of murine CM. It appears to be part of the cascade of pathogenic events probably initiated by products released by activated L3T4$^+$ T lymphocytes. This experimental model may not reproduce human CM, in which accumulations of packed infected erythrocytes but not of macrophages have been observed in cerebral vessels. However, both phenomena may result from a similar mechanism, such as an increased endothelial adhesiveness, which might reflect TNF-α–mediated vascular alterations. Therefore, TNF-α may also be of pathogenic significance in human cerebral malaria, a possibility supported by the observation of increased serum TNF-α in malaria patients (*15*).

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17. We thank R. Ulevitch for suggestions and helpful discussions, F. W. Fitch for providing anti-L3T4 monoclonal antibody (GK 1.5), and C. Briottet, D. Gretener, and C. Magnin for technical assistance. Supported by grants 3.803.0.86 and 3.614.0.84 from the Swiss National Foundation and by the World Bank United Nations Development Program/World Health Organization Special Programme for Research and Training in Tropical Diseases.

2 March 1987; accepted 8 June 1987

Neurosteroids: Cytochrome P-450$_{scc}$ in Rat Brain

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The steroid hormones corticosterone and testosterone are supplied to the central nervous system by endocrine glands, the adrenals and gonads. In contrast, the 3β-hydroxy-Δ5-derivatives of cholesterol, pregnenolone and dehydroepiandrosterone, accumulate in the rat brain through mechanisms independent of peripheral sources. Immunohistochemical studies have been performed with specific antibodies to bovine adrenal cytochrome P-450$_{scc}$, which is involved in cholesterol side-chain cleavage and pregnenolone formation. The enzyme was localized in the white matter throughout the brain. Scarce clusters of cell bodies were also stained in the entorhinal and cingulate cortex and in the olfactory bulb. These observations strongly support the existence of "neurosteroids," which have been posited on the basis of biochemical, physiological, and behavioral studies.

IN THE MALE ADULT RAT, CORTICOSTERONE and testosterone are secreted by endocrine glands, cross the blood-brain barrier, and are found in the central nervous system in concentrations lower than the plasma levels (*1*). Pregnenolone (Δ5-P) and dehydroepiandrosterone are 3β-hydroxy-Δ5-steroids, which derive from cholesterol by side-chain cleavage (*2*) and which are precursors of steroid hormones secreted by steroidogenic glandular cells. These 3β-hydroxy-Δ5-steroids accumulate in brain, in unconjugated, sulfate, and fatty ester forms, and through mechanisms at least partly independent of peripheral sources. Indeed, they persist after surgical or pharmacological elimination of adrenal and gonadal steroid secretion in rats and monkeys (*3*), and their concentration changes do not depend on peripheral glands in a variety of physiological situations, for example, over a 24-hour period (circadian rhythm) (*4*) and during ontogenetic development. In the olfactory bulb of male rats exposed to females, Δ5-P decreases, an effect that is dependent on testicular testosterone, whereas the female signal disappears after ovariectomy (*5*).

Early attempts to demonstrate the steroidogenic pathway from cholesterol in the brain, and particularly the biosynthesis of Δ5-P, were unsuccessful (*6*). We excluded the eventual role of brain storage derivatives and of extraglandular sources by injecting [^{3}H]Δ5-P subcutaneously and by way of an intracardiac or lateral ventricular routes in adult male rats: [^{3}H]Δ5-P was cleared from brain as rapidly as from plasma (*3, 7*). We were unable to demonstrate the biosynthesis of Δ5-P in brain, perhaps because the location of scarce steroid-producing cells was unknown. As an initial step in the demonstration of pregnenolone biosynthesis in brain, we looked for the presence of specific enzymes involved in cholesterol side-chain cleavage to Δ5-P (*2*). Cytochrome P-450$_{scc}$

