L-ASPARAGINASE ACTIVITY IN MARINE SEDIMENTS

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ABSTRACT

L-asparaginase activity and L-asparaginase producing bacteria were estimated in sediment samples collected from three different biotopes—marine, estuarine and the mangroves. In the mangrove areas, the rhizosphere soils of *Rhizophora* and *Avicennia* showed maximum activities. The enzyme showed two pH optima, one at 6·2 and the other at 8·7. The Km value was found to be optimum and further increase, inhibited the enzyme activity. Dialysed samples exhibited slightly higher activity and sodium chloride did not show any effect on the activity even at a concentration of 10%. The occurrence of enteric bacteria in these sediments is also reported. Various factors governing the asparaginase activity are discussed.

INTRODUCTION

BROOME[1] showed that L-asparaginase (L-asparagine amidohydrolase EC 3·5·1·1) was responsible for antitumour activity of guinea-pig serum[2] and further studies showed that this enzyme can effectively be employed in the treatment of acute leukaemia[3,4]. Since then many reported the occurrence and activities of this enzyme in various bacteria[5], yeasts[6], fungi[7], and atinomycetes[8] but it was found that not all the asparaginases from the different sources did exhibit antitumour and anii-leukaemic properties. It is therefore imperative to screen for new strains from different ecological niches for asparaginases having anti-tumour and anti-leukaemic properties and with this in mind, studies on asparaginase from marine environments were initiated in this laboratory. The present report details the occurrence and activity of asparaginase in marine sediments of Porto Novo.

MATERIALS AND METHODS

The sediment samples were collected from ten stations near Parangipettai (Lat. 11° 29′ N; Long. 79° 49′ E) representing different biotopes (Table I) with the help of a Peterson grab. Samples for enzyme analysis were prepared as described earlier[9]. The salinity of the overlying waters was determined with the aid of a salinometer and the pH with a Philips pH meter.

TABLE I

L-asparaginase activities in different sediments

Section No. and Description	Sediment type	Depth (M)	Salinity of the overlying water (%)	Temperature of the overlying water (°C)	pH	L-asparaginase activity (μm NH_3/g)	Enteric Bacterial population	
							NLF ($\times 10^3$)	LF ($\times 10^2$)
1. Sea	Silt	20·00	33·19	25·5	8·0	0·48	—**	—
2. Sea	Silt	14·00	32·83	26·0	8·1	0·16	—	—
3. Mouth of Vellar estuary	Silt	2·00	31·57	26·5	7·8	1·44	10·09	1·31
4. Estuary	Clay	2·00	31·28	26·5	8·0	0·96	9·93	1·49
5. Backwater	Silt	1·20	32·11	26·5	8·0	N.D.*	—	—
6. Mangrove	Clay	0·63	33·73	27·3	8·1	4·80	16·66	22·20
7. Mangrove	Clay	1·10	33·73	27·0	8·0	6·76	—	—
8. Mangrove	Clay	1·14	25·69	30·0	7·5	4·32	18·11	18·12
9. Rhizosphere soil *Avicennia officinalis*	Clay	0·20	31·03	33·0	7·3	5·12	—	—
10. Rhizosphere soil of *Rhizophore mucronata*	Clay	0·30	28·55	32·5	7·6	8·96	—	—

N.D.* Not detectable: ** Not estimated

The enzyme assay was carried out as follows. To 5 g of air dried powdered sediment, 15 ml of 0·1 M Tris-HCl buffer (pH 8·7) containing 0·2 M asparagine and 0·5 ml toluene were added before the mixture was incubated at 40° C for 24 hours. After incubation, 5 ml of N KCl was added and

the mixture was shaken to release the adsorbed ammonia into the solution. The contents were filtered through Whatman No. 1 filter paper and the ammonia content in the filtrate was estimated by the Conway microdiffusion method. The filtrate (1·5 ml) was placed in the outer well and 0·6 ml of 1% boric acid was placed in the central well. To the outer well, 1·5 ml of saturated K_2CO_3 was added, the dishes were covered and incubated at 40° C for 3 hrs. The ammonia absorbed in boric acid was determined by Nesslerization and the extinction was measured at 450 mμ in a Spectronic-20 colorimeter. Suitable controls were maintained throughout the period of investigation.

Total enteric bacteria in sediment samples were estimated by plating with MacConkey agar. The lactose-fermentors (L.F.) and non-lactose fermentors (N.L.F.) were calculated per gram sediment on dry weight basis.

