

## Kernicterus in Asphyxiated Newborn Rhesus Monkeys

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Newborn rhesus monkeys have "physiological jaundice," reticulocytosis, lower level of glucuronyl transferase in the liver and slower bilirubin clearance time than adults. We delivered monkey infants by cesarian section near term and made them hyperbilirubinemic by injecting a solution of indirect-reacting bilirubin every 6 hours from 1 to 3 hours after birth for as long as 4 days. Marked jaundice developed in 6 hours; slight lethargy but no other clinical neurological signs and no kernicterus were seen. Other monkey infants were asphyxiated for 10 or 12 min during cesarian-section birth, resuscitated and then made hyperbilirubinemic. These exhibited neurological deficits clinically, with abnormal EEG, and had kernicterus. The most severely affected ones were lethargic for 12 hours and then developed tremors, seizures and prolonged opisthotonos. Brain slices showed selective canary-yellow staining of certain nuclei of the diencephalon, midbrain, brain stem, spinal cord, cerebellum and some regions in the cerebrum. Microscopical examination of frozen sections revealed bilirubin-pigmented neurons and neuroglia cells. Electron micrographs showed nerve cells in early stages of lysis. Probably asphyxia is only one of

<sup>1</sup> The proposal to explore the possibility of inducing kernicterus in newborn monkeys rendered hyperbilirubinemic experimentally was made by Dr. Lucey, who participated in most of the physiological experiments with Dr. Behrman. The neurological studies were conducted by Dr. Esquivel. All anatomical pathological observations were made by Dr. Hibbard and Dr. Windle; electron microscopy by the former. Dr. Behrman and Dr. Windle principally are responsible for this manuscript. All authors gratefully acknowledge the help of Dr. R. D. Fleischman and that of many other members of the staff of the Laboratory of Perinatal Physiology. Dr. Behrman's present address is The Johns Hopkins Hospital, Baltimore, Maryland. Dr. Esquivel is a NIH Visiting Scientist. Dr. Lucey is a John and Mary R. Markle Scholar in Medical Sciences at the University of Vermont in Burlington, Vermont; while in San Juan he was Visiting Associate Professor of Pediatrics in the University of Puerto Rico.

several agents causing cellular injury to bring about the picture of kernicterus in the presence of excess bilirubin in the blood.

### Introduction

Metabolic disturbances culminating in the pathological condition known as kernicterus have been held responsible for the brain damage in some patients with mental retardation. These have not lent themselves readily to investigation in the clinic because of their variability and complexity, and an understanding of kernicterus has been impeded by lack of a satisfactory experimental model. Indeed, it has not been possible to produce consistently in animals a pattern of canary-yellow pigmentation of brain-stem nuclei comparable to that seen post-mortem in the kernicteric human infant.

Whether antecedent damage to the central nervous system is a prerequisite of the clinical and neuropathological picture observed when hyperbilirubinemia is associated with kernicterus in human infants has been debated for some time. A number of factors—often several in combination—have been closely associated with the incidence of kernicterus. Some of these are the presence of Rh hemolytic disease, prematurity, ABO blood-group incompatibilities, hyperbilirubinemia secondary to deficiency of glucuronyl transferase, prenatal anoxia, sepsis, and the use of such drugs as sulfisoxazole.

We entertained the view that the staining of brain tissue by bilirubin reflects an abnormal *in vivo* cerebral metabolism rather than a simple change in permeability of the blood-brain barrier (8). Neonatal asphyxia thus might be one agent acting in combination with a high serum-bilirubin concentration through a common pathway of neuronal injury to produce the lesions characteristic of kernicterus. Experimentally induced asphyxia during birth of rhesus monkeys results in a neuropathological syndrome (23) involving bilaterally many of the brain-stem nuclei in which bilirubin pigment is encountered in human kernicterus (12, p. 177). This encouraged us to explore the functional and structural changes that occur when asphyxia at birth is followed by hyperbilirubinemia.<sup>2</sup>

The results of studies of intrauterine transport of bilirubin (15), its metabolism after birth (17), and some of the physiological consequences of bilirubin infusion in the newborn monkey (2) have been reported. The monkey has "physiological jaundice" and reticulocytosis at birth. There

<sup>2</sup> Preliminary reports on kernicterus in asphyxiated newborn monkeys have been published (16,24).

are lower levels of glucuronyl transferase in the fetal and neonatal than in the adult liver. The newborn monkey can clear its plasma of a bilirubin load less readily than the adult. Thus it appeared to be a suitable animal for investigation of kernicterus.