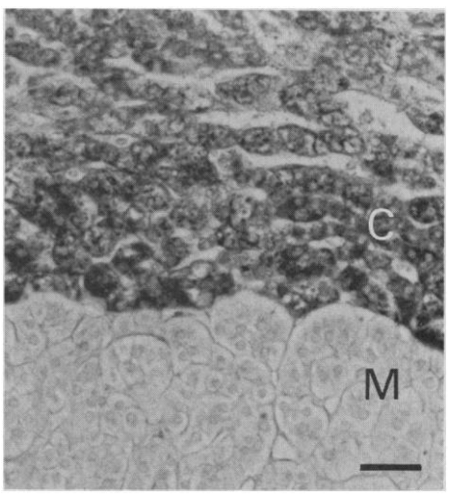

Fig. 1. Immunoperoxidase staining with cytochrome P-450$_{scc}$ antibodies from the adrenal gland of an adult female rat. For the immunohistochemical reaction, deparaffinized 7-μm-thick sections were rehydrated, rinsed in PBS, and incubated in 3% serum from a nonimmune goat. They were then incubated with the rabbit P-450$_{scc}$ antibodies (40 μg/ml, for 2 hours at room temperature). The biotinylated goat secondary antibody was added afterwards (dilution 1:200, 30 minutes), followed by the avidin-biotin-peroxidase complex (dilution 1:100, 30 minutes) (Vectastain reagents, Vector Laboratories). The peroxidase activity was revealed by 3,3′-diaminobenzidine tetrahydrochloride (0.5 mg/ml) in the presence of H_2O_2 (in tris buffer, *p*H 7.4). Sections were not counterstained. They were rinsed, dehydrated, and mounted. Controls were run on adjacent sections placed on the same slide. They included nonimmune rabbit immunoglobulin Gs, dilutions of specific antibody down to extinction of staining, and presaturation of P-450$_{scc}$ antibodies with purified antigen. Scale bar, 40 μm. Abbreviations, C, adrenal cortex (zona reticularis); M, adrenal medulla.

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(side-chain cleavage) is found in mitochondria of all steroidogenic endocrine cells as part of a three-enzyme hydroxylase system, which also includes adrenodoxin reductase and adrenodoxin, an iron-sulfur protein. The presence of the latter has been demonstrated in bovine brain mitochondria together with an undefined cytochrome P-450 detected spectrophotometrically (*8*).

In this work, we have used specific antibodies to cytochrome P-450_{scc} to detect this enzyme in rat brain. There are several cytochromes P-450 involved in steroid hydroxylations, and therefore the antibody specificity had to be carefully checked. Cytochrome P-450_{scc} was purified from bovine adrenocortical mitochondria by the procedure of Seybert *et al.* (*9*), itself based on the method of Suhara *et al.* (*10*). The purified cytochrome P-450_{scc}, which showed a single band after SDS–gel electrophoresis, was injected into the popliteal lymph nodes of rabbits. Booster injections were made subcutaneously at 1-month intervals. The immunoglobulin G fraction of the antiserum was prepared by precipitation with 33% ammonium sulfate. Only one precipitation line was formed in Ouchterlony double-diffusion analysis, indicating homogeneity of the antigen (*11*). Neither purified P-$450_{11\beta}$, the other steroidogenic mitochondrial P-450, nor newly synthesized P-450_{C21}, the microsomal enzyme of 21-steroid hydroxylation, competed with purified P-450_{scc} for binding to antibodies to P-450_{scc}. Western blot analysis of crude preparations with antibodies to P-450_{scc} revealed a protein of a molecular size of 49,000 daltons, corresponding to the molecular weight of purified mature bovine P-450_{scc}, a result confirmed after cell-free translation of adrenocortical polyadenylated RNA followed by maturation of the native protein in adrenocortical mitochondria. The peptide map of P-450_{scc} isolated by immunoprecipitation, determined after radiolabeling of adrenal cell proteins, was the same as that of the purified protein. Finally, several experiments have demonstrated an increased synthesis of the protein recognized by the antibody in steroidogenic tissues after stimulation by the appropriate peptide hormones or by adenosine 3′,5′-monophosphate.

However, even though there are marked structural homologies between bovine and rat cytochrome P-450 and even though immunoblots are positive when rat ovarian enzyme is tested with antibodies specific to bovine adrenocortical P-450_{scc} (*11*), we checked that our antibodies could be used for the immunohistochemical staining of the rat enzyme. Sections of fixed, paraffin-embedded rat adrenals tested by an immunoperoxidase technique showed positive staining of the cytoplasm in all adrenocortical cell types (especially the zonae fasciculata and reticularis) by the P-450_{scc} antibodies; adrenal medulla cells showed no such staining (Fig. 1). This result confirmed the observations made with bovine adrenal cortex (*12*). Antibody-specific staining also occurred in luteal, thecal, and interstitial cells, but not granulosa cells, of rat ovaries.