For the enzyme kinetic studies, sample collected at Station 8 was employed.

Results and Discussion

Asparaginases can be obtained from a variety of sources but the enzyme, obtained from *E. coli*[3], *Erwinia caratovora*[10], *Serratia maceescens*[11], *Mycobacterium tuberculosis*[12], *Azotobacter vinelandii*[13], Guinea-pig serum[14,15] and the serum from a variety of other members of the super-family Caviodea[16], has shown anti-tumour activity. Except for one report on the presence of this enzyme in two marine bacteria *Aeromonas hydrophilla* and *Aeromonas liquifaciens*[5] nothing is known about the other sources from bacteria, fungi and antinomycetes from marine environment. Asparaginase activity could be traced positively in all marine estuarine and mangrove sediments in the present study.

After Drobnik's[17] report Mouraret[18] made an extensive study of asparaginase activity in soils. However higher activities could be recorded in mangrove regions and in the rhizosphere soils of *Rhizophora mucronata* and *Avicennia officinalis*. In general clayey sediments always exhibited higher activities than the silty ones. Asparaginase activity could be recorded in all samples, except at Station 5, irrespective of variations in salinity, temperature and pH.

In order to ascertain the effect of time of incubation on the enzyme activity, the activity was measured at 6 hr. intervals upto 48 hr at 40° C. The results indicated that even a 24 hr. incubation period resulted in as much as 93% of the total activity (Fig. 1).