### Methods

The monkey infants (*Macaca mulatta*) in our experiments were delivered near term by cesarian section under local anesthesia<sup>3</sup> of healthy females with known conceptual dates in a caged breeding colony at San Juan, Puerto Rico.

Hyperbilirubinemia was induced in fourteen of them (one partially asphyxiated) by injecting a solution of indirect-reacting bilirubin (2 mg/100 ml) into umbilical or femoral veins every 6 hours. Crystalline bilirubin (Eastman) was used. The methods for preparing the solution for infusion and estimating the bilirubin concentration have been described (17). Serum samples were obtained before and after each injection. The infusions were started 1 to 3 hours after birth and were continued for as long as 96 hours. Serum levels of 20 to 35 mg of bilirubin per 100 ml were attained promptly in most. Eleven of the animals were killed by perfusion with a solution of formaldehyde after varying durations of hyperbilirubinemia; three others, not until the serum bilirubin had returned to lower levels (Fig. 1). The brains of all were examined.

Six healthy full-term monkeys were asphyxiated at birth. A rubber bag filled with saline solution was placed over the fetal head as it was delivered from the uterus before the first breath. The umbilical cord was then clamped and asphyxiation carried out for 10 or 12 min, after which time the bag was removed, the trachea intubated, and the monkey resuscitated. Thereafter, as in the preceding group, hyperbilirubinemia was induced in the monkey infants. A catheter was placed in the umbilical vein, the infusion started 2 to 4 hours after birth and continued for 30 to 50 hours (Fig. 2). Five were killed by perfusion-fixation and the other was allowed to die without perfusion, the brain being fixed by immersion. A seventh monkey, inadvertently partially asphyxiated during a cesarian section but requiring no resuscitation after delivery, was made hyperbilirubinemic and later killed by perfusion.

During the injection of the bilirubin solution, the animals were re-

<sup>3</sup> The skin of the abdomen and other layers to be incised were infiltrated with a solution of procaine hydrochloride, and the surgical delivery was conducted with less indication of distress or pain than in spontaneous vaginal births of monkeys.

strained on a soft towel placed on a heating pad. Between injections they were cared for around-the-clock in the nursery (13), where they were observed at frequent intervals. They were given no nourishment except glucose and water during the experiments. Neurological examinations, recording of EEG and EKG, and cinematographic recording were conducted as indicated by the condition or behavior of the animals.<sup>4</sup>

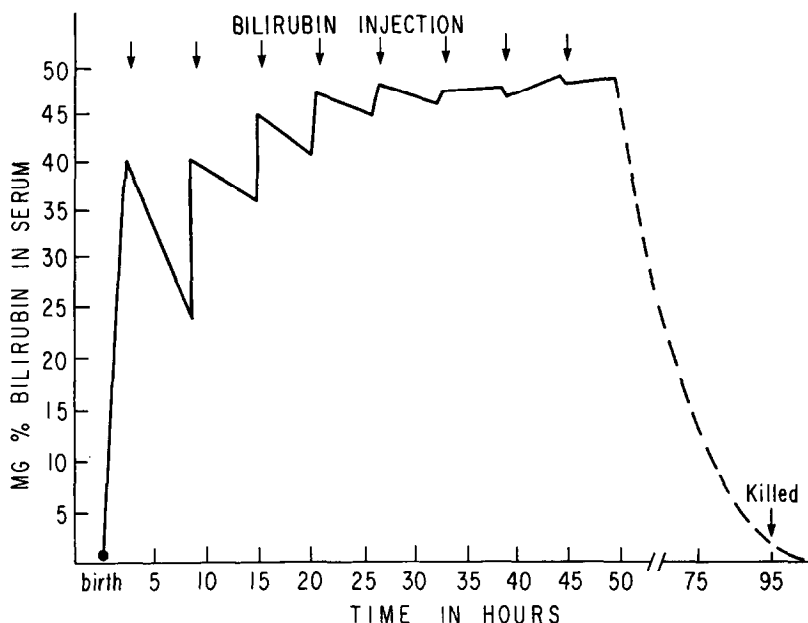


FIG. 1. Serum-bilirubin concentration in the plasma achieved by repeatedly injecting (arrows) a solution of indirect-reacting bilirubin in a healthy female monkey weighing 444.5 gm at delivery by cesarian section at 154 days of gestation. No kernicterus developed.

