component parts for the flowmeter cost less than $100, it is extremely sensitive (about 3.5 cy/sec per millimeter of flow velocity per second), and zero flow level can be easily determined without interfering with actual flow through the vessel (*1*).

DEAN L. FRANKLIN
WILLIAM SCHLEGEL
ROBERT F. RUSHMER
Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle

Note

1. This work was supported in part by a grant (H-4531) from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

10 April 1961

Eastern Equine Encephalomyelitis Vaccine Prepared in Cell Cultures

Abstract. Protection tests in guinea pigs indicate that vaccines prepared from virus propagated in chick embryo cell cultures are as effective as the purified whole chick embryo vaccines which are currently used for human immunization against eastern equine encephalomyelitis.

The extensive distribution of the virus of eastern equine encephalomyelitis, and the occurrence of an increasing number of human cases of this disease, have caused a renewed interest in the preparation of a vaccine for the immunization of those individuals who are exposed to this agent. In 1940, Randall described the preparation of a chick embryo vaccine, inactivated by 0.4 percent formalin, for the immunization of Army horses and mules located in known infected areas (*1*). Considering that for human use it was desirable to reduce the high content of chick tissue and egg components in this vaccine, Randall, Mills, and Engel, in 1947, described a method for purifying this vaccine involving a two-stage differential centrifugation and concentration (*2*). Since 1957 the purified chick embryo vaccine has been produced by the Department of Biologics Research, Walter Reed Army Institute of Research, for the immunization of laboratory personnel and exposed field workers. Most lots of this vaccine, freeze-dried for maximum stability, have been shown to protect a majority of guinea pigs against a challenge of 100 to 1000 intracerebral LD_{50} doses of virus. Serum neutralizing antibody response of humans to an initial series of vaccine inoculations has varied from poor to good.

The use of cell culture methods for the cultivation of arthropod-borne viruses has suggested the possibility of the production of a vaccine against eastern equine encephalomyelitis from virus propagated in chick embryo fibroblast monolayers, rather than in the whole chick embryo. Such a system would result in a relatively pure vaccine without the need for differential centrifugation, a procedure which undoubtedly results in the loss of a considerable amount of viral antigen. This report presents the results of studies on the preparation of experimental vaccines from chick embryo cell cultures (*3*).

Primary cultures of fibroblasts from chick embryos 9 to 10 days old were prepared by seeding Povitsky bottles with 150 ml of trypsinized chick embryo cells (2×10^6 per milliliter) suspended in mixture 199, containing 2 percent calf serum and 100 units of penicillin and 50 μg of streptomycin per milliliter at *p*H 7.4 to 7.6. Uniform monolayers were obtained after incubation at 36°C for 44 to 48 hours. The spent medium was decanted, and the monolayers were washed with 100 ml of Earle's solution containing antibiotics. After thorough draining of the wash solution, the cells were overlayed with 150 ml of a 10^{-4} dilution of chick embryo suspension infected with eastern equine encephalomyelitis in mixture 199 (containing no serum or antibiotics); the bottles were then returned to the incubator. Disintegration of the cells as a result of viral action begins to occur after approximately 20 hours of incubation and is complete after an additional 6 to 10 hours. Since the maximum virus titer ($10^{7.5}$ mouse intracerebral LD_{50} doses per 0.03 ml) appears in the culture fluid after 20 hours of incubation, harvesting of the fluids at this point minimizes the contamination of the culture fluids by material from the damaged cells.

The pooled culture fluids were centrifuged at low speed (2000 rev/min for 30 min) to remove suspended tissue cells, and portions were then filtered through a Millipore filter (HA) to remove particulate matter. Viral titrations in mice indicate that these procedures can be accomplished with less than 0.5 log reduction in virus titer. The virus is inactivated by the addition of neutral formalin to a final concentration of 0.05 percent and by storage of the mixture, with frequent shaking, at 22°C for 4 days and then at 5°C for an additional 10 days. Studies on the rate of inactivation by formalin demonstrated that no active virus could be detected after 48 hours at 22°C when 0.03 ml of the undiluted vaccine was inoculated intracerebrally into each of 20 mice (Fig. 1).

