POLYPHENOL OXIDASE ACTIVITY IN THE DEVELOPMENT OF ACQUIRED AROMA IN TEA (*THEA SINENSIS* VAR *ASSAMICA* L)

R. A. K. SRIVASTAVA
Botany Department, University of Gorakhpur, Gorakhpur 273001, India.
Present address: Division of Biochemistry, Regional Research Laboratory, Jorhat 785006, India.

ABSTRACT

Partially purified polyphenol oxidase from fresh tea leaves was found to form aldehydes from amino acid in the presence of (+)-catechin or other diphenols. These aldehydes contribute to the development of aroma in tea. The formation of aldehydes was proportional to the concentrations of amino acid, polyphenol oxidase and (+)-catechin. However, higher concentrations of (+)-catechin was detrimental to aldehyde formation. Diethyldithiocarbamate inhibited polyphenol oxidase activity and aldehyde formation. The K_m for (+)-catechin was found to be 0.184 mM. Addition of diethyldithiocarbamate, a Cu^{2+} chelating agent, inhibited (+)-catechin oxidation and aldehyde formation.

INTRODUCTION

In processing green tea leaves to form black teas, a number of chemical and biochemical changes take place during fermentation resulting in the development of aroma[1]. One of the important reactions in the development of flavour in tea leaves is the formation of aldehydes from amino acids[2]. Interaction of amino acids with sugars at higher temperatures[3,4] and with polyphenol oxidase at normal temperature[5] results in the formation of aldehydes. Bokuchava and Popov[6] and Nakabayashi[7] working with crude preparations have found that volatile aldehydes, important to black tea aroma, are formed from amino acids by Strecker degradation during the tea fermentation process. A similar phenomenon was reported to occur in coffee[8] and wine[9]. Preliminary studies of Wikremsinghe and Swain[10] indicated that leucine and isoleucine were involved in flavour development in tea.

Bokuchava and Popov[8] found that addition of amino acids to tea infusion developed a characteristic flavour. In the macerated tea leaves, addition of phenyl alanine resulted in the formation of phenyl acetaldehyde, but studies have not been conducted to show the formation of aldehydes from amino acids by purified polyphenol oxidase of tea leaves. Of late purified polyphenol oxidase from microbial sources[12] has been reported to cause aldehyde formation from amino acids, but its mechanism of action is still little understood. Chakraborty *et al*[13] obtained a relationship between amino acid content and the quality of black teas. Aldehyde formation from amino acids by partially purified polyphenol oxidase and flavour development with respect to amino acid concentration are discussed in the present paper.

MATERIALS AND METHODS

Extraction and partial purification of PPO

Polyphenol oxidase (PPO), E.C. 1.10.3.$^-$, was partially purified from the leaves of Tocklai released clone TV-1. Fifty grams of the young fresh shoot (two leaves with a bud) were macerated with acetone and the powder was dried in vacuum at 0°C. Fresh leaves contained PPO activity of 0.015 unit/mg fresh weight but the acetone dried powder contained an activity of 0.08 unit/mg weight. One unit of PPO activity is defined as the amount of enzyme required to consume 1 µl oxygen/minute. The enzyme was purified by ammonium sulphate fractionation. The precipitate formed between 60 and 80 % saturation of ammonium sulphate contained the highest PPO activity. The enzyme thus obtained was dissolved in tris-acetate buffer (0.1 M; pH 5.6) and was dialyzed for 15 hr against distilled water at 4°C. The dialyzed solution was concentrated in a Toshniwal lyophilizer.

In vivo formation aldehydes: To study the fermentation in the presence and absence of added amino acids, 100 g of freshly plucked shoot were allowed to wither for 16 hr and were then rolled mildly for 1 hr. The sample was divided into two equal parts. To one part 0.1 ml of phenyl alanine (0.1 M) was added and

the other part was an untreated control. Fermentation in leaves was allowed to proceed in the treated and the untreated leaves at 25°C in an incubator for 3 hr. The presence of phenyl acetaldehyde was detected by gas chromatography and hydrazone formation[11].

