28. Ponten, J., XVI International Cancer Congress, New Delhi, Oct.–Nov. Abstracts, 1994, p. 388.
29. Nair, M. K. K., XVI International Cancer Congress, New Delhi, Oct.–Nov. Abstracts, 1994, p. 319.

ACKNOWLEDGEMENTS. We thank Mr Arun Kumar, Ms Yasoda for their assistance, Mr Gopal Krishna Nayak and Ms Daisy Thomas for computer programming, Mr Nayak for medical records, assistance. We thank all the patients as well as the heads of the departments of Medicine, Medical Oncology, Radiation Oncology, Surgery, Surgical Oncology and Urology for cooperation. Financial support from Manipal Academy of Higher Education is acknowledged.

Received 21 July 1997; revised accepted 28 October 1997

Optimal pH conditions for the growth, release and stability of Japanese encephalitis virus in PS cell line

S. V. Gangodkar, S. P. Paranjape, V. D. Kadam and R. P. Deolankar

National Institute of Virology, Dr Ambedkar Road, Pune 411 001, India

Effects of pH of the medium on the growth, release and stability of Japanese encephalitis virus (JEV) were studied with an aim to optimize the conditions for the virus stock preparation. Eagle's minimum essential medium (MEM) was modified by addition of 30 mM HEPES and reduction of $NaHCO_3$ to 0.85 g/l. The pH values were adjusted to 7.2, 7.4, 7.6, 7.8 and 8.0 by 1.0 N HCl or 1.0 N NaOH. The study revealed that the optimum pH values for the growth, release and stability of JEV were 7.4, 7.8 and 7.6 respectively.

THE significance of optimum nutrient composition for the growth of Japanese encephalitis virus (JEV) in PS cell cultures has been reported earlier by us[1]. Further optimization of conditions for the release and the stability of the progeny virions is of utmost importance, especially if preparation of the vaccine is intended from this stock.

There are reports that among other factors, the viral integrity, stability and infectivity are dependent on the pH of the medium. Purified JEV loses its structural integrity at pH 6.2–6.6, the pH at which the haemagglutination test is carried out. Russel *et al.* found excellent preservation of infectivity of JEV during purification procedure using tris-saline EDTA buffer pH 8.2 (ref. 2). The stability of Dengue virus was found to be optimum at pH 8.0 (ref. 3). Fusion of virion envelope with the plasma membrane has been proposed as being involved in the infectious entry of enveloped viruses[4]. Fusion of viruses with liposomes containing phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol was found to be pH-dependent, which may finally be accounted for the infectivity of the virus. Fusion of enveloped viruses with cellular or artificial lipid target membrane can occur either in neutral pH (Paramyxo and Herpes) or can be triggered by mildly acidic pH (Togaviridae, Rhabdoviridae, Bunyaviridae)[5]. Flavivirus infectivity and haemagglutinin are stable at pH 8.0 to 8.4 (ref. 6). It is also possible to achieve significant increase in amount of virus release per cell by increasing ionic strength of the medium[7].

With this background, the present study was carried out to know the effect of pH on the growth, release and stability of JEV (Nakayama strain).

PS cell line at passage number 79 through 87 was grown in Eagle's minimum essential medium [MEM (E)] supplemented with 10% goat serum (GS) and confluent monolayers were maintained in the same medium without GS. Subcultures were prepared by splitting the trypsinized monolayer in 1:4 ratio. Viable cell count was determined by Trypan blue dye exclusion method.

The medium M-8 (ref. 1) was used with a few modifications. HEPES buffer was added at a concentration of 30 mM and $NaHCO_3$ was reduced to 0.85 g/l. The pH values of the media were further adjusted by addition of 1.0 N HCl or 1.0 N NaOH. The test media having pH 7.2, 7.4, 7.6, 7.8 and 8.0 at 37°C were prepared.

JEV Nakayama strain which underwent 52 mouse brain passages and 5 PS cell culture passages was plaque-purified thrice in PS cells. The titre of the stock was $10^{7.5}$ PFU/ml.

