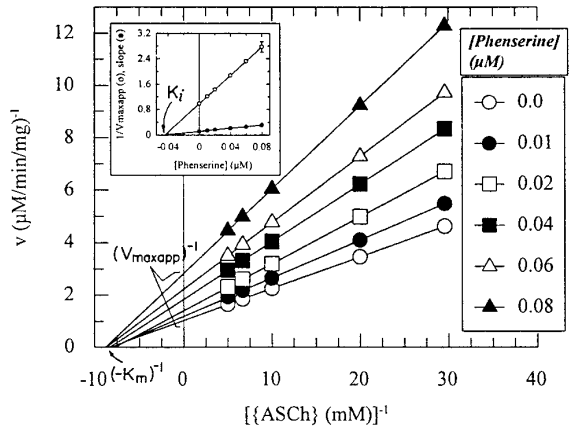


FIG. 2. Lineweaver-Burk plots representing reciprocal of initial enzyme velocity versus reciprocal of ASCh concentration in the absence and presence of phenserine **(inset)** Secondary replots of Lineweaver-Burk plot, i.e. $1/V_{maxapp}$ (○) and slope (●) versus phenserine concentrations, where correlation coefficient are 0.9956 and 0.9950 for $1/V_{maxapp}$ and slope, respectively.

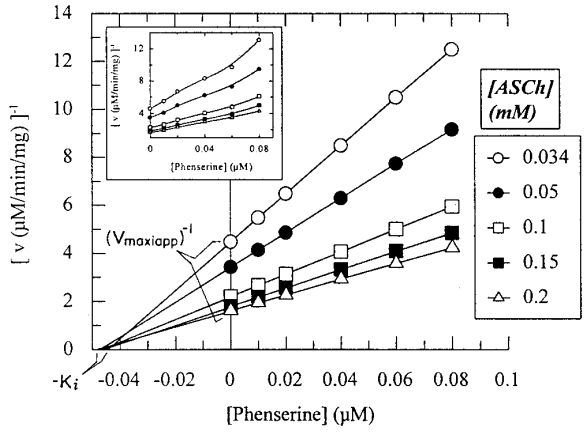


FIG. 3. Dixon plot for human erythrocyte AChE at five ASCh concentrations as indicated in legend box. The correlation coefficient were 0.9889, 0.9934, 0.9964, 0.9955 and 0.9986 for ○, ●, □, ■ and △ respectively. **(inset)** Same data in same Dixon plot but before linear regression analysis to expose actual linearity of the lines in this plot.

of Figure 3. Similarly, $V_{maxiapp}$ was determined by the intersection of the line for each substrate concentration on the Y-axis and presented in Table 2. Over the range of ASCh concentration used (0.034-0.20 mM) there was an increase in $V_{maxiapp}$ of between 30.49 and 177.6 % while there was no significant change in the K_i value. The inhibition constant, K_i was also calculated by four other ways. Firstly, the slope of each Lineweaver-Burk plot (Fig. 2) was plotted against phenserine concentration. Secondly, $1/V_{maxapp}$ was calculated at each concentration of phenserine (Fig. 2) and then plotted against phenserine concentration; the K_i was calculated from the abscissa (Fig. 2 inset). Thirdly, the K_i was calculated by linear regression analysis from data in the Dixon plot (Fig. 3) and replotted against 1/[ASCh] as presented in Figure 5A. While in the fourth method, the K_i was replotted against ASCh concentration as presented in Figure 5B. It should be noted that Figure 5 is composed of a novel relationship between K_i and substrate concentrations. These five values for the K_i, calculated by the described methods, are shown in Table 3.

TABLE 1

Effect of Phenserine on the K_m and V_{maxapp} of Human Erythrocyte AChE

Phenserine (μM)	K_m (mM)	V_{maxapp} (U/mg)	% decrease
0	0.1155	0.955	0
0.01	0.1196	0.826	13.51
0.02	0.1272	0.71	25.65
0.04	0.1181	0.539	43.56
0.06	0.1131	0.447	53.19
0.08	0.1114	0.349	63.46

Note. The K_m and V_{maxapp} were determined by their respective regression equations (Fig. 2).