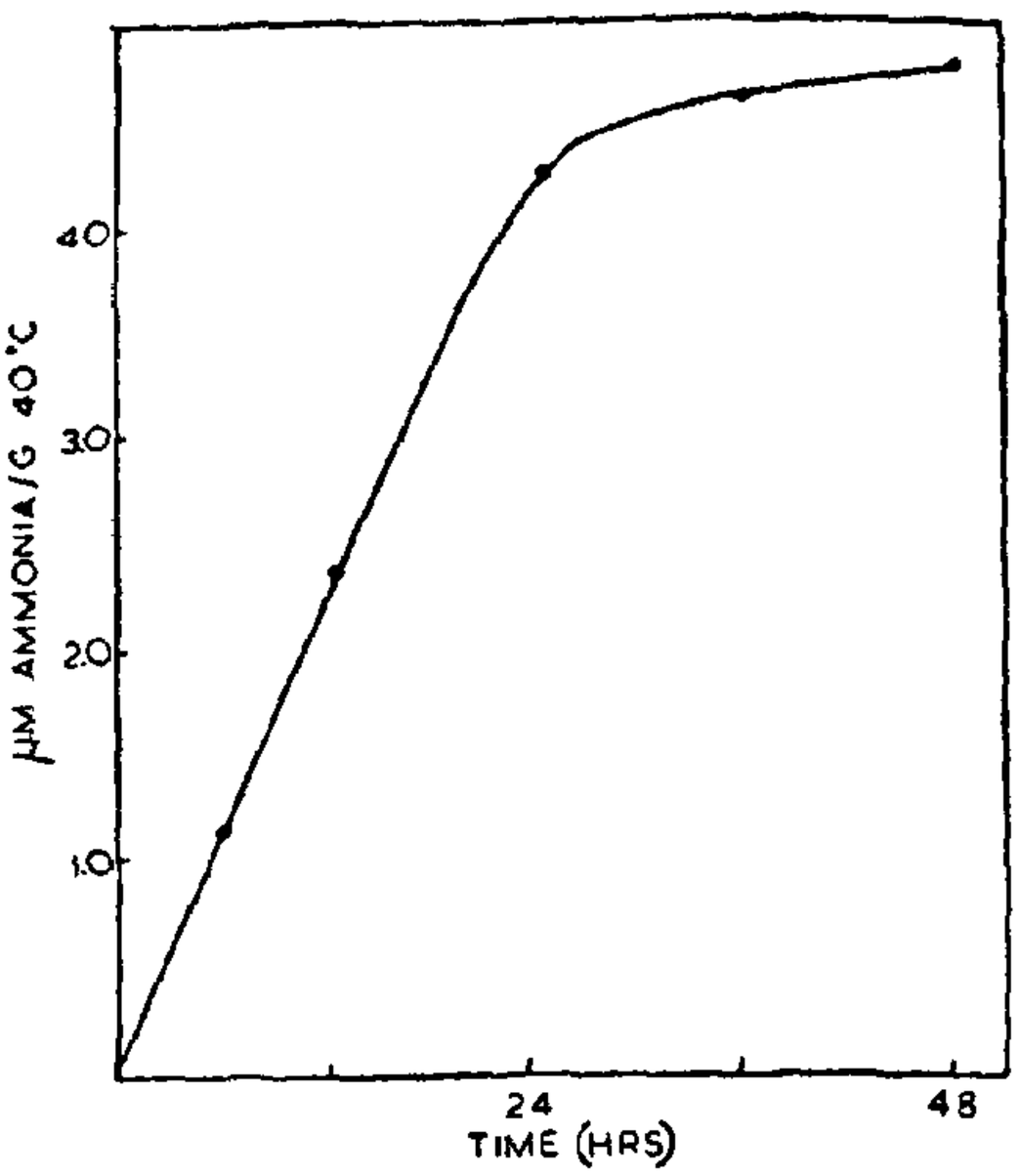

Fig. 1

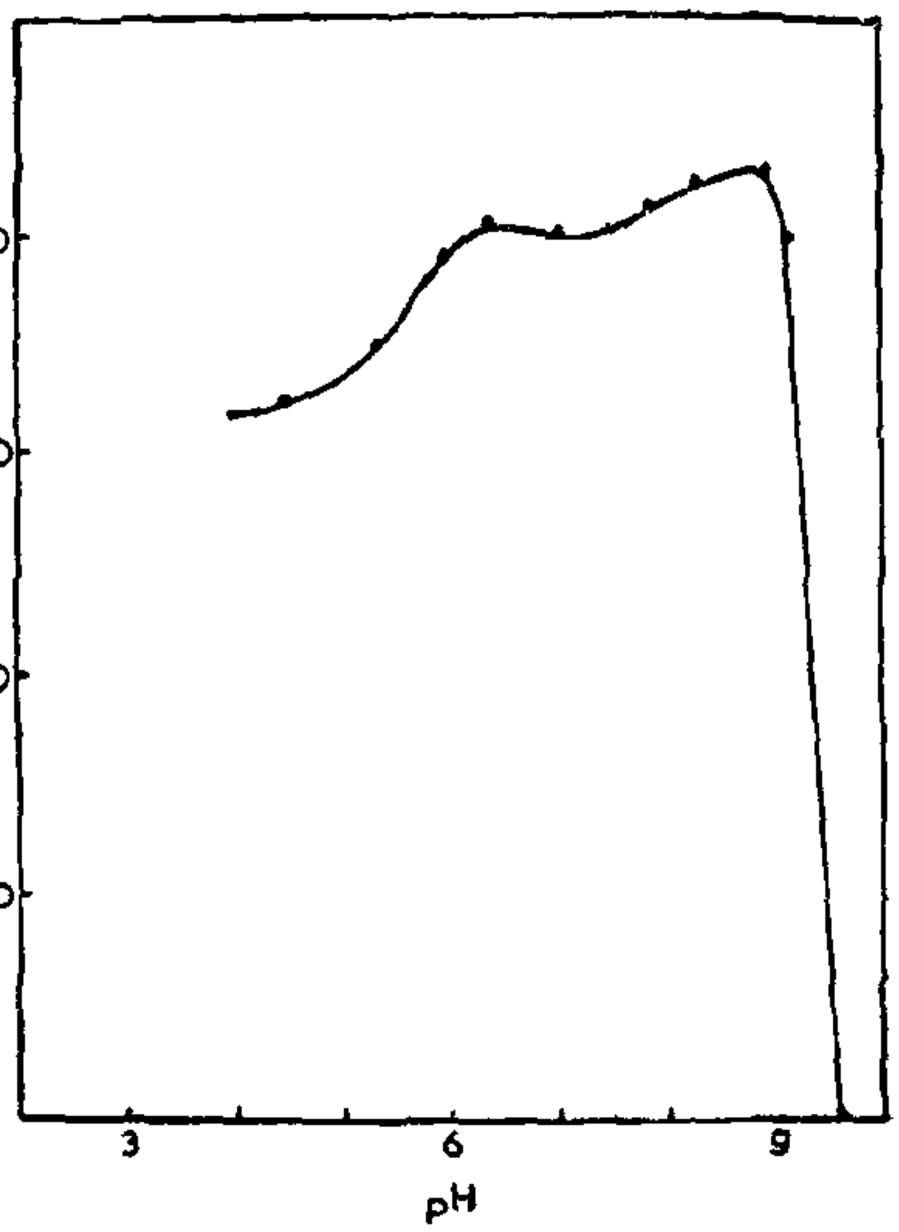

Fig. 2

Asparaginase activity as a function of pH at 40° C is shown in Fig. 2. The buffers used were (*i*) 0·1 M Tris-HCl (pH 7·2–8·9); (*ii*) 0·1 M Citrate buffer (pH 4·0–6·2); and (*iii*) 0·1 M Glycine–NaOH (pH 9·6–10·0). The activity curve showed two pH maxima, one at 6·2 and the other at 8·7. Possibly the marine sediments contain