In vitro formation of aldehydes: Three grams of green tea were extracted with 50 ml boiling distilled water for 10 min. In 10 ml of this extract, formation of aldehyde was studied after the addition of suitably diluted PPO enzyme and the formation of aldehyde was detected by gas chromatography.

In vitro aldehyde assay: For the reaction with partially purified PPO enzyme, the assay system contained, 0.5 ml of (+)-catechin (0.1 M), 5.0 ml amino acid (0.1 M) and tris-acetate buffer pH 5.6 to make the total volume to 10 ml. The reaction was carried out at 30°C for 2 hr in a reciprocal shaker. The reaction mixture in which amino acids were not added was taken as control. The reaction was stopped by adding 0.5 ml of TCA and the aldehyde formed was detected by gas chromatography at the column temperature of 42°C. The method of aldehyde detection was the same as reported by Motoda[12]. For the detection of phenyl acetaldehyde, the reaction mixture was kept at 60°C for 1 hr and then analyzed by gas chromatography at a column temperature of 120°C. Quantitative analysis of only phenyl acetaldehyde was done and in the case of the other aldehydes their presence was qualitatively detected by gas chromatography. *n*-Propanol was used as an internal standard but in the case of phenyl-acetaldehyde the internal standard used was benzaldehyde.

In vitro polyphenol oxidase activity: Polyphenol oxidase activity was studied both in the presence and in the absence of diethyldithiocarbamate (2.5 mM) in a Warburg respirometer at 30°C. The assay mixture contained 5.0 to 10.0 mM (+)-catechin, 0.1 ml phenyl-alanine (0.1 M); 0.1 ml PPO and tris-acetate buffer (0.1 M, pH 5.6) to make the final volume to 3.0 ml. The volume of oxygen consumed (μl) was recorded every 10 min.

Isoleucine, leucine and alanine were the products of Reanal Company, Budapest, Hungary; l-valine and phenylalanine were procured from B.D.H., England; (+)-catechin was purchased from Fluka Chemische Fabric, Switzerland; and chlorogenic acid from Koch Light Laboratories, Colnbrook, England.

RESULTS AND DISCUSSIONS

Alanine, valine, leucine, in the reaction mixture were found to be converted into its corresponding aldehydes (table 1), which have been reported as constituents of the aroma of black teas[6,10,11].

The formation of phenylacetaldehyde was proportional to (+)-catechin concentration up to 0.3 mM (figure 2), but at higher concentrations substrate saturation occurred. The value of K_m for (+)-catechin was 0.184 mM. Phenylacetaldehyde formation was linear with time upto 2 hr 40 min. The enzyme and the amino acid concentrations affected oxidation of (+)-catechin. Aldehyde formation was dependent on the concentration of amino acids added, but their higher concentration (100 mM) did not result in proportional increase in aldehydes.

(+)-Catechin oxidation and aldehyde formation were inhibited by the addition of diethyldithiocarbamate (figure 1) but addition of Cu^{2+} restored them. Cu^{2+} may, thus, play an important role in the oxidation process. Diethyldithiocarbamate inhibited PPO activity non-competitively (figure 1).

Addition of partially purified PPO to dried green tea leaf infusion resulted in the formation of aldehydes as already reported by Saizo and Takeo[11]. Results presented here with pure assay mixture also accord with the above (figure 3). Addition of phenylalanine during fermentation led to the formation of phenylacetaldehyde, detected by gas chromatography and the formation of a yellow derivative (dinitrophenyl hydrazone) with a melting point of 119°C. It is generally believed that the aldehyde formation is a two-step reaction, *viz*, (i) quinone formation by PPO and (ii) aldehyde formation from quinones[11,12]. Aldehyde formation is supposedly non-enzymic and is catalyzed by quinones[11,12]. It has been observed that higher

Table 1. *In vitro formation of aldehydes from amino acids by polyphenol oxidase system*

Amino acid addition (50 mM)	Aldehyde detected by gas chromatography
Alanine	Acetaldehyde
Valine	Isobutaraldehyde
Isoleucine	2-Methyl butanol
Leucine	Isobutaraldehyde
Phenylalanine	Phenylacetaldehyde

Reaction mixture contained 0.1 ml (40 units) of PPO, 0.5 ml (0.1 M) of (+)-catechin, 5.0 ml (0.1 M) amino acids and tris-acetate buffer (0.1 M, pH 5.6).

