STUDIES ON THE PATHOGENESIS OF KERNICTERUS

WITH SPECIAL REFERENCE TO THE NATURE OF KERNICTERIC PIGMENT AND ITS DEPOSITION UNDER NATURAL AND EXPERIMENTAL CONDITIONS*

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PLATE 46

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Yellow pigment is deposited in focal regions of the central nervous system in a small proportion of newborn infants with hyperbilirubinemia, as is well known, the lesion characterizing the disease state known as kernicterus (1–4). The nature of kernicteric pigment has not heretofore been defined, though several observations previously made by others have indicated that it differs from bilirubin. For example, it gives to cerebral tissues a lighter yellow color than bilirubin gives to jaundiced tissues, while furthermore it does not change to biliverdin when the tissues containing it are exposed to air or are kept in 10 per cent formalin, as does the bilirubin of jaundiced tissues held under similar conditions; on the contrary, under such circumstances kernicteric pigment gradually fades away, so that after a week or 2 it is no longer visible in the tissues formerly stained by it (5). The effects of kernicteric pigment upon the central nervous system likewise remain obscure; for while it is generally assumed on clinical grounds that kernicterus often is followed by mental retardation and other neurologic abnormalities (6) it is also known that in the kernicterus of human beings, deeply pigmented neurons often fail to show other cytological changes (3), though in some instances they have been found to display varying degrees of chromatolysis, liquefaction necrosis, hydropic change, or atrophy (3).

The studies now to be described provide evidence that kernicteric pigment is comprised of mesobilirubin, a reduction product of bilirubin. They also show that mesobilirubin, injected intracerebrally in newborn kittens, promptly gives rise to a focal pigmentation of brain tissues that resembles naturally occurring kernicterus, while bilirubin has no such effect. From microscopic studies of the experimental lesions it has become plain that mesobilirubin is readily taken up by the neurons of the central nervous system and that it regularly stains the

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nerve cells deeply without inducing other cytological changes in them. In addition to throwing light on the nature of kernicteric pigment and the manner of its deposition in the brain, the findings suggest that some factor other than the pigment itself should be sought to account for the degenerative changes that are sometimes seen in neurons in the naturally occurring kernicterus.

Materials and Methods

The general plan of the experiment was as follows. Kernicteric pigment was extracted by means of chloroform from the brains of 3 infants, and its nature determined by means of the diazo reaction and spectrophotometric analysis, chloroform solutions of commercially prepared, purified mesobilirubin and of bilirubin being employed for comparison. Attempts were then made to reproduce kernicterus experimentally by injecting solutions of mesobilirubin, and of bilirubin for control purposes, intracerebrally into newborn kittens, with gross and microscopic studies of the brains at appropriate times thereafter.

Extraction of Kernicteric Pigment.—Kernicteric pigment was obtained for analysis from the brains of three infants, each of which manifested hyperbilirubinemia with jaundice during life. At postmortem examination, the viscera of two of the infants presented the characteristic lesions of erythroblastosis fetalis, while those of the third showed immaturity and multiple pulmonary hemorrhages. The lenticulate, hippocampal, subthalamic, pontine, and cerebellar nuclei of the brains of all three infants were characteristically colored by a canary-yellow pigment which did not turn green but gradually faded when the tissues containing it were stored in 10 per cent formalin. Histological examinations showed the yellow cerebral pigment within the neurons, as well as in the interstitial tissues, in the brains of each of the 3 infants, the findings as a whole being characteristic of kernicterus (2).

Approximately 5 gm. of the deeply pigmented cerebral tissues was removed at postmortem examination from the basal ganglia and hippocampal regions of the brains of each of the 3 infants. The tissues, sectioned into blocks 2 to 5 mm. across, were placed in 5 cc. of reagent grade chloroform. The pigment came away readily into the solvent and in each instance yielded a clear, stable, canary-yellow solution. The deeply pigmented solutions were washed with distilled water, then dehydrated with anhydrous sodium sulfate, and filtered. The pigment was crystallized by evaporation and redissolved in chloroform.