DISCUSSION

The IC_{50} value for phenserine against rat and human AChE has been reported to be 0.024 μM (5-7, 21) which is in accord the current study in which we determined a value of 0.045 μM for a different source of human AChE. The results of Lineweaver-Burk analysis indicate that phenserine inhibits AChE activity non-competitively (Fig. 2). The decrease in V_{max} in the presence of phenserine without any change in K_m of AChE suggests that phenserine produces a conformational change in the enzyme. A Noncompetitive type of inhibition has two sub-types: partial and pure, which can be distinguished on the basis of replots of $slope_{1/ASCh}$ and $1/V_{maxapp}$ versus phenserine concentration. In this study we found linear plots (Fig. 2 inset) which indicate pure noncompetitive, instead of hyperbolic plots, as would be case for a partial noncompetitive subtype. One point of confusion remained, in that for a pure competitive inhibition, the $slope_{1/ASCh}$ plot is also a linear function of the inhibitor concentration, but this is overcome by a secondary replot of the Dixon plot, i.e. slope versus 1/[ASCh], in which the straight line does not pass through the origin as it does with a replot in the case of a pure competitive inhibition (Fig. 4A). These results reflect a conformational change in the enzyme by binding phenserine with the AChE-ASCh complex, yielding a non-productive AChE-ASCh-phenserine complex. In this way, phen-

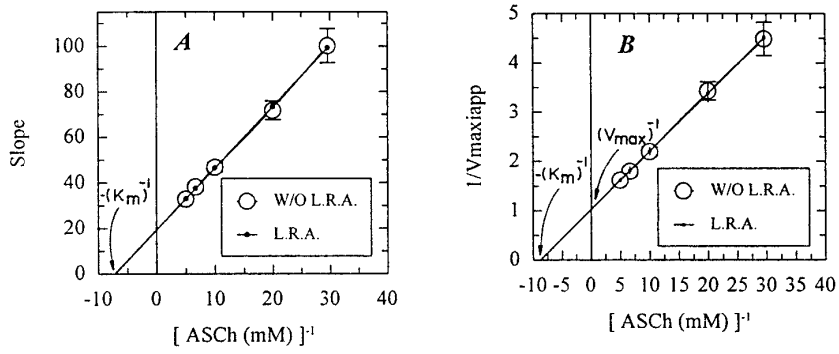
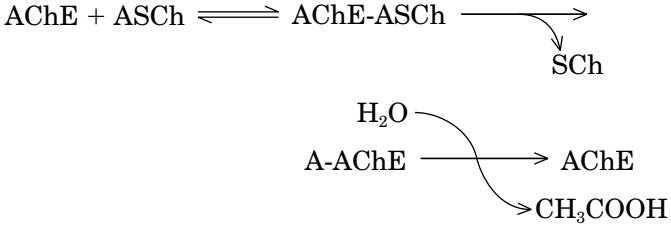


FIG. 4. Secondary replot of the Dixon plot: Slope (A) and $1/V_{maxiapp}$ (B) of the lines in Dixon plot versus reciprocal of the ASCh concentrations, where the correlation coefficient were 0.9993 and 0.9997, respectively. Each point represents the mean ± S.D. (obtained by regression analysis). W/O stands for "without", and L.R.A. for "linear regression analysis".

serine decreased the activity of the enzyme. Moreover, phenserine and ASCh are not mutually exclusive and both ligands bind independently to each other.