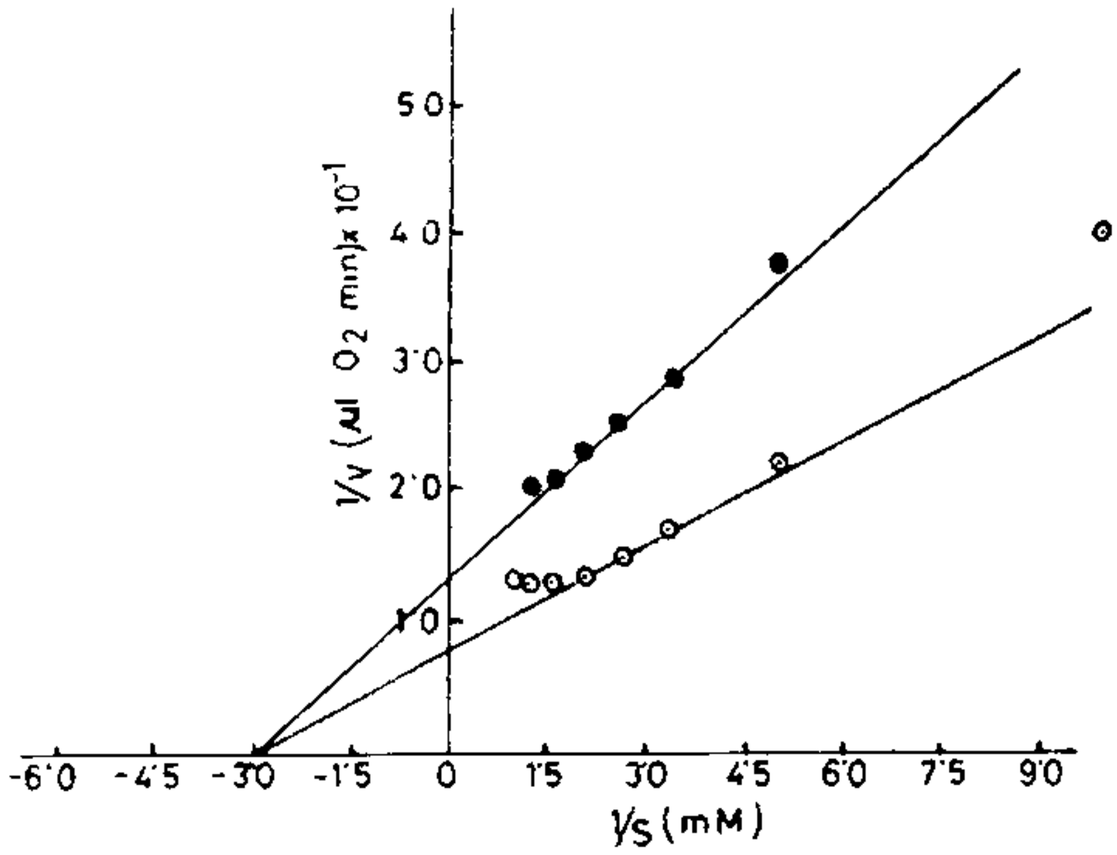

Figure 1. Relation between (+)-catechin concentration and reaction velocity in presence and absence of inhibitor. No inhibitor (o–o), with 0.25 mM inhibitor, diethyldithiocarbamate (●–●). The K_m value, as calculated from the Lineweaver-Burk double reciprocal plot, is 0.184×10^{-2} M.