Mesobilirubin and Bilirubin.—The mesobilirubin used for control purposes and for intracerebral injection into animals was procured from the Bios Laboratories, Inc., New York, N. Y. It was a dark blue crystalline substance that dissolved readily in chloroform and in rabbit serum, yielding in each instance a bright, canary-yellow solution. The bilirubin was a product of the Eastman Kodak Co., Rochester, N. Y. It was a reddish brown compound that yielded golden yellow solutions in chloroform and in serum.

Diazo Reaction.—The diazo reagent was prepared as follows: diazo A: 1 gm. sulfanilic acid in 15 cc. of hydrochloric acid with distilled water to make 1 liter; diazo B: 0.5 gm. sodium nitrite with distilled water to make 100 cc., mixed in proportions of 10 cc. of diazo A to 0.3 cc. of diazo B. The tests were carried out in duplicate in the following manner: 0.8 cc. of the kernicteric pigment extract in chloroform from each of the three cases, 0.8 cc. of purified bilirubin in chloroform, and 0.8 cc. of crystalline mesobilirubin in chloroform were each added to two test tubes containing 0.3 cc. of freshly prepared diazo reagent and 1 cc. of methyl alcohol. A series of color changes from yellow to colorless to pink-violet, occurring within 2 minutes, constituted a positive diazo reaction (8).

Spectrophotometry.—The light absorption of the kernicteric pigment, of mesobilirubin, and

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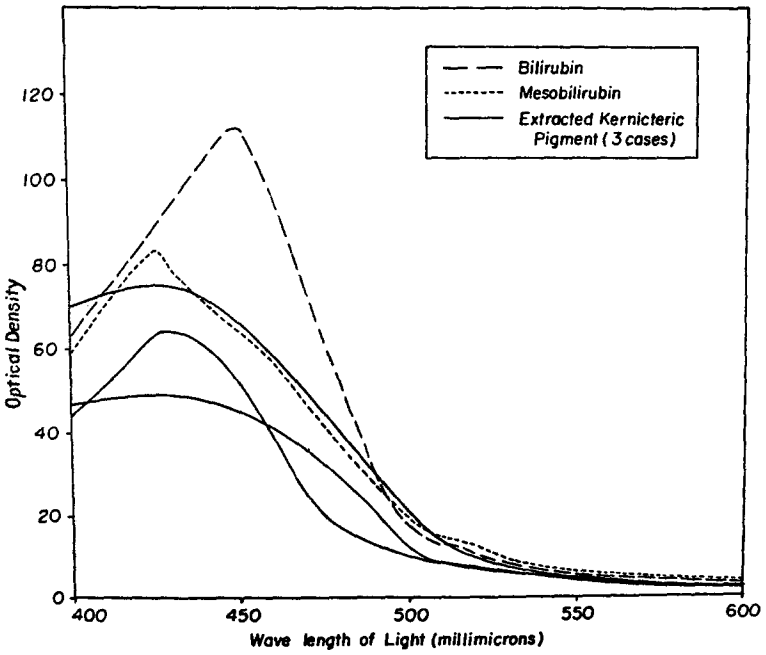
of bilirubin were determined by means of the Coleman electrophotometer, the conventional methods being used with reagent grade chloroform serving as the blank (7, 9).[1]

Intracerebral Injections of Mesobilirubin and Bilirubin.—Solutions containing 10 to 30 mg. of mesobilirubin or of bilirubin were prepared by grinding the pigment in the presence of 1 cc. of fresh, sterile, rabbit serum. All intracerebral injections were made slowly, by means of a 22 gauge needle inserted through the intact calvarium into the frontoparietal regions of 1 to 5 day old kittens under ether anesthesia. The animals were returned to the mothers and were examined frequently thereafter for abnormal neurological signs or other manifestations of toxicity. They were killed by nembutal, given intraperitoneally, 3 hours to 15 days later.