Twelve hours or more were permitted to elapse after killing the monkeys by perfusion with a solution of formaldehyde before performing the autopsy. This permitted fixation of brain tissue to take place in situ from the fluid-filled vascular bed (4). The brain and spinal cord were then removed, placed in a small quantity of the fixing solution, and shipped by air-express to our laboratory in Bethesda, Maryland, where further studies were conducted. The brain stem with cerebellum attached was removed

<sup>4</sup> Details of individual experiments of the present series will be provided by the Laboratory of Perinatal Physiology upon request.

by a cut at the rostral end of the midbrain and subdivided by several transverse slices; the cerebrum was cut into six or more coronal sections and photographed (Fig. 5). Frozen sections were prepared and examined microscopically, first without staining and then stained by various techniques including hematoxylin and thionine.

One monkey was perfused with a solution of glutaraldehyde (21) and the brain was removed and sliced. Small pieces then were removed from

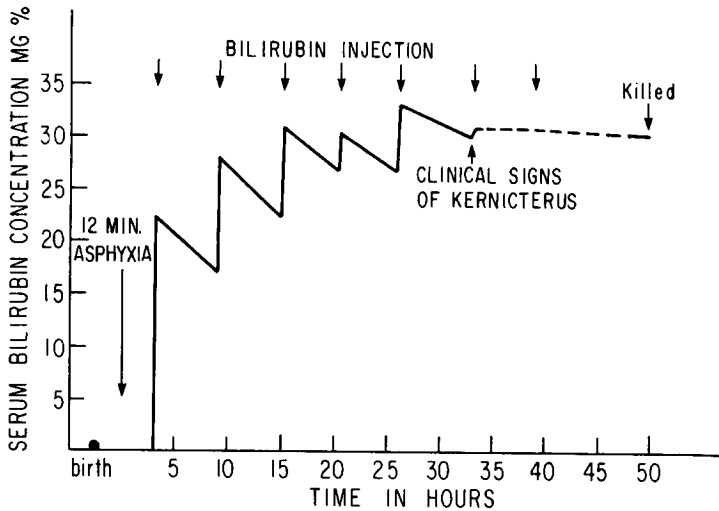


FIG. 2. Serum-bilirubin concentration in the plasma achieved by repeatedly injecting a solution of indirect-reacting bilirubin in a male monkey (Fig. 3) weighing 548 gm, asphyxiated for 12 min during delivery by cesarian section at 161 days of gestation. Clinical neurological signs of kernicterus appeared at 33 hours. The EEG records of this monkey are illustrated in Fig. 4; neuropathology, in Figs. 5A-D.

regions showing yellow pigmentation; these were osmicated and thin sections were stained with lead hydroxide or potassium permanganate for study with the electron microscope (Zeiss EM9).

## Results

*Clinical Observations.* All the newborn monkeys that were made hyperbilirubinemic showed marked yellow coloration of skin and mucous membranes within 6 hours. Most nonasphyxiated ones became slightly lethargic but none developed other clinical or neurological signs. No abnormalities of the EEG were encountered in the ten in which recordings

were made. The bilirubin solvent alone induced no changes in the EEG in two that were tested.

Three of the monkey infants in which birth asphyxia preceded induction of hyperbilirubinemia were moderately lethargic during the first 12 hours and then suddenly developed tremors, focal seizures, jerks which rapidly progressed to generalized tonic spasms, and prolonged periods of opisthotonos (Fig. 3). The monkeys were weak and flaccid between seizures; later opisthotonos could be elicited by pinching the skin of the back. A fourth animal developed a similar syndrome without focal seizures. It was more lethargic than the others, but became hyperirritable intermittently;



FIG. 3. Newborn monkeys: normal control on right, with kernicteric animal during an opisthotonos seizure on left. See Figs. 2, 4 & 5A-D.

the periods of opisthotonos were shorter and could be provoked by lightly pinching the skin of the back. The course in two other animals was characterized only by severe lethargy and alternating periods of hyperirritability; no other neurological signs appeared. One was hyperbilirubinemic for 2 days but the serum-bilirubin concentration rarely exceeded 15 mg per 100 ml. The partially asphyxiated monkey was depressed, but showed no marked clinical neurological deficits.