asparaginases from different sources; from extracellular secretions of microbial cells or from intracellular enzymes released after the death of the cells, or again the observed peak at pH 6·2 may be due to the buffer effect. It has been reported earlier that most of the enterobacterial asparaginases have pH–optima around 8·5[5]. The sediment samples, in the present study harboured members of *Enterobacteriaceae* (Table I), and selected isolates of *Escherichia coli* produced asparaginase at an optimum pH of 8·7. Thus asparaginase activity measured at pH 8·7 in marine sediments may be due to *E. coli*, though the possibility of similar pH–optima for other microbial asparaginases cannot be ruled out. Asparaginase of *Streptomyces griseus* has a pH-optimum of 8·5[8]. It is interesting to note that the marine sediments also harbour members of *Streptomycetaceae*[19,20].

The effect of substrate concentration on the activity was studied by incubating the sediment samples with varying concentrations of asparagine. Maximum activity was reached at a concentration of 0·233 M and no inhibition occurred at higher concentrations (Fig. 3). Similarly, Mouraret[18] and Drobnik[17] used a substrate concentration of 2·5% and 3% respectively to study the asparaginase in soils. The Km value was calculated by means of Lineweaver-Burk plot (Fig. 4) and it was found to be $2{\cdot}17 \times 10^{-3}$ M. Similar Km values for asparaginases have already been reported for certain bacteria (Table II) and this indicates that marine sediments may also harbour similar asparaginase producers.