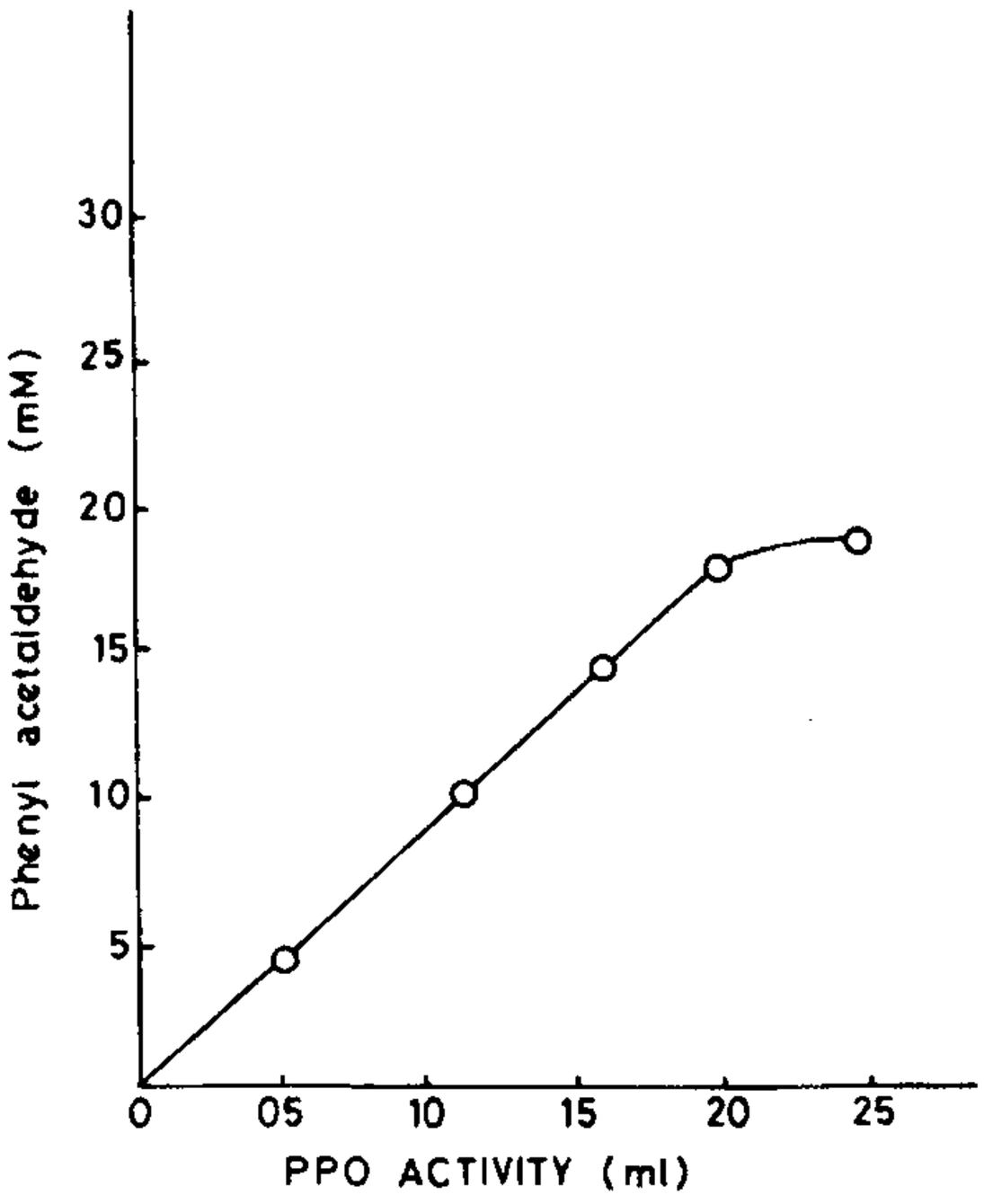

Figure 3. Effect of PPO concentration on phenylacetaldehyde formation. Concentration of phenylalanine 50 mM, PPO activity 40 unit per ml.

