## A DENSITY GRADIENT METHOD FOR FRACTIONATION OF BUFFALO SPERMATOZOA

In an earlier report[1], fractionation of buffalo spermatozoa was achieved using the basic principles of homogenization and fractionation with different molar concentrations of sucrose. However, the procedure was inconvenient and amenable to morphological alterations due to longer time of homogenization. In the present communication, a modified and simple procedure is described which is based on the principles of ultrasonication and density gradient centrifugation.

Approximately 2 ml of freshly collected buffalo semen was centrifuged at 1400 × g for 10 minutes. Seminal plasma was decanted. The sperm pack was resuspended in an equal volume of $Ca^{++}$ -free Ringer solution, and centrifuged again for 10 minutes at 1400 × g. The process was repeated twice. The washed sperm pack was diluted three fold with distilled water and disintegrated with the aid of Vibronics ultrasonicator (model VPL-P1) for 60 seconds at a frequency of 25 kilocycles/second.

A density gradient of sucrose was prepared by layering 1 ml each of 50%, 40%, 30% and 20% sucrose in the ascending order in the centrifuge tube (29 mm × 116 mm). An amount of 0·7 to 1 ml of ultrasonicated sperm was layered on the top of sucrose gradient and centrifuged at 1050 × g for 8 minutes. After centrifugation, two distinct layers were obtained and sizable amount settled at the bottom of centrifuge tube. The two layers were carefully removed with the aid of pasteur pipette.

Smears of the two layers and sediment were stained with Rose Bengal and examined microscopically. The first layer consisted of tails (tail fraction) which were fragmented almost to collodial dimensions and hence its morphological entity could not be maintained. The second layer comprised of mid pieces (mid piece fraction) and the sediment consisted of sperm heads (head fraction). In order to ascertain the purity of different fractions, smears were prepared from each fractions and cytochemically assayed for the succinic dehydrogenase activity by the method of Hrudka[2]. Microscopical examination revealed that in the ultrasonicated material and the different fractions, only the mid piece showed an intense activity of succinic dehydrogenase which was in agreement with the findings of Hrudka[2]. The purity of different fractions was more than 95%. In some samples, however, it was observed that the contamination of mid pieces in head fraction was more than 10%. In such cases, the head fraction was resonicated for 15 seconds and the same procedure was followed in order to achieve purity of more than 95%.

An attempt was made to study the recovery of protien from standard preparation (S–P), *i.e.*, ultrasonicated sperm, in the different fractions. The protein was estimated by method of Lowry *et al.*[3]. Table I shows the relative distribution of protein in different fractions as compared to S–P. The recovery was 95·1%.

TABLE I

*Recovery of proteins in different fractions*

| | Mg of protein | Relative % |
|---|---|---|
| S-P | 10·010 | 100 00 |
| Head | 1·732 | 17·30 |
| Mid piece | 0·998 | 9·97 |
| Tail | 6·790 | 67·83 |
| Recovery | 9·520 | 95·1 |

The optical density of the S–P and different fraction was determined at 260 *mμ* and 280 *mμ*. Table II gives the ratio 280/260. A relatively lower value of 280/260 ratio for head fraction further confirms the purity of the fraction since the head comprised mainly of nucleic acids.

TABLE II

*Ultraviolet absorption of different fractions*

| | $O.D._{260m\mu}$ | $O.D._{280m\mu}$ | 280/260 ratio |
|---|---|---|---|
| S-P | 0·585 | 0·244 | 0·417 |
| Head | 0·668 | 0·194 | 0·290 |
| Mid piece | 0·721 | 0·620 | 0·859 |
| Tail | 0·658 | 0·585 | 0 88 |

Department of Biochemistry, Y. S. RAJPUT.
College of Vaterinary Science R. K. SRIVASTAVA.
and Animal Husbandry,
Jabalpur (M.P.), India, *July* 12, 1975.

1. Chauhan, R. A. S. and Srivastava, R. K., *Ind. J. Exptl. Biol.*, 1974, 12, 501.
2. Hrudka, F., *J. Reprod. Fertil.*, 1965, 10, 15.
3. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, 193, 265.

## CYCLIC NUCLEOTIDE PHOSPHODIESTERASE OF *HARTMANNELLA CULBERTSONI*

PREVIOUS studies from this laboratory have implicated cyclic 3′ : 5′ adenosine monophosphate (cyclic AMP) through its intracellular concentration to regulate the biochemical events leading to the differentiation of trophic *Hartmannella culbertsoni* into viable cysts[1-3]. The intracellular concentration of cyclic AMP at any phase of cellular life reflects the turnover of this regulatory

nucleotide involving adenylate cyclase mediating its synthesis and cyclic nucleotide phosphodiesterase catalysing its degradation[4]. Biogenic amines which trigger encystation of amoeba also activate its adenyl cyclase[5]. Some properties of the cyclic nucleotide phosphodiesterase are described in this communication.