Confluent monolayers of PS cells were employed for growth of virus. Milk dilution (MD) bottles of PS culture were infected with 0.1 PFU of virus/cell. Virus inoculum (0.4 ml) was allowed to adsorb on the monolayer for half an hour at 37°C. The monolayers were then washed with PBS, fed with test media having different pH and incubated at 37°C. The pH values were monitored visually using a phenol red pH indicator. Ten culture bottles were used to test each medium. Extracellular tissue culture fluids (ETCF) were collected at 24, 40 and 48 h post infection (hpi). The progeny viral content of pooled ETCF was assayed by end point dilution method and the 50% tissue culture infective dose ($TCID_{50}$) was calculated[8]. The experiment was repeated thrice and averages were compared using *t* test[9].

MD bottles were infected with JEV as described above and fed with M8 medium. ETCF of infected monolayers were discarded after 40 h post infection. Five groups of 10 MD bottles each were fed with the five test media having different pH and incubated for 1 h at 37°C. The virus released during 1 h was assayed by end point dilution method. Experiments were repeated thrice and averages were compared using *t* test.

Aliquots of the seed virus suspension having titre 7.5 log $TCID_{50}$/0.1 ml were diluted 1:10 to required pH val-

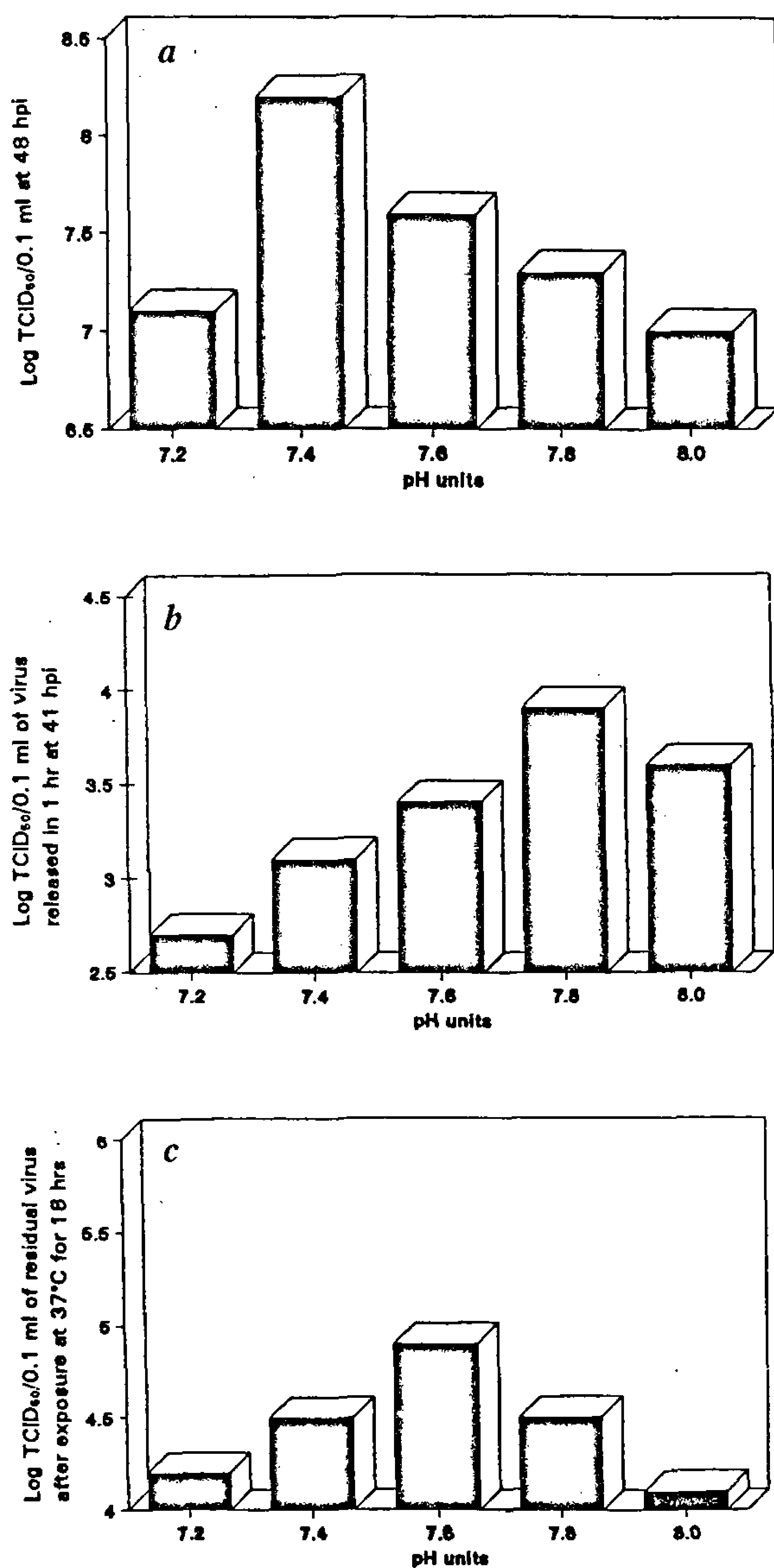

Figure 1. Effect of pH on (*a*) the growth of JEV; (*b*) the release of JEV; and (*c*) the stability of JEV.

ues ranging from 7.2 to 8.0, and incubated at 37°C for 18 h. The $TCID_{50}$ of the residual virus was determined and the results were compared using *t* test.

The effects of pH on growth, release and stability of JEV in PS cells using media of pH level ranging from 7.2 to 8.0 were studied. The data on virus titre was transformed by subtracting 6.5, 2.5 and 4 for growth, release and stability respectively for better graphical presentation. Observations are given in Figure 1 *a–c*.

From Figure 1 *a*, it was observed that the titre of ETCF from culture bottle containing medium of pH 7.4 was maximum at 48 hpi. The optimal pH requirement of 7.4 appears to be stringent as deviation of as little as 0.2 pH units on either side resulted in a considerable drop in the yield of the virus ($P < 0.01$, Figure 1 *a*).

Optimum release was observed in medium having pH 7.8. Significant reduction in release of virus was observed in media having pH range 7.2 and 7.4 ($P < 0.01$; Figure 1 *b*).