The immunohistochemical experiments with rat adrenals and ovaries were also useful to optimize the fixation conditions, the working dilutions, and exposure times of the primary and secondary antibodies, and the final peroxidase reaction. The most criti-

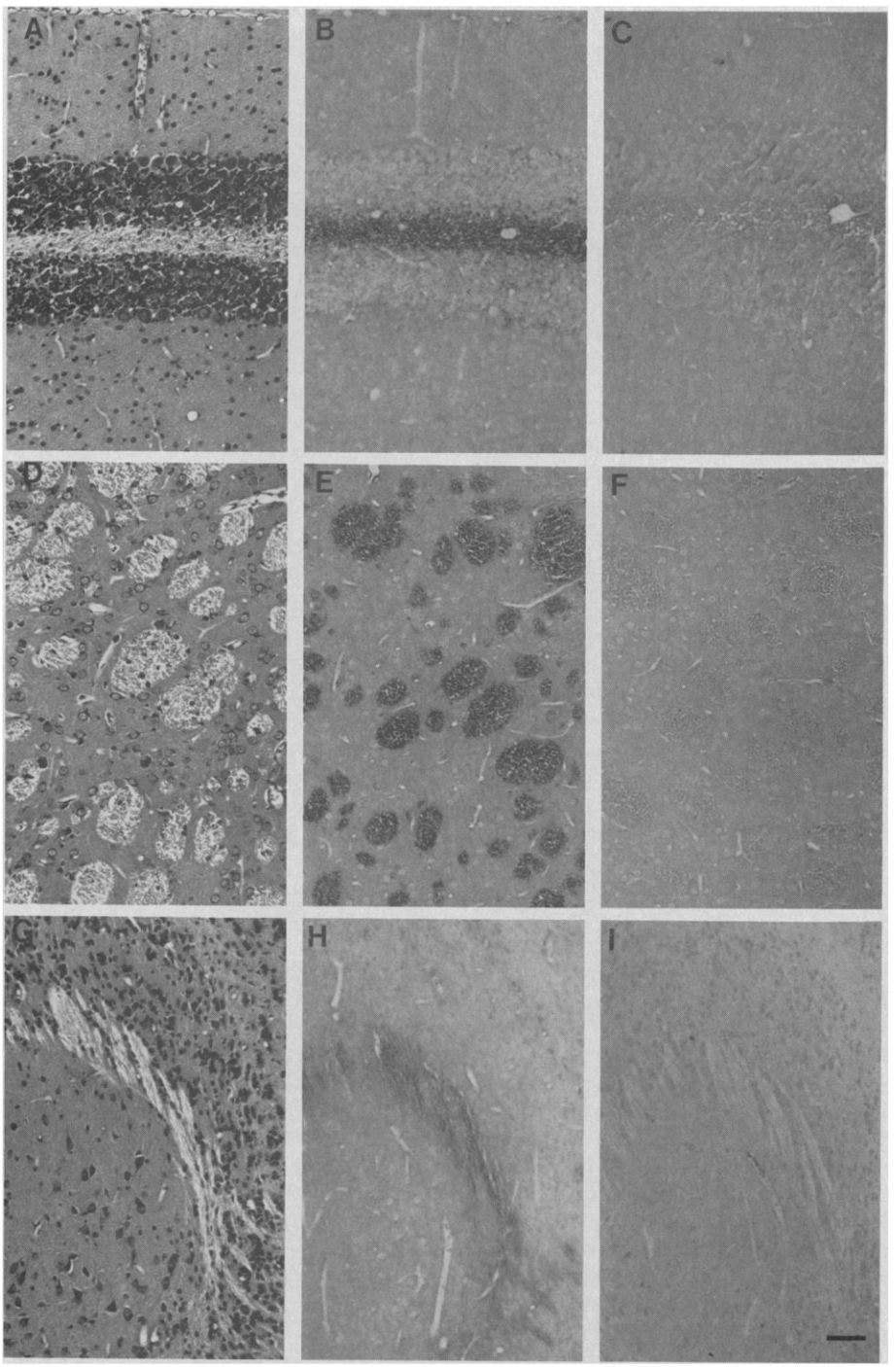

Fig. 2. Immunoperoxidase staining with cytochrome P-450_{scc} antibodies of myelinated regions in the rat brain. (**A**, **B**, and **C**) Cerebellum; (**D**, **E**, and **F**) caudate nucleus; (**G**, **H**, and **I**) olfactory bulb. (A, D, and G) Histological staining with Masson's trichrome; (B, E, and H) immunoperoxidase staining (the immunohistochemical technique is as in Fig. 1); (C and F) the P-450_{scc} antibodies were preincubated with a saturating concentration of purified antigen; (I) nonimmune immunoglobulins have been substituted for the specific immunoglobulins. Scale bar, 50 μm.

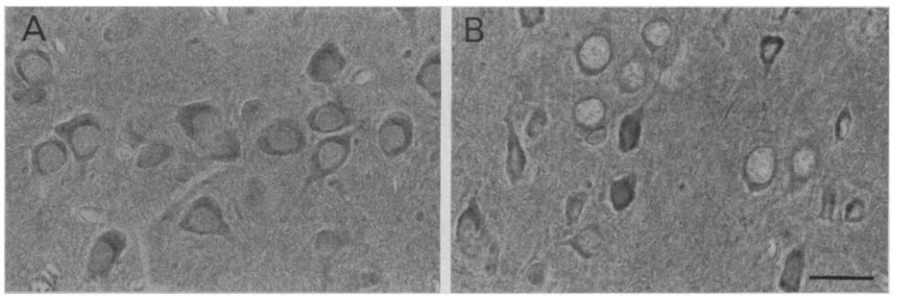

Fig. 3. Immunoperoxidase staining with cytochrome P-450_{scc} antibodies of cell bodies in the cerebral cortex. (**A**) Entorhinal cortex; (**B**) cingulate cortex. The immunohistochemical technique is as in Fig. 1. Scale bar, 20 μm.

cal point was to determine which fixation procedure could provide both preservation of the tissue and the best immunohistochemical detection of the enzyme. A rapid perfusion of the brain with formaldehyde followed by Carnoy's fixative was chosen. Three young adult (4 to 6 weeks old) male Sprague-Dawley rats were anesthetized with pentobarbital. Formaldehyde [3.7% in phosphate-buffered saline (PBS)] was perfused through the left ventricle. The brain was removed, divided in several parts, and fixed in Carnoy's