*Hartmannella culbertsoni*[6], obtained from the collection of Dr. B. N. Singh, of this Institute was grown in 1% (w/v) proteose peptone, 1% (w/v) tryptone and 0·5% (w/v) NaCl, pH 6·8 as described elsewhere[7]. Six-day growth of cells was harvested by centrifugation at 800 g and washed by dispersal in sterile 150 mM NaCl and centrifugation at 800 g. Homogenates (10% w/v) were prepared in cold 250 mM sucrose by grinding the cells for 1–2 min in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle and setting the motor speed at 4,000 rpm. The homogenates were subjected to differential centrifugation.

cAMP phosphodiesterase was assayed according to the procedure described by Butcher and Sutherland[8] and inorganic phosphorus estimated in the supernatant by the procedure described by Fiske and Subba Row[9].

By differential centrifugation about 60% of the enzyme activity was recovered in the 100,000 g supernatant (organelle free cytosol) and the rest of the activity was distributed almost equally among fractions sedimenting at 800 g, 15,000 g and 100,000 g.

The crude enzyme was active on the free acid form of cyclic AMP as well as on its dibutyryl derivative. On preincubation, theophylline exerted 40% inhibition at 1 mM and 80% inhibition at 10 mM. The enzyme was stimulated 40% by 40 mM imidazole.

The activity of the enzyme was followed in cells as well as the medium used for starving and triggering differentiation. Some typical results are summarised in Table I. The activity of cells exposed to taurine or epinephrine during starvation was significantly less than of the control cells in plain starvation medium. Some amount of enzyme was also secreted into the medium irrespective of whether the cells were in plain starvation medium or in the taurine or epinephrine supplemented medium.

The decay of cAMP phosphodiesterase under starvation conditions suggests that intracellular concentration of cyclic AMP could increase. The decay of the enzyme is faster when cells are committed to differentiation in the starvation medium by the triggering action of taurine or epinephrine. Earlier it has been shown that the activity of adenyl cyclase of *H. culbertsoni* remains unchanged during six hours of starvation whereas it is stimulated 2–3 fold in the same cells undergoing differentiation[1]. Thus the stimulated adenylate cyclase and the fast decaying cAMP phosphodiesterase together appear to be responsible for the build-up of a relatively high intracellular level of cAMP in the differentiating amoeba as compared to merely starving amoeba.

TABLE I

*cAMP phosphodiesterase activity in cells and medium*

| Time of exposure (hr.) | Control medium | | Encystment medium | | | |
|---|---|---|---|---|---|---|
| | | | Taurine medium | | Epinephrine medium | |
| | C | M | C | M | C | M |
| | Enzyme units* | | | | | |
| 0 | 262 | 0 | 262 | 0 | 262 | 0 |
| 1 | 240 | 19 | 217 | 15 | 229 | 15 |
| 2 | 217 | 30 | 172 | 26 | 165 | 30 |
| 3 | 157 | 30 | 131 | 23 | 101 | 23 |
| 4 | 135 | 34 | 101 | 19 | 75 | 23 |
| 5 | 127 | 30 | 90 | 23 | 68 | 19 |
| 6 | 124 | 24 | 75 | 15 | 56 | 15 |

Assay mixtures in a final volume of 0·8 ml contained 0·36 $\mu$ mole cyclic AMP, 36 $\mu$ moles acetate buffer, pH 5·6, 1·8 $\mu$ moles $MgSO_4$ and enzyme. After 60 min. incubation at 37° C the contents were held at the temperature of boiling water for 2 min. and cooled. The pH of reaction mixture was adjusted to 7·5 with 0·1 N NaOH and 0·1 ml of a solution of cobra venom (Naja naja; from Haffkine Institute, Bombay) (1 mg/ml of Tris HCl buffer, pH 7·5) was added and incubated for 30 min, and the reaction stopped by the addition of 0·1 ml 55% w/v trichloroacetic acid, centrifuged and supernatant used for Pi estimation.

* Values are enzyme units in $1 \times 10^7$ cells (1 unit ≡ amount of enzyme that cleaves 1 *n* mole of cyclic AMP in 60 min).