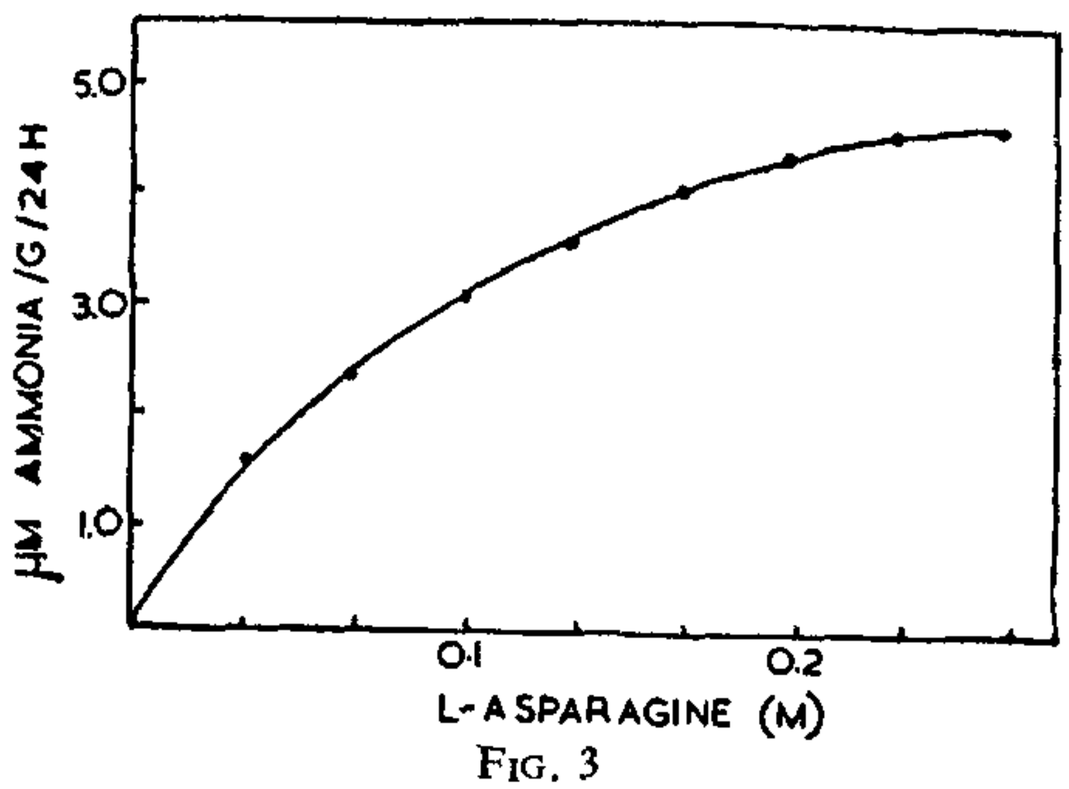

FIG. 3

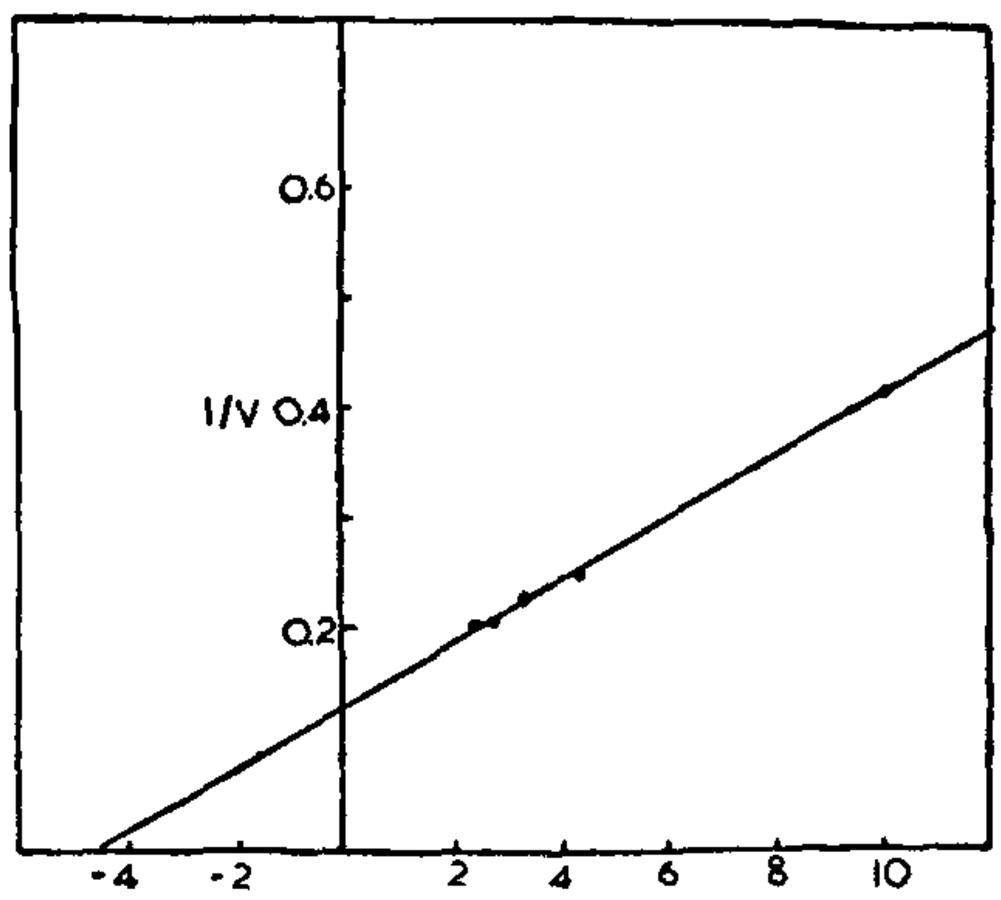

FIG. 4

TABLE II

K_m values of different L-asparaginases

Source	K_m (M)
Guinea-pig serum[16]	$7{\cdot}2 \times 10^{-5}$
Escherichia coli[26]	$1{\cdot}25 \times 10^{-5}$
Erwinia aroideae[27]	3×10^{-3}
Bacillus coagulans[28]	$4{\cdot}7 \times 10^{-3}$
Fusarium tricinctum[29]	$5{\cdot}2 \times 10^{-5}$
Proteus vulgaris[30]	$2{\cdot}6 \times 10^{-5}$
Vibbrio succinogenes (anaerobic)[31]	$1{\cdot}7 \times 10^{-5}$
Candida utilis[6]	$1{\cdot}1 \times 10^{-4}$
Hansenula jadini[6]	$1{\cdot}3 \times 10^{-4}$
Rhodotorula rubra[6]	$5{\cdot}5 \times 10^{-5}$
Marine sediment (present study)	$2{\cdot}17 \times 10^{-3}$

The effect of temperature on the stability of the enzyme is given in Fig. 5. Sediment samples *plus* buffer were incubated at 10, 20, 30, 40, 50,