Residual virus was maximum in media having pH 7.6. Decline in residual virus was observed in media of pH 7.2 and 7.8 ($P < 0.01$; Figure 1 *c*).

It is evident from $TCID_{50}$ titres that yield of JEV was maximum in medium having pH 7.4. Gollins and Porterfield[10] observed uncoating of West Nile virus with 1 min lag phase and RNA remained infectious in medium having pH 7.6, whereas, in the medium having pH 6.2, the viral uncoating occurred rapidly without lag phase and uncoated RNA appeared less infectious. Our observation on less infectivity in acidic pH supports the above report.

From the study on release of virus, it can be seen that the maximum release of virus within 1 h was observed when the infected culture bottles were fed with the medium of pH 7.8 and 8.0 which were slightly more alkaline than the optimal pH for the growth, indicating that slight alkalinity favours the release of the virus.

It is evident that the optimal stability could be obtained in pH 7.6. Bloedhorn[11] observed maximum stability of Grazi strain in Tick Born Encephalitis (TBE) virus (an arbovirus), at pH 7.5 and the virus was more stable in Tris buffer than in phosphate buffer. Our observation on optimal pH for stability is comparable to the above report.

From the present study it can be concluded that there are different pH optima, for the growth, release and stability of JEV and it would be better to maintain the pH conditions, wherever possible, to get the optimal results while cultivating JEV *in vitro*. A popular practice emerged from these observations in our laboratory to grow JEV in PS cell line is to (a) maintain the pH to 7.4 during the growth of the virus and (b) raise the pH to 7.8 one hour prior to harvest of the ETCF. These observations are useful for mass cultivation of virus in biofermentors.

1. Kadam, V. D., Paranjape, S. P., Gangodkar, S. V. and Deolankar, R. P., *Acta Virol.*, 1995, **39**, 287–289.
2. Russel, P. K., Brandt, W. E. and Delrymple, J. M., in *The Togaviruses, Biology, Structure and Replication* (ed. Schledginger, R.), Academic Press, New York, 1980, pp. 510–529.
3. Manning, J. S. and Collins, J. K., *J. Clin. Microbiol.*, 1979, **10**, 235–239.
4. Marsh, M., *Biochem. J.*, 1984, **218**, 1–10.
5. Gollins, S. W. and Porterfield, J. S., *J. Gen. Virol.*, 1986, **67**, 157–166.