solution for 2 to 24 hours. After dehydration in graded ethanol series and in 1-butanol, each part was separately embedded in paraffin. Deparaffinized 7-μm-thick sections were obtained and treated as indicated in Fig. 1. In the brain, as in steroidogenic organs, the specific immunoreaction was inhibited by preincubation of P-450_{scc} antibodies with purified bovine adrenal P-450_{scc}. Nonimmune rabbit immunoglobulin Gs did not give any staining at concentrations up to 80 μg/ml. Routinely, P-450_{scc} antibodies were used in the range of 10 to 40 μg/ml.

The myelinated regions of white matter were characterized by selective staining with Luxol fast blue and with Weigert's hematoxylin containing lithium carbonate, whereas the histological staining of the cerebellum with Masson's trichrome (Fig. 2) showed the white matter as a white area on the photomicrograph, delineated on both sides by the dark granular layers and more distantly by the molecular layers. The immunoperoxidase staining exhibited an intense reaction restricted to the white matter zone. Similarly, in frontal sections of the caudate nucleus, the tracts of myelinated fibers, cut perpendicularly, appeared relatively clear after the histological staining, whereas they were intensely stained by the immunohistochemical reaction. In the olfactory bulb, the myelinated fibers were obliquely sectioned, and again the immunohistochemical staining for cytochrome P-450_{scc} was restricted to the areas of myelinated fibers. Similar results were obtained in the lateral portion of the olfactory tract, in the anterior commissure, in the outlets of cranial nerves, and more generally in the white matter throughout the brain.