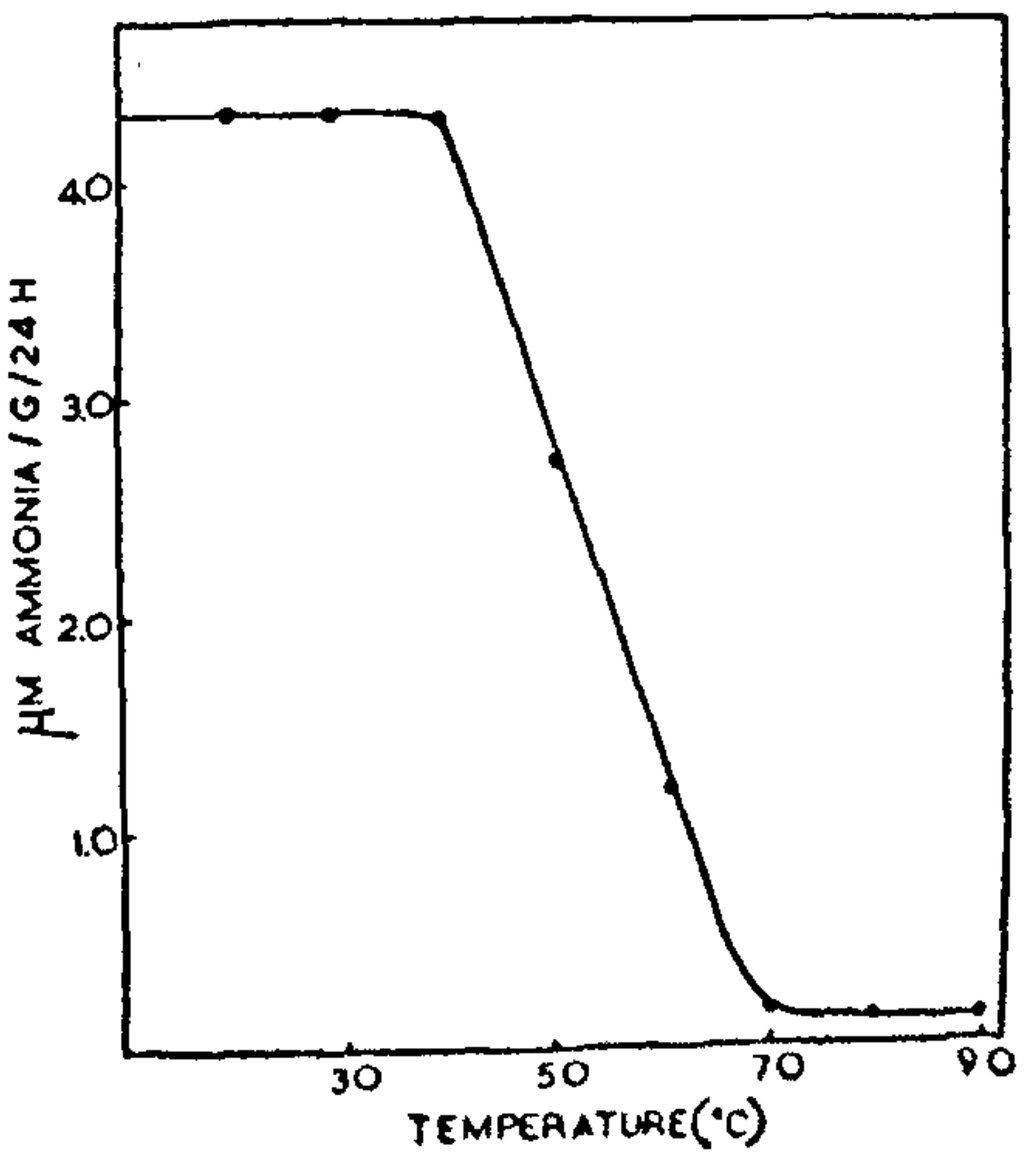

FIG. 5

60, 70, 80, 90° C for one hour. After incubation the substrate solution was added to each sample and the incubation continued at 40° C for 24 hrs. The enzyme remained unaffected upto 40° C but further increase in temperature resulted in loss of activity. Maximum activity could be recorded at 40° C. At 70° C almost 97% of the activity was lost and at 90° C, total loss of activity was recorded. Rotini[21] while examining urease activity at higher temperatures (58° C), registered a high activity and suggested the contribution of urease by lysis of micro-organisms. He observed that the increase in activity was more at this temperature in the presence of toluene. When the heat stability of the enzyme was studied it was found to retain its total activity only up to 40° C and at 70° C nearly 97% of the total activity was lost. Galstyan[22], while examining the effect of temperature on the inactivation of soil enzymes, reported that the inactivation of enzyme in soil occurs at 10° C higher than that in solution[23]. This indicated that when the heat stability of the enzyme was studied, the effect of temperature would be only on the free enzymes of the sediment and since the sediments were exposed to different temperatures for only one hour, there is not much contribution by lysis of the microbial cells. Drobnik[17] and Mouraret[18] also employed higher temperatures (42° C and 49° C respectively) for estimating asparaginase activity in soils.