C = cells ; M = medium ; Control cells in 86 mM NaCl. Encystment medium = cells in medium containing 15 mM taurine or 0·5 mM epinephrine, 20 mM $MgCl_2$ and 86 mM NaCl.

Aliquots of cells ($2 \times 10^7$) were taken at indicated intervals, separated from medium and both cells and medium used in assay of enzyme activity.

The authors are grateful to Dr. C. R. Krishna Murti for his interest in this work.

Division of Biochemistry, Central Drug Research Institute, Lucknow 226 001, *June* 17, 1975.

D. C. KAUSHAL.
A. K. VERMA.*
M. K. RAIZADA.**

* Present Address : Department of Microbiology, University of Alabama in Birmingham, The Medical Centre, Birmingham, Alabama 35294, U.S.A.

** Present Address : Lady Davis Institute for Medical Research of Jewish General Hospital, 3755 Chemin Cote St., Catherine Road, Montreal, Quebec H3T JE2, Canada.

1. Raizada, M. K. and Krishna Murti, C. R., *J. Cell. Biol.*, 1972, 52, 743.
2. Krishna Murti, C. R., *Curr. Sci.*, 1971, 40, 589.
3. —, *Biochem. Soc. Trans.*, 1973, 1, 1104.
4. Robison, G. A., Butcher, R. W. and Sutherland, E. W., *Cyclic AMP*, Acad. Press, New York, 1971, p. 84.
5. Verma, A. K., Raizada, M. K. and Krishna Murti, C. R., *Biochem. Pharmacol.*, 1974, 23, 57.
6. Singh, B. N. and Das, S. R., *Phil. Trans. Roy. Soc.* (Lond.), 1970, 259 B, 435.
7. Razada, M. K. and Krishna Murti, C. R., *J. Protozool.*, 1971, 18, 115.
8. Butcher, R. W. and Sutherland, E. W., *J. Biol. Chem.*, 1962, 237, 1244.
9. Fiske, C. H. and Subba Row, Y., *Ibid.*, 1925, 66, 375.

## FOSSIL VERTEBRATES FROM MARUVATTOOR (TIRUCHCHIRAPPALLI DISTRICT, TAMIL NADU)

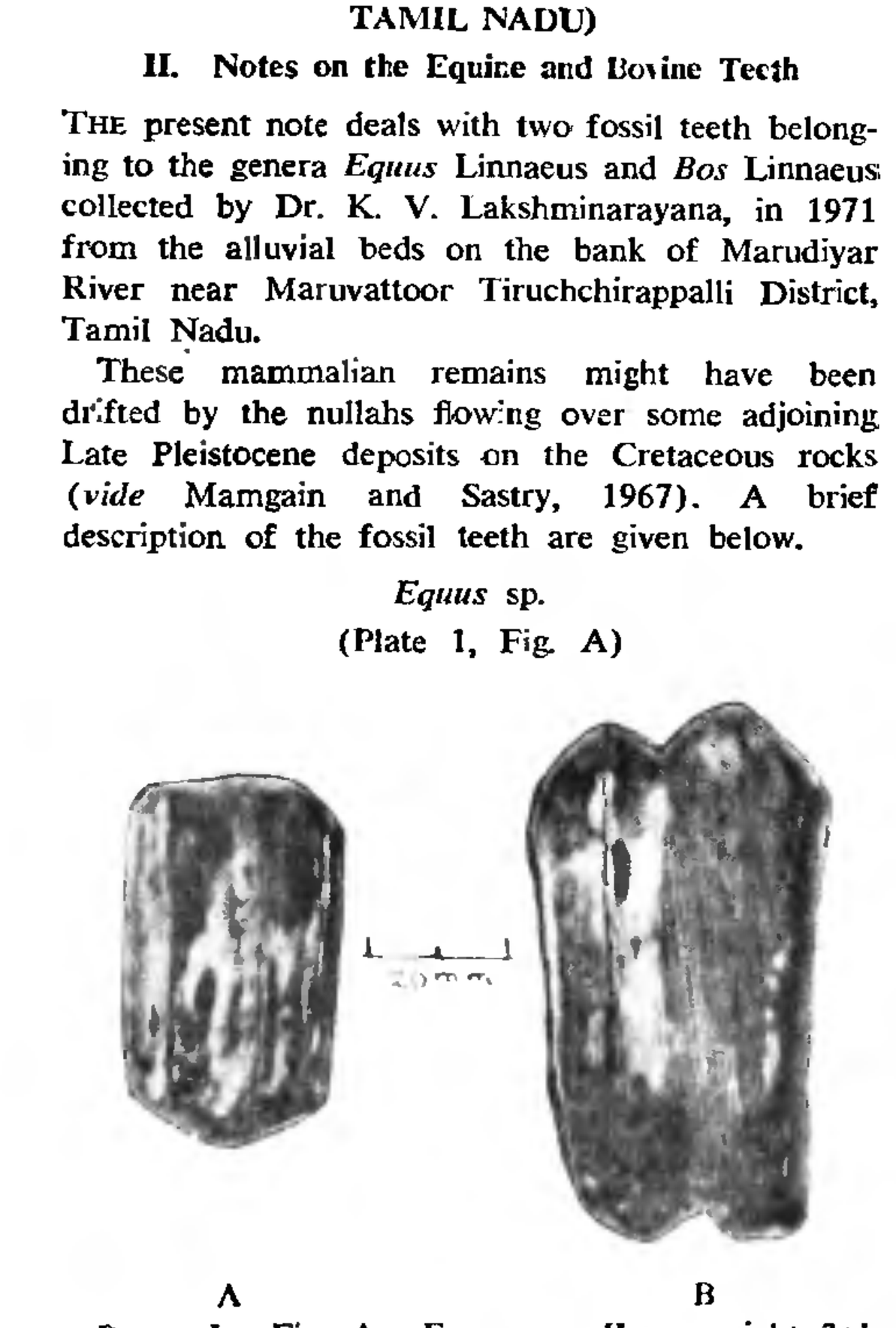
### II. Notes on the Equine and Bovine Teeth