6. Karabatsos, K., in *St. Lous Encephalitis Washington D C.* (ed. Monach, T. P.), American Public Health Association, 1980, pp. 105–158.
7. Matsumura, T., Shikari, E., Hotta, S. and Sashikara, T., *Proc. Soc. Exp. Biol. (N.Y.)*, 1972, **141**, 599–605.
8. Reed, L. J. and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
9. Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, Oxford and IBM, Calcutta, 6th edn, 1967, pp. 120–122.
10. Gollins, S. W. and Porterfield, J. S., *J. Gen. Virol.*, 1986, **67**, 1941–1950.
11. Bloedhorn, H., *Zentralbl. Bakteriol. Parasitenkl. Infektionskr. Hyg. Abt. I*, 1963, **190**, 149–153.

ACKNOWLEDGEMENTS. We thank Ms C. A. D'Mello, Mr M. P. Rajarshi and Mr K. Vijaya Simha for their skilled technical assistance.

Received 9 September 1997; accepted 17 October 1997

A new human fossil find from the Central Narmada Basin and its chronology

Anek Ram Sankhyan

Anthropological Survey of India, 27 Jawaharlal Nehru Road, Calcutta 700 016, India

We report here a clavicle fossil finding of the Early Stone Age Man from the Central Narmada basin in its bio-cultural context that sheds new light on the evolution of *Homo sapiens* in South Asia.

FOLLOWING the discovery of a partial cranium[1] of Fossil Man from the Central Narmada Valley, the Anthropological Survey of India launched an extensive hunt for the fossil and lithic relics of the Palaeolithic Man in the Central Narmada basin. Explorations were conducted during 1983 to 1992 between Jabalpur and Punasa (Khandwa) covering 50 sites that yielded a rich assemblage of over 700 lithic artifacts, mammalian fossils and a hominid clavicle. A monograph on these findings is in preparation. A brief reporting of the important hominid fossil finding in its bio-cultural context is made here retaining the detailed account slated for elsewhere.

The present hominid fossil (Figure 1) was recovered from the unit I of the Boulder Conglomerate[2] bed at Hathnora that previously yielded the hominid partial cranium. Hathnora is located 22 km crow-flight distance north-east of the town of Hoshangabad along the right bank of the Narmada in Sehore District (Madhya Pradesh). The specimen is a fully fossilized right hominid clavicle and is well preserved except for a minor erosion near the medial end. The robust and rugose character of the specimen and the fused medial epiphysis[3] indicate an adult person in the age group of about 25 to 30 years.

The maximum total length of the fossil clavicle is 90 mm, which characterizes it as the shortest clavicle among the Middle to Late Pleistocene hominids, including East African *Homo erectus*, European and Middle East Neanderthals/early *Homo sapiens*. It is comparable only to the modern human pygmies, the Onges and the Andamanese in clavicular length. However, in its highly robust character (with very high caliber index of 45.6) it has no parallels among the known extinct and extant hominid clavicles. Coupled with other unique morphological characters like very rugose and rugged subclavian area, less-expanded medial epiphysis, well-developed rhomboid fossa, less axial torsion and relatively-flattened diaphysis, the Narmada hominid clavicle characterizes an archaic, robust, short and stocky hominid. On these counts, it stands uniquely different from African *Homo erectus* as well as from the Neanderthals and western archaic *Homo sapiens*. The present post-cranial evidence thus opens up a new dimension for understanding the phylogenetic relationships of the Narmada hominid, which is hithertofore considered either to be similar to the South-East Asian *Homo-erectus*[4] or to an European Neanderthal/archaic *Homo sapiens* on partial cranial[5] or cultural[6] evidence.

The lithic cultural evidence recovered by us from Hathnora comprises of 36 Late Acheulian to Microlithic tools. But most of these are surface collections from the top of the Boulder Conglomerate platform and probably was derived from the upper Concretionary Clays and Black Cotton Soil. Only 11 tools chiselled out from the unit I Boulder Conglomerate bed may be considered of *in situ* nature. These are assignable to the terminal phase of the Acheulian culture and include two small handaxes, one flattish and another miniature biconvex, two small V-shaped cleavers, one flattish bifacial chopper, a small discoid, an awl, a backed knife, two side-cum-end scrapers and a notched one, all made on reddish, purplish and greenish quartzite pebbles. Late Acheulian tools have also been reported by other workers[6–9] from the Hathnora Boulder Conglomerate as well as from the

Figure 1. Inferior view of the Narmada hominid right clavicle.