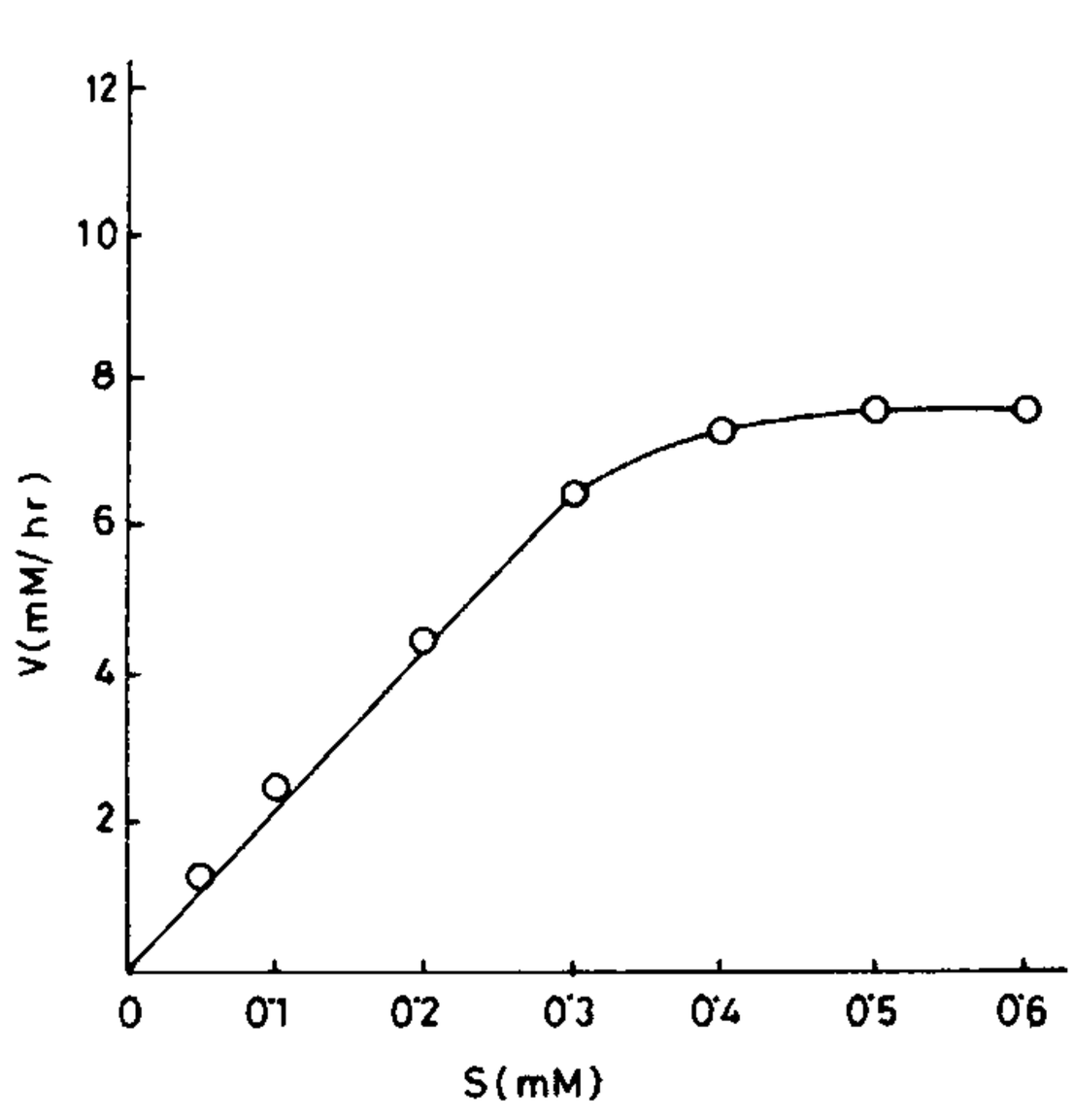

Figure 2. Effect of (+)-catechin concentration on the formation of phenyl acetaldehyde from phenylalanine by polyphenol oxidase enzyme. The system contained, in a total volume of 10 ml, 5.0 ml phenylalanine (0.1 M), 0 to 1 mM (+)-catechin, 0.1 ml PPO enzyme and tris-acetate buffer (0.1 M, pH 5.6).

concentration of catechin adversely affects the formation of aldehyde (figure 2). Following evidences suggest that aldehyde formation not only proceeds non-enzymatically, but also *via* some enzymic route involving polyphenol oxidase.