All the hyperbilirubinemic asphyxiated monkeys, including the one requiring no resuscitation, showed abnormalities in the EEG.<sup>5</sup> The normal maturation of the EEG in rhesus monkeys had been determined (19), providing a reference for comparison with the records from the

<sup>5</sup> We could find no descriptions of EEG abnormalities during acute stages of human kernicterus. Cruz Hernández and his associates (6) have reported changes in the recordings from some newborn infants with jaundice not associated with kernicterus.

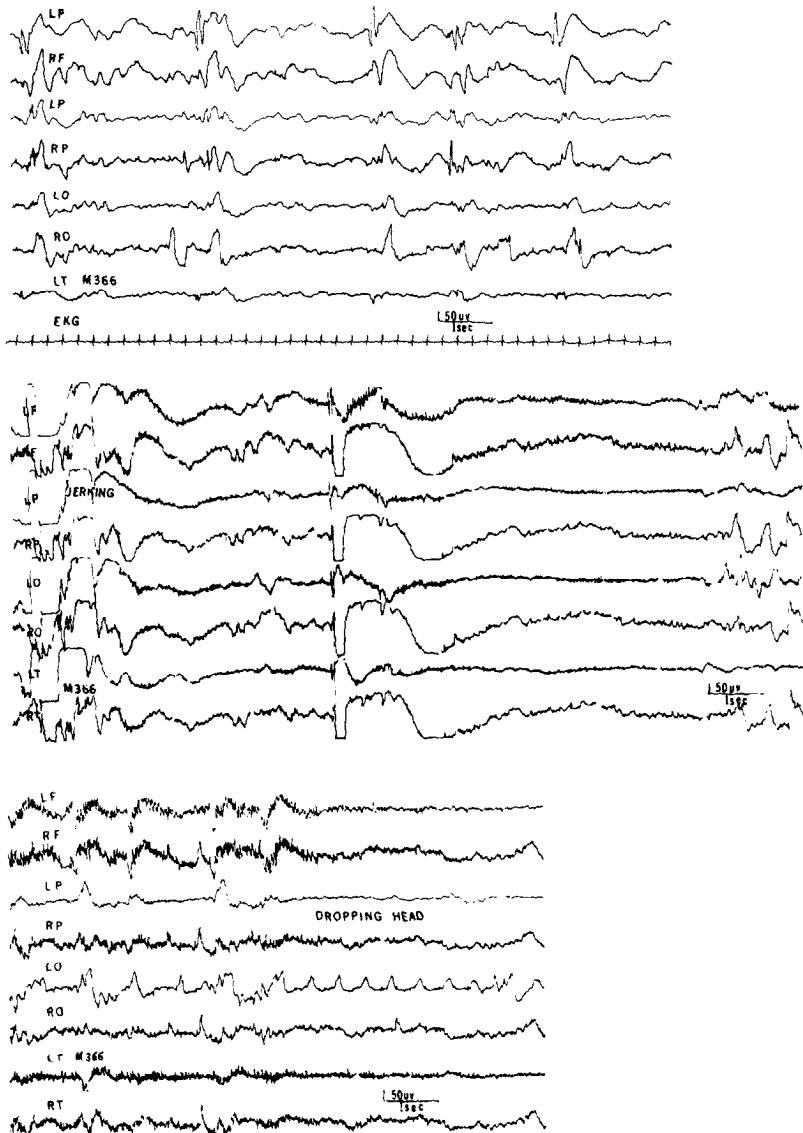


FIG. 4. Electroencephalograms from the asphyxiated hyperbilirubinemic monkey shown in Fig. 3. Upper: during a resting period before a crisis. Middle: beginning and during an opisthotonos seizure. Lower: after the seizure. Explanation in text.

icteric animals. Figure 4 is representative of events seen in the EEG of newborn monkeys with opisthotonos seizures. The animal from which these records were taken was in status epilepticus during which seizures occurred one after another. Suddenly opisthotonos began with movement of the head to one side followed by hyperextension of the head and back. Respiration was light and the EKG was normal during the seizure. The head dropped forward at the end of the seizure, and jerking movements occurred in the intervals between crises. The EEG records were characterized by flattened traces from all leads, interrupted by paroxysmal discharges of diphasic or triphasic spikes or sharp and slow wave complexes which were more marked from the right central frontal region. The discharges came singly or in bursts and the background rhythm was depressed between them. Muscle artifacts appeared just before and at the end of the crisis and were followed by flattened traces. A generalized depressed pattern characterized by desynchronization and disappearance of the paroxysmal discharges was seen during the tonic phase of the opisthotonos seizure. Photoc stimuli inhibited seizures. Loud sounds elicited a reaction from the animal but did not provoke a crisis.