THE present note deals with two fossil teeth belonging to the genera *Equus* Linnaeus and *Bos* Linnaeus collected by Dr. K. V. Lakshminarayana, in 1971 from the alluvial beds on the bank of Marudiyar River near Maruvattoor Tiruchchirappalli District, Tamil Nadu.

These mammalian remains might have been drifted by the nullahs flowing over some adjoining Late Pleistocene deposits on the Cretaceous rocks (*vide* Mamgain and Sastry, 1967). A brief description of the fossil teeth are given below.

*Equus* sp.

(Plate 1, Fig. A)



A B

PLATE I. Fig. A. *Equus* sp. [Lower right 3rd premolar]. Fig. B. *Bos* sp. [Upper left 2nd molar].

*Material*

Lower right 3rd premolar (Reg. No. V.P. 3).

*Description*

Angle between metaconid and metastylid V-shaped, protoconid and hypoconid rather straight; parastylid not reaching metaconid; length of the crown 25·5 mm; total height 42 mm; maximum breadth of the crown 14·5 mm.

*Remarks*

The structure of the tooth resembles largely that of *Equus namadicus* Falc. and Caut., but nothing definite can be said about its specific status due to the absence of upper tooth and other supporting data. However, Rao and Seshachar (1927) and Rao (1927) have also reported fossil equine tooth from Ariyalur, Tamil Nadu.

*Bos.* sp.

(Plate 1, Fig. B)

*Material*

Upper left second molar (Reg. No. V.P. 4).

*Description*

Hypsodont; erupting tooth having basal pillar, tip of the basal pillar 12 mm below the tip of the crown; length of crown 33 mm; height of tooth 64 mm; maximum breadth of the crown 17·2 mm.

*Remarks*

Mamgain and Sastry (1967) reported the occurrence of *Bos* sp. from Ariyalur, based on lower jaw and limb bones. The present is, therefore, the first record of an upper tooth, hence interesting.

The author is thankful to Drs. S. Khera, S. K. Bhattacharyya, and K. V. Lakshminarayana, of Zoological Survey of India, for providing facilities and guidance and Shri A. K. Dutta, Palaeontology Division, Geological Survey of India, for suggestions.

Zoological Survey of India, K. D. SAHA.
8, Lindsay Street,
Calcutta 700 016, *September* 23, 1974.

1. Mamgain, V. D. and Sastry, M. V. A., *Indian Miner.*, 1967, 21 (1), 61.
2. Rao, C. R. N. and Seshachar, B. R., *Half-yearly J. Mysore Univ.*, Bangalore, 1927, 1 (2), 144.
3. Rao, L. R., *Ibid.*, 1972, 1 (2); 153.

## A NOTE ON THE SMALLER FORAMINIFERA FROM THE BARIPADA BEDS, MAYURBHANJ DISTRICT, ORISSA

THE present note deals with the foraminifera obtained from the Baripada Beds exposed on the eastern bank of the Buhabalang river. The writers collected the samples from the Baripada Beds during 1970 and observed the following stratigraphic sequence of the rocks :