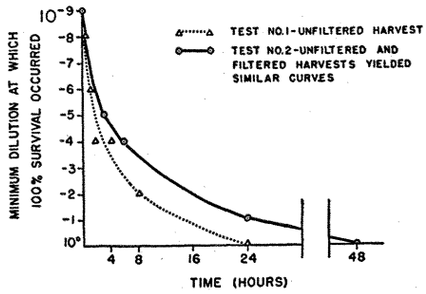

Fig. 1. Inactivation of chick embryo cell culture propagated eastern equine encephalomyelitis virus by 0.05 percent formalin at 22°C, as measured by intracerebral inoculation of white mice.

For those preparations which were freeze-dried, the formalin was neutralized with sodium bisulfite after the inactivation period and the product was freeze-dried in a chamber dryer. Inoculation of 0.03 ml of the fluid or rehydrated final product intracerebrally into each of 50 white mice failed to detect any live virus. Sterility and toxicity tests were also satisfactory. Potency tests in guinea pigs demonstrated that the cell culture vaccines are at least as effective as the chick embryo vaccines in protecting the animals against an intracerebral challenge of 100 to 1000 LD_{50} doses of virus (Table 1).

Sufficient encouragement is derived

Table 1. Protection of guinea pigs immunized with eastern equine encephalomyelitis vaccine against intracerebral challenge with 100 to 1000 intracerebral LD_{50} doses of virus (S/T, number of survivors per total number challenged).

Lot	Type of vaccine			
	Fluid		Dried	
	S/T	%S	S/T	%S
Whole chick embryo vaccine (purified)				
R-A	3/6	50	2/6	33
R-B	5/6	83	4/6	67
R-C	4/6	67	—	—
R-D	—	—	4/6	67
R-E	9/15	60	11/21	52
Total	21/33	64	21/39	54
Cell culture vaccine (unfiltered)				
CC-A	6/6	100	—	—
CC-B	5/6	83	5/6	83
CC-C	9/10	90	9/10	90
CC-D	6/6	100	—	—
CC-E	5/6	83	4/6	67
CC-F	5/6	83	5/6	83
Total	36/40	90	23/28	82
Cell culture vaccine (filtered)				
CC-D	4/6	67	—	—
CC-E	4/6	67	4/6	67
CC-F	5/5	100	5/6	83
Total	13/17	76	9/12	75

from data for laboratory animals to justify further study of a vaccine prepared from virus propagated in chick embryo cell cultures, for use in the immunization of humans against eastern equine encephalomyelitis.

JOSEPH P. LOWENTHAL
SANFORD BERMAN
EARL W. GROGAN
Department of Biologics Research, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D.C.

References and Notes

1. R. Randall, *Vet. Bull.* **34**, 7 (1940).
2. ———, J. W. Mills, L. L. Engel, *J. Immunol.* **55**, 41 (1947).
3. The excellent technical assistance of Miss Mette Strand and Mrs. June Cole is acknowledged.

9 May 1961

Calcification of the Permanent First Mandibular Molar in Rhesus Monkeys

Abstract. Evidence suggests that in rhesus monkeys calcification in the mandibular permanent first molar commences much earlier than has been previously reported. This tooth is the first secondary dental element to calcify, as it is in man.

To my knowledge there are no studies relating directly to the calcification of either the deciduous or permanent teeth in the rhesus monkey. Perhaps the only exception to this is in the widely used *Anatomy of the Rhesus Monkey* (1933), which has recently been reprinted (*1*). In the chapter on the teeth, Marshall discusses rather briefly the complex problem of tooth calcification. Because of the obvious importance this subject has for the dental researcher, and since there are several inconsistencies in Marshall's presentation, it seems appropriate to present some new observations on the calcification of the permanent first mandibular molar.

In his study of the development of the deciduous and permanent teeth, Marshall made the following comments:

1) In still-born animals of a gestation period ranging from 153 to 169 days, x-rays indicated, "The calcification of the deciduous second molars and of the permanent first molars is just beginning" (*1*, p. 85).