According to the accepted hydrolysis scheme for acetate substrate by AChE (22), phenserine can interact with AChE at either AChE-ASCh complex stage or at a regulatory site of the free AChE to form a phenserine-AChE complex and thus decrease acylation as well as deacylation, because absorption of the color product (thiocholine; SCh) was decreased at 412 nm with increasing phenserine concentration. In considering the case of an AChE-ASCh complex, the anionic site is occupied by the cholinic part of the ASCh and thus is not available to bind a second ligand. It is, however, exposed and accessible to such binding during the next step, i.e. as an acetylated AChE intermediate complex (A-AChE), although phenserine does not appear to bind at this step. If it did bind to A-AChE, then SCh would be unaffected according to the following reaction for AChE:



A question arises, which is illustrated in Figure 5. Theoretically, in the case of a noncompetitive type of inhibition, K_i should be identical at all concentrations of substrate in the Dixon plot. However, in the current study, as illustrated in Figure 3, all lines did not 100% intersect at one point on the x-axis. Hence there was potential doubt about the specific type of inhibition. To resolve this doubt, we at first plotted K_i values at each substrate concentration against the reciprocal of the substrate concentration (Fig. 5A). This, however, did not resolve the problem because its linear regression analysis gave a slope with a value of -0.1143 ± 0.0599. The K_i was not significantly dependent on the reciprocal of the substrate concentration, with a correlation coefficient of -0.7403. Interestingly, when K_i values were replotted against substrate concentration (Fig. 5B), its linear regression analysis gave zero slope value (0.0000 ± 0.0000). Although apparently here, also, K_i appeared to non-significantly increase with an increase in substrate concentrations; with a correlation coefficient of 0.7251. This novel analysis is noteworthy due to two points. First, whenever there is confusion regarding the type of noncompetitive inhibition, it can be solved by applying this novel analysis. Second, if this analysis is applied in each of the other types of inhibition systems, such as competitive, un-competitive and mixed type of inhibitions (whether pure or partial), six new kinetic constants can be calculated. In the present study, only four could be determined as a consequence of the zero value of the slope in Figure 5B. These are the following:

TABLE 2

Effect of Phenserine on K_i and $V_{maxiapp}$ of Human Erythrocyte AChE

[ASCh] (mM)	K_i (μM)	$V_{maxiapp}$ (U/mg)	% increase
0.034	0.045	0.223	0
0.05	0.048	0.291	30.49
0.1	0.048	0.455	104.04
0.15	0.047	0.556	149.33
0.2	0.049	0.619	177.58

Note. The K_i and $V_{maxiapp}$ were determined by their respective regression equations in the Dixon plot (Fig. 3). The K_i is equal to x-axis intersection, whereas $V_{maxiapp}$ is equal to the reciprocal of the y-intersection of each line for each ASCh concentration in the Dixon plot (each point in this figure represents the mean of four determinations).

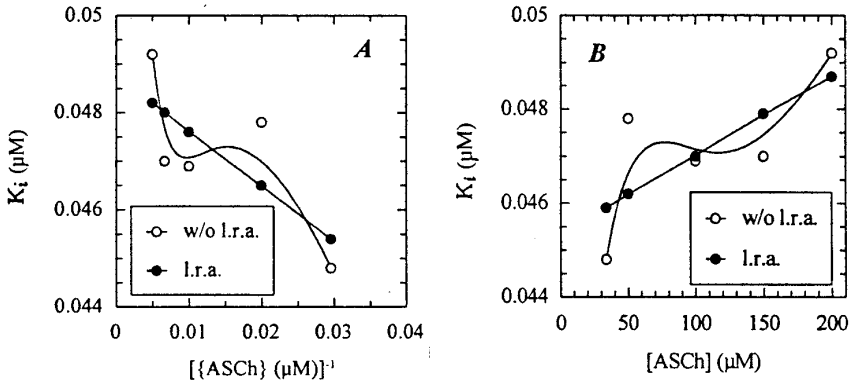


FIG. 5. A novel relationship of K_i with substrate concentration: **A.** K_i values at each substrate concentration against reciprocal of the substrate concentrations. **B.** K_i values at each substrate concentration against the substrate concentrations.