Scarce cell bodies were labeled with P-450_{scc} antibodies in the olfactory bulb. Clusters of positively stained cells were observed in two other regions of the cerebrum: the entorhinal cortex and the cingulate cortex (Fig. 3). The cells of the entorhinal cortex were large, with a big spherical nucleus. In the cingulate cortex, immunoreactive cells were fewer than in the entorhinal cortex, and they were smaller with an irregular shape. In all immunoreactive cells, the staining was restricted to the cytoplasm. No region of the central nervous system outside the cerebrum has been studied so far. The assignment of these cell bodies to a specific brain cell type (neuronal or glial) awaits further investigation.

The immunohistochemical detection of adrenodoxin has also been observed in the same brain areas as that of P-450_{scc} with the use of specific antibodies.

These immunohistochemical results support the existence of neurosteroids and imply that the persisting presence of Δ5-P in the brain that occurs after the removal of adrenals and gonads, results from a process intrinsic to the central nervous system. Indeed, we have previously shown [^{3}H]Δ5-P formation by the rat C6 glioma cell line, incubated with [^{3}H]mevalonolactone or [^{3}H]cholesterol (*4*). Interestingly, under identical incubation conditions, negative results were obtained with two neuroblastoma and one fibroblast cell lines. While this report was being prepared, preliminary but direct evidence has been obtained, indicating that the mitochondria of oligodendrocytes indeed can make Δ5-P from cholesterol (*13*).

The concentration of Δ5-P in different parts of the adult male rat brain is of the order of 50 pmol per gram of tissue for both the unconjugated plus sulfate and for the lipoidal fraction. It is larger in the olfactory bulbs and hypothalamus and lower in the cerebral cortex but still remains within the same order of magnitude. These data are consistent with an ability of oligodendrocytes to make Δ5-P and may suggest a paracrine modulation of neuronal functioning for this steroid.

However, regulatory changes of Δ5-P concentration selectively occur in defined parts of the brain (*5*). Both the cellular distribution reported here for cytochrome P-450_{scc} and the Δ5-P changes recorded in different parts of the brain of male rats exposed to other male or female rats (*6*) might suggest the involvement of this steroid in an olfactory pathway related to sex recognition. There are at least three possible mechanisms for Δ5-P action. (i) It could be inserted into cell membranes and change their functioning. (ii) It may be transformed into progesterone or other steroidal derivatives, which themselves could be active (*5*). (iii) It could bind to an as yet uncharacterized intracellular or membrane receptor. The latter site of action is suggested by the recent demonstration that Δ5-P sulfate interacts with the γ-aminobutyric acid (GABA) receptor and behaves as an antagonist of GABA-mediated neurotransmission (*14*), under conditions that may have physiological significance.

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25 February 1987; accepted 9 June 1987

An Upper Eocene Frog from the Dominican Republic and Its Implication for Caribbean Biogeography

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A frog of the leptodactylid genus *Eleutherodactylus* is reported from Eocene amber found in the Dominican Republic. It is the first described amphibian fossil in amber, and the oldest complete lissamphibian fossil from Mesoamerica (Central America and Mexico). Dating of the amber matrix indicates that by the end of the Eocene a diverse fauna was present in the Antilles, much earlier than has generally been proposed. The presence of this and other amber fossils from this same age suggests that Tertiary patterns of landmass movements were significant in determining the present distribution of species.

THE ORIGIN AND EVOLUTION OF THE highly endemic biota of the West Indies has been under debate in recent years. Two principal models, vicariance and dispersal, have been proposed to account for present faunal distributions. Under the vicariance model, the distribution of the present-day biota among the Caribbean islands is the result of a breakup of a contiguous "proto-Antillean archipelago" that was located between North and South America in the Mesozoic; the biological history of the Antilles is closely linked with its historical geology. An early formulation of this hypothesis held that ancestors of the Recent fauna were present in the "proto-Antilles" by the Late Cretaceous or early Tertiary (*1*). Current applications of the vicariance theory to Caribbean biogeography lead to testable predictions about the relations of biotas of the various islands on the basis of a reconstruction of landmass fragmentation and accretions throughout the past 65 million years (*2*).