*Pathological Findings.* Hyperbilirubinemia alone did not result in selective staining of nuclei in the brain, such as is associated with human kernicterus. After perfusion-fixation, the brains had a diffuse, faint to moderate, yellow color, but no extravascular bilirubin was seen histologically. In some specimens a few crystals of the pigment were trapped inside vessels at junctures of arterioles and capillaries. No cellular alterations such as chromatolysis were encountered in the brains. These observations were consistent in all of the thirteen nonasphyxiated monkeys.

All of the seven monkeys asphyxiated prior to induction of hyperbilirubinemia exhibited selective canary-yellow staining of nuclei in the

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FIG. 5. Representative photographs of specimens from asphyxiated hyperbilirubinemic monkeys illustrating the neuropathology of kernicterus; bilaterally symmetrical canary-yellow staining of nuclei characterized all asphyxiated hyperbilirubinemic newborn monkeys. A-D: brain of monkey shown in Fig. 3 (also Figs. 2 and 4), killed by perfusion after exhibiting marked clinical signs of kernicterus. E: section through the cerebrum of an asphyxiated icteric monkey allowed to die during an experiment. F: brain slices from another kernicteric monkey killed by perfusion. G: yellow-staining astrocyte from the medial vestibular nucleus of a kernicteric monkey; frozen section stained with thionine. H: neurons of the dentate nucleus of a kernicteric monkey (same as E), showing bilirubin in the cytoplasm; frozen section stained with hematoxylin.



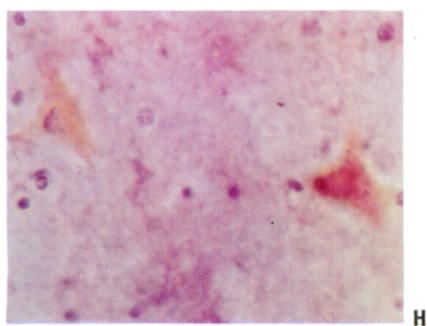
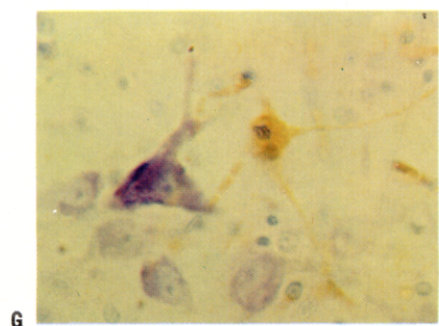
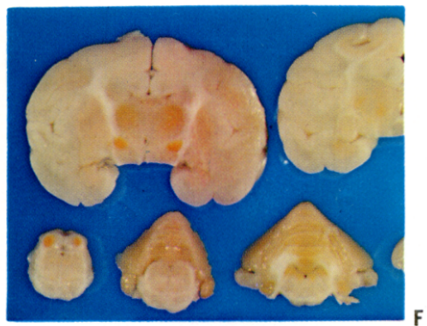
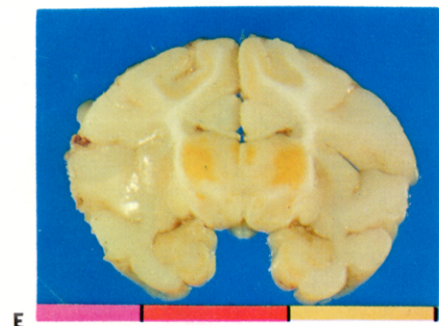
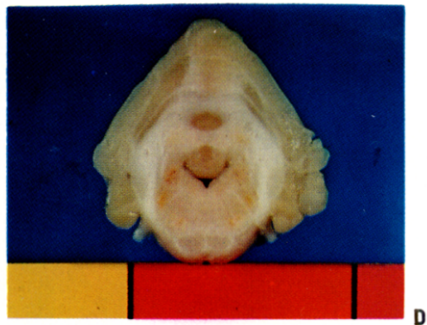
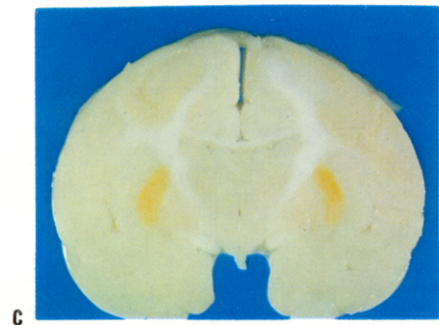


Figure 5

brain. The one that had been lightly asphyxiated and required no resuscitation presented the least impressive picture. Grossly the brain was pale yellow and slices revealed yellowing of the dentate nucleus, cerebellar vermis, oculomotor-interstitial nucleus complex, lateral thalamic nuclei, and medial geniculate body, but none was apparent in the basal ganglia; the inferior colliculi and some of the other commonly affected nuclei were not examined in gross sections. Frozen sections showed no bilirubin in cells, but did reveal crystals in blood vessels within the nuclei that were selectively stained.