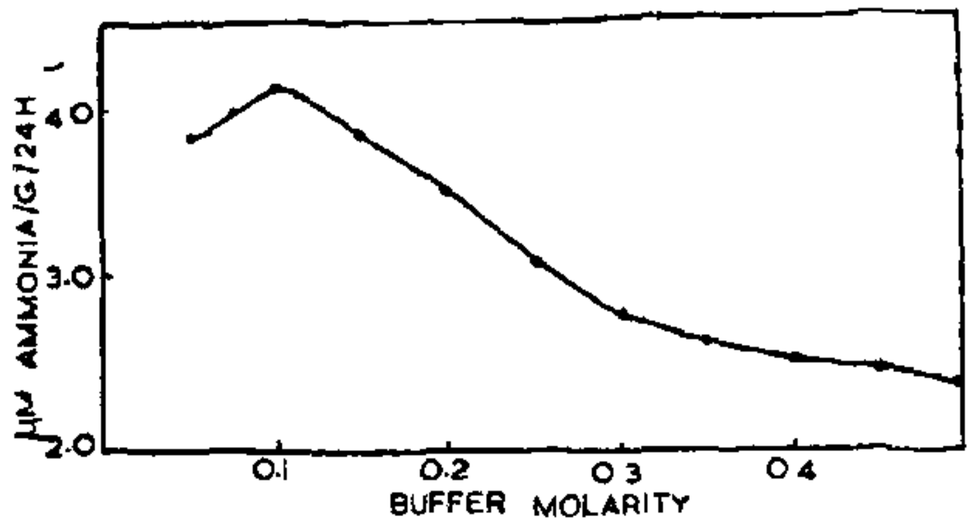

FIG. 6

The effect of buffer molarity on enzyme activity was also studied (Fig. 6). Tris-HCl buffer (pH 8·7) was employed at various concentrations to study its influence on enzyme activity. Maximum activity could be observed at 0·1 M and any further increase in molarity inhibited the enzyme activity.

Effect of sodium chloride on asparaginase activity is shown in Fig. 7. The sediment samples were dialysed against distilled water at 4° C for 24 hrs and the samples were air dried at room temperature (28 ± 2° C) in the laboratory. The dried samples were again powdered and used for the enzyme assay. To the enzyme substrate mixture sodium chloride was added at varying concentrations upto 10%. The results indicate that NaCl even at a concentration of 10% did not have any effect on enzymes activity. This suggests that the asparaginase activity is not affected by changes in salinity under natural conditions. Dialysed samples always showed slightly higher activity than the undialysed ones. This may be due to the removal of some inhibitory factors along with sodium chloride during dialysis. It may be mentioned here that phosphates and nitrates, at higher concentrations, are known to inhibit soil asparaginases[18].