In contrast, the more traditional dispersal model argues that the islands were colonized recently from the mainland (*3*). Although this model predates the general acceptance of plate tectonics (*4*), advocates of the dispersal model do not dispute the evidence for continental drift. Rather, the timing of the immigrations is thought to be post-Eocene and therefore relatively recent because the mainland fossil record for extant genera is not earlier than the Oligocene (*5*). Therefore, the fossil record suggests a mid-Tertiary date for the appearance of modern genera on the continents, and subsequent colonization of the Antilles. Additional evidence supports the dispersal viewpoint. The Antilles have been islands probably throughout most of the Cenozoic (*6*); this suggests that their inhabitants immigrated over water. Although the Pleistocene fossil record is quite extensive, there are almost no fossil terrestrial vertebrates older than the Pleistocene in the West Indies (*7*). The two exceptions are lizards of the genera *Anolis* and *Sphaerodactylus* in amber from the Dominican Republic. The *Anolis* was reported as Miocene (*8*), and the *Sphaerodactylus* as Oligocene (*9*), but new data have refined the date of these fossils.

A nearly complete fossil of the frog genus *Eleutherodactylus* was found in amber from the Dominican Republic, on the island of Hispaniola. The major source of Dominican amber is a leguminous tree, *Hymenaea*, which may have become established in the Caribbean from African relatives in the Tertiary (*10*). The specimen (*11*) was collected from the La Toca amber mine, located between Santiago and Puerto Plata in the Cordillera Septentrional. The mine is in the Altimira facies of the El Mamey Formation (upper Eocene), which is shale-sandstone interspersed with a conglomerate of well-rounded pebbles (*12*). In contrast, the presence of the marine foraminiferan *Miogypsina* in a matrix sample from another amber mine (Palo Alto) suggests a lower Miocene to upper Oligocene age (20 million to 23 million years) (*13*); analysis of foraminifera counts from the same mine also indicates a minimum age of lower Miocene (*14*). Thus, sedimentary and geological evidence indicate a range of upper Eocene to lower Miocene for various amber mines in the Dominican Republic.

Differences in the magnitude of absorption peaks of the exo-methylene group of amber from different mines in the Dominican Republic have been shown by nuclear magnetic resonance spectroscopy. From the 20-million- to 23-million-year-old figure obtained for Palo Alto amber as a calibration, a range of 15 million to 40 million years was determined for various specimens of Dominican amber, with that from the La Toca mine being the oldest, 35 million to 40 million years old (lower Oligocene to upper Eocene (*15*). This date agrees with the upper Eocene age assigned to the El Mamey Formation on the basis of the stratigraphic evidence mentioned above. Amber from different mines also varies in certain physical properties, such as color and hardness; the order of hardness and color scale of La Toca amber correlates well with chronological sequence established by analysis of nuclear magnetic resonance spectra (*15*). In summary, the evidence unequivocally indicates the presence of a fauna in the Antilles at a date much earlier than that postulated by the dispersal model.

A series of chemical and physical tests (*16*) performed on a small portion of the amber piece verify that it is authentic. In addition, the specimen has all of the visual characteristics of natural Dominican amber, as judged by the examination by G.O.P. of more than 15,000 pieces of amber with biological inclusions. The piece of yellow, transparent amber containing the frog weighs 36 g and is 5 cm by 5.5 cm (Fig. 1). The specimen measures 22.2 mm from the tip of the snout to the end of the urostyle (the approximate location of the vent). The right arm is broken at the distal end of the humerus and the left leg is broken at the tip of the tibiofibula. Events leading to the entombment of the frog were probably traumatic. Possibly the frog was captured by a predator (possibly a bird) and brought back to a nest in the cavity of a resin-producing tree. The frog came into contact with the resin before being eaten, and a portion of the predator's nest was also covered by the resin. The latter statement is suggested by the fact that in the piece of amber opposite the frog were three decomposing leg bones of another individual of *Eleutherodactylus* (Fig. 1). Numerous fly maggots and the remains of a large centipede were also preserved. Portions of the skin and the eyes of the frog are intact, although much of the skin has become transparent, rendering fine details of the skeleton visible (Figs. 2 and 3).

Low-voltage radiographs of the specimen indicate that the frog was an adult, as evidenced by the degree of ossification of the skeleton. Details of the osteology (shape of the vomer and its dentition, features of the vertebral column, pectoral and pelvic gir-

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