Grossly the brains of the five other monkey infants killed by perfusion had a light yellow color with darker areas in some places (Fig. 5 A). That of the one permitted to die was darkly and evenly pigmented with bilirubin. Brain slices revealed selective staining of certain nuclei bilaterally in the diencephalon, mesencephalon, medulla oblongata and cerebellum. The neocortex (precentral gyrus) and hippocampus (zones H<sub>2</sub> and H<sub>3</sub>) were involved in some of the brains. The most striking canary-yellow color was seen in the basal ganglia, thalamus, subthalamic and inferior-collicular nuclei, certain cranial-nerve nuclei (e.g., trigeminal afferent, medial vestibular and spinal vestibular), cuneate nuclei, superior and inferior olivary complexes, dentate and roof nuclei and vermis of the cerebellum. Not all of these were found in each specimen, partly because the brain knife did not pass exactly through the same regions in each case. There was also individual variation; for example, the putamen was more affected than the globus pallidus in one, while the reverse was the case in another. Some centers were affected in only one or two specimens, e.g., the lumbar spinal gray columns. Figure 5 B-F shows representative gross sections through the brains of three kernicteric monkeys.

Microscopical examination of frozen sections revealed yellow coloration of the brain tissue. Bilirubin appeared to be nonspecifically and diffusely distributed in some regions, but elsewhere was concentrated in cells. It was deposited in the cytoplasm of some of the nerve cells (Fig. 5 H). However, neuroglia cells, especially astrocytes, were more brilliantly colored than nervous (Fig. 5 G).

Photomicrographs and electron micrographs were obtained from tissues removed from yellow-stained regions of the brain of the animal perfused with a solution of glutaraldehyde. (This kernicteric monkey infant had survived for 48 hours after asphyxiation.) Light-microscopy revealed a microglial response to the asphyxial damage (Fig. 6). Electron micrographs showed an early stage of cytolysis in the affected inferior colli-

culus (Fig. 7) and subthalamic nucleus. Neuronal degeneration was indicated by swollen neuronal cytoplasm with disorganized and dispersed endoplasmic reticulum, vesiculated golgi complex, scattered filamentous components and irregularly swollen cell processes. Mitochondria and the nucleus appeared to be intact. Perivascular microglia cells, seen here and there, contained large dark masses in the cytoplasm resembling lipid



FIG. 6. A section through the lesion in the inferior colliculus of a monkey 48 hours after subjection to asphyxia followed by hyperbilirubinemia. Neurons in the center have become hypochromatic or atrophic and a marshalling of microglia has occurred around the periphery. Electron micrographs shown in Fig. 7 are of tissue taken from the corresponding region of the opposite side of this animal.