1. $K_{os} = 0.0454 \pm 0.0011\ \mu M$
2. $K_{os'} = 0.0488 \pm 0.0010\ \mu M$
3. $K_s = -0.1143 \pm 0.0599\ (\mu M)^2$
4. $K_{is} = 0.42695\ (\mu M)^{-1}$

It should be emphasized that the current study is restricted to characterizing the mechanism of action of phenserine, its K_i and related measures, for inhibition of human AChE derived from erythrocytes. An extensive review of the biochemical and molecular polymorphism, and genomic origin of both AChE and BChE is provided by Soreq and Zakut (23). AChE exists in a number of forms that can be separated by sucrose density gradient centrifugation. The globular tetramer (G4) form predominates within human and mammalian brain, and is selectively lost in AD [24]. In contrast, the G4 form represents a relatively minor component in muscle which, like erythrocytes, possess AChE in a globular dimmer (G2) form (25). The catalytic subunit of erythrocyte G2 AChE differs from the major brain G4 AChE subunit primarily in its mode of assembly and its association with solid support, i.e. its anchoring (23). Interestingly, the IC_{50} values of a variety of carbamate derivatives of physostigmine are similar for AChE derived from either human erythrocytes or brain (26). This therefore opens the potential for similar characteristics for phenserine's action in brain as described herein for erythrocyte; however, only further studies utilizing brain and other tissues will be able to assess whether or not the described results are tissue and /or species dependent.

TABLE 3

Kinetic Constants Estimated by Various Plots (p) and Replots (r)

Plots and replots	K_i (μM)	K_m (mM)	V_{max} (U/mg)
Primary p	0.047	0.118	0.955
Secondary r^{1LBP}	0.044	—	1.011
Secondary r^{2LBP}	0.052	—	—
Secondary r^{1DP}	—	0.138	—
Secondary r^{2DP}	—	0.115	0.975
Secondary r^{3DP}	0.049	—	—
Secondary r^{4DP}	0.045	—	—
Mean value	0.047	0.124	0.9803
S.E.M.	0.0014	0.0125	0.0284

Note. The details of primary plot and secondary replots have been given in the text while ^{LBP} means Lineweaver-Burk plot and ^{DP}means Dixon plot. ^{1LBP}represents replot of $1/V_{maxapp}$ versus phenserine concentration and ^{2LBP}represents replot of slope from Lineweaver-Burk plot versus phenserine concentration (Fig. 2). ^{1DP}represents replot of slope from Dixon plot versus 1/[ASCh], ^{2DP} represents replot of $1/V_{maxiapp}$ versus I/[ASCh] (Fig. 4), ^{3DP}represents replot of K_{iapp} from Dixon plot versus 1/[ASCh] (Fig. 5A) and ^{4DP}represents replot of K_{iapp} versus ASCh concentration (Fig. 5B).

Phenserine is clearly an interesting third-generation anticholinesterase that combines a long duration of action (>8 hours) with a high brain uptake (brain:plasma concentration ratio 10:1) and a selectivity for AChE versus BChE (65-fold) inhibition (6, 7). These characteristics, together with its unusually wide therapeutic window, high bioavailability and rapid body clearance in animals (6, 21, 27), may prove advantageous in AD treatment to maximize central nervous system cholinergic augmentation and minimize augmentation elsewhere. Like other anticholinesterases, it s also possible that phenserine may prove of value in the treatment of myasthenia gravis and glaucoma. Such assessments must, however, await the results of present clinical trials. Nonetheless, phenserine and like compounds (28, 29) may be of immediate value in understanding the catalytic activity, size of the active site gorge and the kinetics of the binding sites of the various forms of AChE and BChE found in nature.

ACKNOWLEDGMENT

This work was supported in part by a Research Grant by British Aerospace, British Council post-doctoral Summer Research Program.

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