TEXT-FIG. 1. Electrophotometric determinations of bilirubin, mesobilirubin, and extracted kernicteric pigment.

Nature of the Kernicteric Pigment

A strongly positive diazo reaction was regularly given by the kernicteric pigment, extracted as described in the previous sections from the brains of the three infants with kernicterus, and identical reactions were also given by chloroform solutions of the purified bilirubin and mesobilirubin compounds employed in the work. This chemical reaction characterizes a group of pyrrolic compounds as is well known (10), and the test herein described serves to identify the kernicteric pigment as one of these compounds.

Text-fig. 1 shows the results of the electrophotometric determinations of the

[1] The assistance of Dr. Donald B. Melville in the performance of the electrophotometric determinations, and in the interpretation of the results, is gratefully acknowledged.

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three samples of kernicteric pigment and also those of mesobilirubin and bilirubin. It is plain that the kernicteric pigment extracted from each of the three cases gave a maximum absorption of light having a wave length of 425 mμ, in this property being identical with the crystalline mesobilirubin used for comparison, while the bilirubin gave a maximum absorption of light having a wave length of 450 mμ. The question remained open whether the light absorption of the kernicteric pigment might have been altered by the presence of chloroform-soluble components of the brain that could have been extracted from the cerebral tissues together with the kernicteric pigment. To investigate this possibility, the soluble components of brain tissue were brought into solution by storing 5 gm. of cerebral tissue from a newborn infant in 5 cc. of reagent grade chloroform for 48 hours. The solvent was washed with distilled water, then dehydrated with anhydrous sodium sulfate, and filtered. When purified bilirubin and mesobilirubin were dissolved in aliquots of this chloroform, they gave maximum absorption of light having wave lengths of 450 and 425 mμ respectively, proving identical in this property with solutions of these pigments in reagent grade chloroform, the findings providing evidence that the light absorption of the kernicteric pigment was not significantly altered by the presence of soluble components of the cerebral tissue. The findings as a whole provide strong evidence that the kernicteric pigment extracted from the brains of the three infants consisted of mesobilirubin, a reduction product of bilirubin (7, 10).

Experimental Kernicterus Induced by Means of Mesobilirubin Injected Intracerebrally

To learn whether kernicterus can be induced experimentally by means of mesobilirubin, solutions containing 1 to 3 mg. of this compound dissolved in 0.1 cc. of rabbit serum were injected intracerebrally in 16 newborn kittens. For control purposes, comparable amounts of bilirubin were likewise injected intracerebrally in 6 additional animals. The kittens were returned to their mothers, as already remarked, and were examined frequently thereafter for abnormal neurological signs or other manifestations of toxicity. These did not develop in any instance; the kittens generally nursed well and often gained weight before they were killed. The essential findings of these experiments are given in Table I. From this it can be seen that the intracerebral injection of 1 to 3 mg. of mesobilirubin was regularly followed by the appearance of focal areas of bright yellow pigmentation in the brains of the 16 animals. The pigmented areas were localized about the injection sites, extending outwards from them for distances up to 5 mm., and uniformly involving both the grey and the white matter (Fig. 1). The focal areas of pigmentation appeared promptly following the injections, being present in the brains of 6 animals killed 2 to 7 hours afterwards, and they persisted, being present in all the 6 animals that