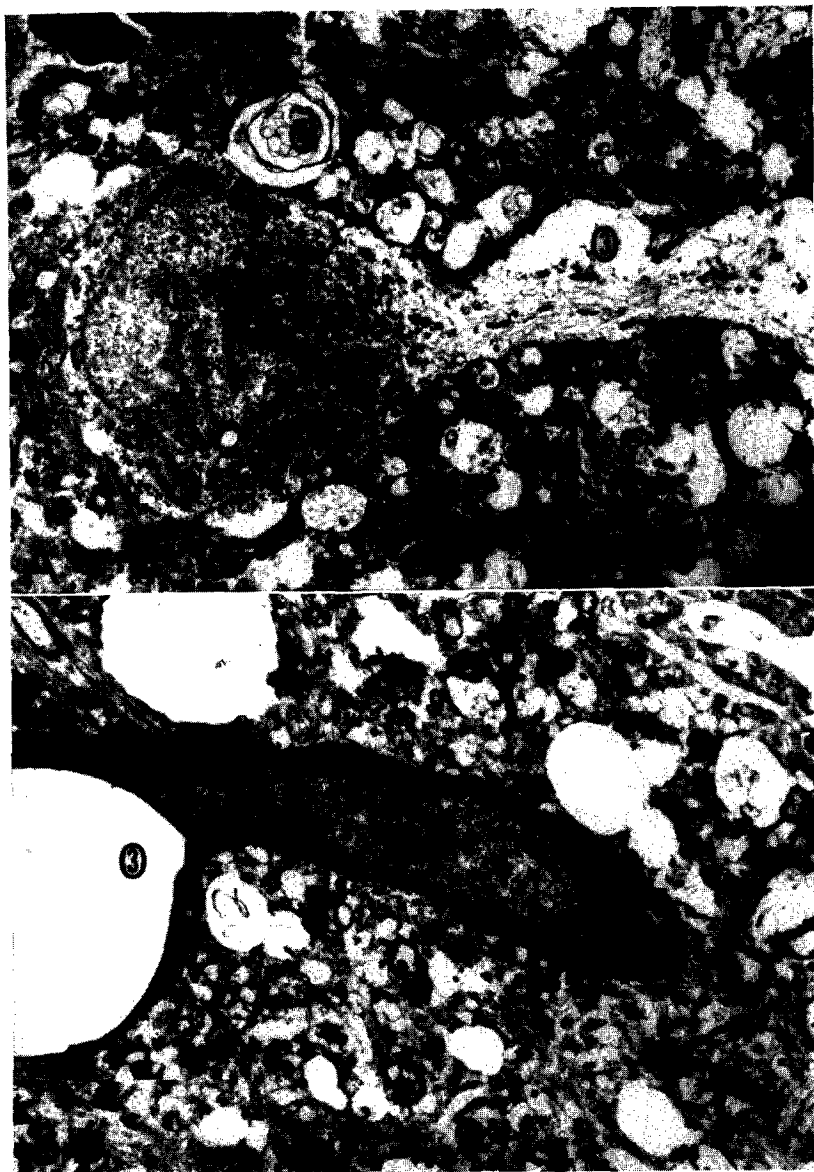


FIG. 7. Low power electron micrographs of a lesion in the inferior colliculus of a monkey subjected to asphyxia and hyperbilirubinemia. A: a damaged neuron (1) with Nissl-free blebs at the periphery of the perikaryon (2) and along one of the processes (3). A degenerating myelin sheath is seen at (4). B: a phagocytic microglia cell with an elongated nucleus (1) and containing a large lipid inclusion (2) is found in the necrotic area. Its close relationship to the adventitia of a capillary (3) is evident. Magnifications: A, 3200 $\times$ ; B, 3400 $\times$ .

inclusions, possibly from ingestion of breakdown products of the tissues. Bilirubin could not be identified with certainty in the electron micrographs.

Organs other than the central nervous system were examined after perfusion-fixation of the monkeys. Except the widespread staining with bilirubin, no pathology was noted. There was nothing resembling liver disease that differentiated the jaundiced asphyxiated from the jaundiced nonasphyxiated monkeys.

### Discussion

One may question whether the nuclear jaundice seen in monkeys asphyxiated at birth and then made hyperbilirubinemic is the same as kernicterus in human infants. Certainly not all instances of human kernicterus result from the combination of factors that produced it in the monkeys. We chose to employ only the factors asphyxia and hyperbilirubinemia for this study.

What are the basic diagnostic features of kernicterus? Other investigators (14) have applied the following criteria: There is distinctive evidence of central nervous system abnormality during life in the presence of jaundice; canary-yellow staining of nuclear masses of the central nervous system clearly visible in gross specimens; persistence of the stain when the tissue is fixed in formalin; pigment within neuroglia and nerve cells microscopically; and neuronal degeneration in chronic preparations even after bilirubin staining may no longer be evident. Our experimental animals fulfilled these criteria, although the neuronal degeneration was not followed into the posticteric phase.

A second question is whether hyperbilirubinemia plays a role other than marking the site of cellular injury, as Polani (18) suggested. Little of a decisive nature can be learned from reviewing reports of earlier animal studies, or for that matter, from attempts to evaluate kernicterus in the human infant.

A condition resembling kernicterus occurs spontaneously in the Gunn rat (11) which has received attention as an experimental model. An inherited deficiency of glucuronyl transferase appears to be responsible for the condition. Bilirubin deposition was found in nearly all the regions of the brain that are affected in one or another human subject. A number of technical problems limit the usefulness of rats, especially young ones, for studying the many factors involved in pathogenesis of kernicterus. The most obvious of these is their small size.