1. Aldehyde formation was found to be proportional to PPO enzyme concentration (figure 3) showing that for each unit of aldehyde formed, one unit of PPO was required.
2. Formation of aldehyde was proportion to (+)-catechin concentration indicating that aldehyde formation was subject to (+)-catechin oxidation, but higher concentration of (+)-catechin was detrimental to aldehyde formation.
3. Cu^{2+} chelating agent diethyldithiocarbamate inhibits (+)-catechin oxidation and aldehyde formation which indicate that Cu^{2+} plays an important role.

The present studies indicate that polyphenol oxidase is involved in the formation of aldehydes that induce flavour in tea leaves during the fermentation process, but more work is needed to elucidate the mechanism.

ACKNOWLEDGEMENTS

The author wishes to thank Professor I. S. Bhatia, Department of Biochemistry, Punjab University for a critical reading and correction of the manuscript. Thanks are also due to Dr N. K. Jain, Director, CSIR Complex, Palampur, Himachal Pradesh who always encouraged the author.

19 January 1985; Revised 28 October 1985

1. Wikremsinghe, R. L., *Tea Q.*, 1967, **38,** 205.
2. Eskin, N. A. M., Henderson, H. M. and Townsend, R. J., *Biochemistry of foods*, Academic Press, New York, 1965, p. 88.
3. Rohan, T. A., *J. Food. Sci.*, 1964, **29,** 456.
4. Printo, A. and Chichester, C. O., *J. Food Sci.*, 1966, **31,** 726.
5. Saijo, R. and Takeo, T., *Agric. Biol. Chem.*, 1970, **34,** 222.
6. Bokuchava, M. A. and Popov, V. R., *Chem. Abstr.*, 1955, **49,** 3439c.
7. Nakabayashi, T., *J. Agric. Chem. Soc. Jpn*, 1958, **32,** 941.
8. Bailey, S. D., Mitchell, D. G., Bazinet, M. L. and Weurman, C., *J. Food Sci.*, 1962, **27,** 165.
9. Singleton, V. L. and Esau, P., *Phenolic substances in grapes and wines and their significance*, Academic Press, New York, 1969, p. 197.
10. Wikremsinghe, R. L. and Swain, T., *J. Sci. Food Agric.*, 1965, **16,** 57.
11. Saijo, R. and Takeo, T., *Agric. Biol. Chem.*, 1970b, **34**, 227.
12. Motoda, S., *J. Ferment. Technol.*, 1979, **57,** 395.
13. Chakraborty, S., Srivastava, R. A. K. and Derchoudhury, M. N., *Two & A Bud*. 1978, **25,** 17.

ANNOUNCEMENT

INDIAN NATIONAL SCIENCE ACADEMY, NEW DELHI-LECTURE AWARDS

Nominations are invited for the award of the following Lecture awards, due in 1986: (i) Prof. B. D. Tilak Lecture, (ii) Prof. R. K. Asundi Memorial Lecture, and (iii) Prof. T. S. Sadasivan Lecture.

Details of the Lecture Awards: (i) *Professor Bal Dattatraya Tilak Lecture Award*: The selection of scientist is to be made from amongst the persons who have made outstanding contributions to rural economy and life through innovative and effective application of science and technology. The lecture is to be given every year. The first award was made in 1983. The lecturer is paid honorarium depending upon the income besides travelling and halting expenses for journeys performed to deliver the lecture. (ii) *Professor Rango Krishna Asundi Memorial Lecture*: The selection of the scientist is to be made from amongst the persons who have made outstanding contributions in the field of spectroscopy. The lecture is to be given once in two years alternately with Professor K. Rangadhama Rao Memorial Lecture. The first award was made in 1984. The Lecturer is paid honorarium depending upon the income besides travelling and halting expenses for journeys performed to deliver the lecture. (iii) *Professor Toppur Seethapathy Sadasivan Lecture Award*: The selection of the scientist is to be made from among the persons who have made outstanding contributions in any field of Botany. The lecture is to be given once in two years. The first award was made in 1982. The lecturer is paid honorarium depending upon the income besides travelling and halting expenses for journeys performed to deliver the lecture.

The nominations may please be sent so as to reach the Executive Secretary, Indian National Science Academy, Bahadur Shah Zafar Marg, New Delhi 110 002