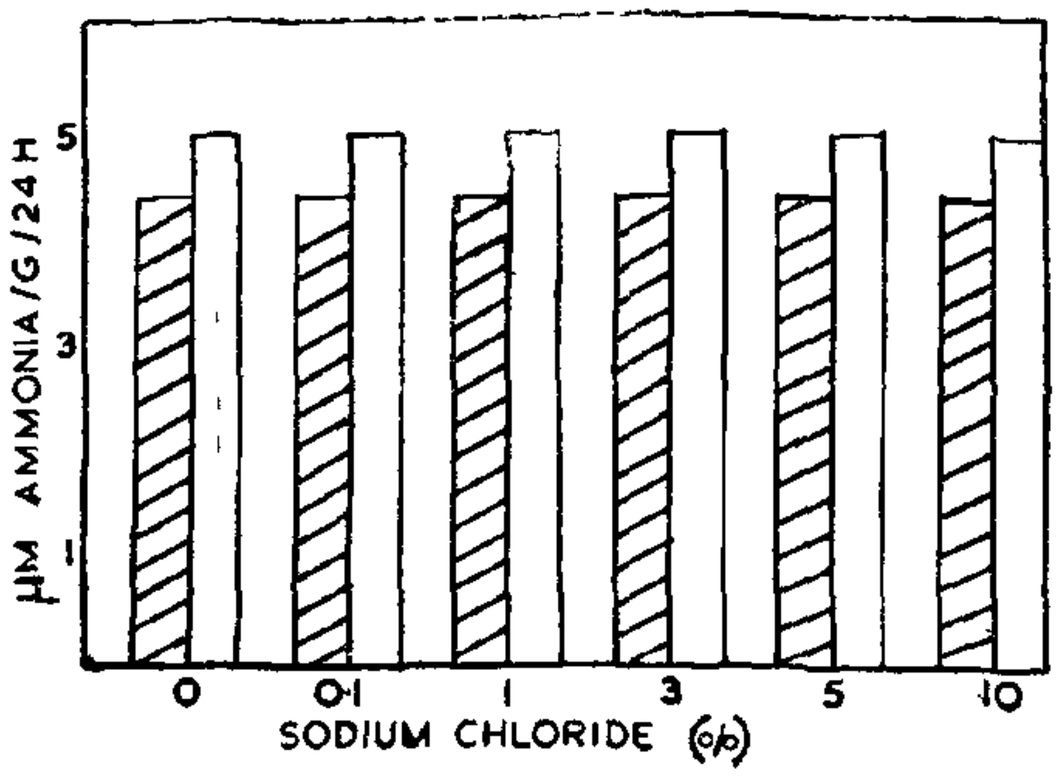

FIG. 7

Previous reports on phosphatase[24], arylsulfatase[9] and nuclease[25] activities in marine sediments indicate the possible role of free enzymes in regeneration of nutrients in marine environment. Asparaginase may also have a similar role, at least to a limited extent, by releasing ammonium for nitrification. In addition, the partial purification and the antitumour property of the enzyme from one culture (MEB–130) has been completed and the results will be published elsewhere.

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PREPARATION AND NMR STUDIES OF BENZO (*h*) QUINOLINE DERIVATIVES

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MONO nitration of 2, 4-dimethyl benzo (*h*) quinoline has been achieved by the use of nitric acid. NMR studies have served to establish the structures of the two products as the 9 and 7-nitro, 2, 4 dimethyl benzo (*h*) quinolins. The NMR spectra of benzo (*h*) quinoline as reported by E. Vender Donckt[1] is very complicated. The spectrum could be simplified by preparing its suitable derivatives. Although many of the reactions of benzo (*h*) quinoline and its derivatives have been thoroughly studied[2], nitration has received only limited attention. Baltrop and McPhee[3] recently investigated the nitration of 1-azaphenanthiene by Haid's[4] directions and separated the isomers on an alumina column monitored by ultraviolet light. These authors have showa that in 1-azaphenanthrene, electrophilic substitution takes place preferentially at the 9 and 7 positions. There is no report of an electrophilic reaction of 1-azaphenanthrene containing a functional group in the heteroaromatic ring. This investigation was undertaken in order to develop a practical method for the preparation of these compounds

Results and Discussion

Although several workers have claimed to have isolated 2, 4-dimethyl benzo (*h*) quinoline, no direct evidence for its formation has been obtained. Melting point also varies as reported in the literature. Vasserman[5] reported a melting point of 44°, as did Combes[6] and Reed[7]. Johnson and Mathews[8], reporting 51·5–53°, agreed with Van Braun[9] at 52° but Schenk and Baily[10] gave the melting point as 55–56°.

Definite evidence for its structure was, therefore, needed. The NMR spectra were consistent with all the intermediates and end product. The H–10 signal in the NMR spectrum at 60 M/C of 2, 4-dimethyl benzo (*h*) quinoline arising from the ring protons is very little informative due to its complexity. This is caused by the coincidence of most of the chemical shifts resulting in strongly overlapping signals. H-3 proton and 2 and 4 methyl groups on ring containing hetero atom are easily detectable. The difficulties in the interpretation of the very complex pattern of the low field H–10 signal could be alleviated by a further increase of the spectrometer frequency, because this leads to a corresponding increase of the chemical shift differences and hence to a simplification of the analysis of spectra.

An important progress in this connection has been achieved by the introduction of high resolution 100 MHz NMR spectrometer using magnets of high magnetic field strength. A comparison of the 60 and 100 MHz spectra shows that the former already reveals all spectral subtleties of the ring containing nitrogen atom whereas the low field signal of H-10 is not very