2) In 14 animals between 1 and 2 months old, "There was no evidence at this age of even the beginning of calcification of the permanent teeth" (*1*, p. 85).

3) "The calcification of the second set of teeth was not demonstrated roentgenographically until after the sixth month. At this time the permanent upper central incisors are just beginning to be formed" (*1*, p. 86).

It seemed advisable, therefore, to reexamine these statements in the light of our observations in the developing deciduous and permanent teeth of rhesus monkeys.

To date, 13 animals have been born in our colony at Charleston. Of these 13 animals, four had x-rays taken on the day of birth, whereas the initial x-rays of the other animals were taken at various ages (see Table 1). One male rhesus fetus of approximately 129 days gestation (170 days average gestation period) (*2*) was dissected, cleared, and stained with alizarin red S, the method of Noback and Noback (*3*) being used.

Table 1 presents the findings on the live animals via roentgenography. For two animals the lower molar is listed under "questionable," because the identification was not positive. I believe that the crown tips are present; however, I prefer to record the animals in the "not sure" category. Animal number 109 did not have jaw films taken again until he was 1 year old, at which time the permanent first mandibular molar was well formed. Number 119 was 1 day old at the time this report was written. It should be mentioned that No. 113 has a large, well-formed crown which is clearly visible on the x-ray film. It is evident, therefore, that the present x-ray evidence does not substantiate statements 2 and 3 of Marshall; on the contrary, it suggests a much earlier calcification time for the permanent first mandibular molar, more in line with Marshall's first pronouncement. Incidentally, I have never observed calcification occurring in the permanent maxillary central incisors earlier than in the permanent first mandibular molars, as noted by Marshall in his statement 3. The present findings indicate that the permanent first mandibular molar is the first permanent tooth to calcify, and in this respect the rhesus monkey is similar to man.

The fetal monkey that was dissected would seem to confirm this. While all the deciduous teeth were present and well developed morphogenetically, the crypts for the mandibular and maxillary permanent first molar were discovered. The crypts for the upper molars contained only a membranous dental organ at a stage preceding the formation of hard structures, whereas the mandibular crypts had the membranous dental organ with two of the four crown tips beginning to calcify. X-rays were unable to detect this early formation of calcified material but alizarin red S was absorbed by these minute cusp tips. (For a more elaborate comment relating to this question, see *4*.) These primordial dental organs were characterized by their bilophodont appearance, two mesial and two distal cusps connected by a transverse ridge (*5*). The two calcified cusps would correspond to the mesial buccal (protoconid) and distal buccal (hypoconid) cusps of dental terminology. It is interesting to note that Kraus found the mesial buccal cusp the first to calcify in his investigations of human teeth (*6*, *7*).

Table 1. Calcification of permanent first mandibular molar. In column three, zero indicates that the first x-ray was made at birth.

Animal No.	Sex	Postnatal age at first x-ray (days)	Lower molar
108	F	14	Present
110	F	21	Present
112	F	0	Present
117	M	14	Present
104	F	32	Present
106	F	44	Present
109	M	3	Questionable
111	M	113	Present
113	M	170	Present
115	M	56	Present
119	M	0	Questionable
121	M	0	Present
114	F	0	Present

DARIS R. SWINDLER
Department of Anatomy, Medical College of South Carolina, Charleston

References and Notes

1. C. G. Hartman and W. L. Straus, Jr., Eds., *The Anatomy of the Rhesus Monkey* (Macaca mulatta) (Hafner, New York, 1933, reprinted 1961).
2. J. A. Gavan and G. van Wagenen, *Am. J. Phys. Anthropol.* **13**, 399 (1955).
3. C. R. Noback and E. Noback, *Stain Technol.* **19**, 51 (1944).
4. B. S. Kraus, *J. Am. Dental Assoc.* **59**, 1128 (1959).
5. W. E. Le Gros Clark, *The Antecedents of Man* (Quadrangle Books, Chicago, 1960).
6. B. S. Kraus, personal communication.
7. This research was supported by grants (C-2663 and H-2417) from the U.S. Public Health Service.

1 May 1961