Conditions filling most of the criteria for kernicterus have been met in other experimental animals. Rozdilsky (20) attempted to induce kernicterus in kittens, puppies and rabbits with intravenous injections of a solution of bilirubin with albumin. Some of the animals developed clinical signs of neural damage. The neuropathological findings varied in the three species.

He encountered selective gross nuclear staining of thalamic and sub-thalamic nuclei, inferior colliculi, various brain-stem nuclei and the ventral columns of the spinal cord of some of the newborn and young kittens. Pigmentation of nerve cells was found only in animals surviving over 18 hours, at which time they were deteriorating physiologically. He saw neuronal chromatolysis only in one that survived for 36 hours (the longest period of time); this was in the lateral cuneate nucleus, a common site of asphyxial lesions in newborn monkeys.

In one-third of Rozdilsky's puppies infused with bilirubin plus albumin, pale yellow color was noted in brain tissues, but no pigmented nerve cells were found in frozen sections. On the other hand, severe insulin-induced hypoglycemia followed by bilirubin injections did result in intense nuclear staining of the inferior colliculi, and the nerve cells there contained the pigment. Inferior-collicular lesions were always found after asphyxia at birth of monkeys.

In only two of the twenty-eight rabbits was nuclear jaundice associated with repeated intravenous injections of the bilirubin-albumin solution by Rozdilsky and in these instances hemorrhagic lesions, probably of traumatic origin, occurred.

In kittens, puppies, rabbits and Gunn rats, the nuclei which were stained with bilirubin have varied from animal to animal within the same species and the intensity of color has varied even when the same nuclei were stained in two animals of the same species. The intensity of the anatomical response has been grossly proportional to the physiological symptoms. This, too, appears to be the case in the monkeys asphyxiated and given injections of bilirubin solution.

Other investigators have produced conditions suggesting human kernicterus. Eyquem (9) injected into puppies and kittens less than 3 months old, serum of rabbits immunized against dog or cat red blood corpuscles. Polani (18) used rats in a similar experiment, concluding that "the neurological damage may be associated principally with hepatic damage," and that "it is unlikely to be produced by bilirubin or similar pigment."

Clinical and neuropathological reports of human subjects provide no unequivocal proof that kernicterus has resulted simply from elevation of the serum-concentration of bilirubin. Some other factors clearly were present in many instances. In others there was inadequate clinical history to allow one to be certain. Lack of a "standard" description of the pathology of kernicterus in human brains and disagreement about a correlation between the yellow staining and neurocytological changes has been decried (3). Claireaux (5) pointed out that there are many regions involved; the broad spectrum includes basal ganglia, hippocampus, thalamus, mesencephalon, medulla oblongata, spinal cord, cerebellum and cerebral cortex. Generally, some nuclei are stained in one brain and other nuclei in other brains. Haymaker and his colleagues (12) emphasized the hippocampus ( $H_2$  and  $H_3$ ), hippocampal rudiment, globus pallidus, subthalamic nucleus, interstitial nuclei of Cajal and red zone of the substantia nigra as most severely damaged regions, but many other nuclei were found to be affected in one or another specimen. This too was the situation in the monkey infants of our series. The role of antecedent anoxia in the pathogenesis of kernicterus has been disputed largely because there have been few well controlled human studies. Full-term human infants with history of anoxia but having normal serum-bilirubin concentration do not develop kernicterus, and there is no correlation between erythroblastosis fetalis and anemia (7). Some studies have linked anoxia with kernicterus in premature human infants (1, 10), but other investigators (22) failed to find kernicterus associated with asphyxia either during or after birth. Furthermore, kernicterus in Gunn rats was not exacerbated by experimentally induced anoxia (14).

The experiments in monkeys demonstrated for the first time a clear relationship between asphyxia and hyperbilirubinemia in production of kernicterus. However, they did not exclude other causative factors. Comparison of the clinical picture of monkeys asphyxiated during birth (23) and that in the present experiments leaves no doubt that kernicterus was associated with the more devastating neurological deficits.

When asphyxia is viewed as just one of a number of agents—some unknown—acting probably in various combinations to damage neuroglia and nerve cells, our findings can be put into some perspective. Antecedent experimental asphyxia is one of the factors that can be critical during marked and continuing hyperbilirubinemia. Thus, the monkey preparation provides a model for further study of the nature of the abnormality in

cerebral metabolism and an opportunity to investigate experimentally various methods of treating this disorder.

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