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TABLE I

Effects of Mesobilirubin and of Bilirubin Injected Intracerebrally in Kittens

Experimental groups	Age of kitten	Amount of mesobilirubin or bilirubin injected (in 0.1 cc. serum)	Interval between injection and sacrifice	Essential gross and microscopic changes in brains
Animal No.	*Days*	*mg.*		
I. Kittens injected with mesobilirubin intracerebrally 1	5	3	2 hrs.	One focal area of canary-yellow pigmentation approximately 3 by 5 by 5 mm. in white and grey matter of right frontal lobe, linearly arranged along needle tract. Microscopic examination showed canary yellow pigment within the interstitial tissues and in the cytoplasm of approximately one-fourth of the neurons. Neurons appeared otherwise intact. Few leukocytes present at injection site.
2	5	2	3 "	Small focal area of canary-yellow pigmentation approximately 3 by 3 by 2 mm. about injection site in grey matter of right frontal lobe. Particulate yellow pigment in interstitial tissues and in approximately one-fifth of the neurons which were otherwise unaltered. Few leukocytes.
3	1	2	3 "	A focal area of canary-yellow pigmentation 3 mm. across at injection site in white and grey matter of right parietal cortex. Histological examination disclosed that somewhat less than one-fifth of the neurons were pigmented. Neurons intact. Few leukocytes.
4	1	3	4 "	Area of canary-yellow pigmentation approximately 5 by 5 by 3 mm., linearly arranged about needle tract in right frontal cortex. Histological sections showed yellow pigment in interstitial tissues and in approximately one-fourth of the neurons.
5	5	2	6 "	A focal area of canary-yellow pigmentation 3 mm. across at injection site in grey matter of right parietal lobe. Microscopic examination disclosed yellow pigment in approximately one-fifth of the neurons.

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TABLE I—*Continued*

Experimental groups	Age of kitten	Amount of mesobilirubin or bilirubin injected (in 0.1 cc. serum)	Interval between injection and sacrifice	Essential gross and microscopic changes in brains
Animal No.	*Days*	*mg.*		
6	5	2	7 hrs.	Linear area of canary-yellow pigmentation approximately 2 by 6 by 3 mm. in right frontal lobe. Approximately one-fourth of the neurons contained particulate yellow pigment without showing other cytological changes.
7	5	3	1 day	A focal area of canary-yellow pigmentation 4 mm. across in the white and grey matter of right parietal lobe. Approximately one-fourth of the neurons were pigmented.
8	3	3	2 days	A focal, pyramidal area of canary-yellow pigmentation approximately 5 by 5 by 4 mm. in grey and white matter of right frontal lobe (Fig. 1). Approximately one-third of the neurons contained finely particulate yellow pigment without showing other cytological changes (Fig. 2).
9	3	3	3 "	Linear area of canary-yellow pigmentation 4 mm. long in right frontal lobe, in which approximately one-fifth of the neurons were pigmented.
10	5	3	3 "	Focal area of canary-yellow pigmentation approximately 5 by 3 by 2 mm. in white matter of right frontal lobe; yellow pigment present in approximately one-fourth of the neurons.
11	1	1	5 "	Small focal area of canary-yellow pigmentation approximately 2 mm. across in grey matter of frontal lobe. One-fifth or less of the neurons were pigmented. A few astrocytes and microglia were present about injection site.
12	1	2	5 "	Linear area of canary-yellow pigmentation approximately 3 mm. across in frontal cortex, in which approximately one-fifth of the neurons were pigmented. A few astrocytes and pigment containing microglia.

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TABLE I—*Concluded*

Experimental groups	Age of kitten	Amount of mesobilirubin or bilirubin injected (in 0.1 cc. serum)	Interval between injection and sacrifice	Essential gross and microscopic changes in brains
Animal No.	*Days*	*mg.*		
	13	5	3	8 days.	Small focal area of canary-yellow pigmentation approximately 2 by 2 by 2 mm. in frontal cortex. Yellow pigment present in approximately one-tenth of the neurons. A moderate astrocytic gliosis and many microglia.
	14	2	3	9 "	Like those in animal No. 13.
	15	2	3	13 "	A large ovoid area of yellow pigmentation in frontal and parietal lobes approximately 10 by 6 by 5 mm. Yellow pigment present in approximately one-tenth of the neurons. Neurons intact. Moderate numbers of astrocytes and microglia.
	16	4	3	15 "	A minute area of canary-yellow pigmentation approximately 2 by 2 by 1 mm. in white matter of frontal lobe. Microscopic examination showed the neurons intact with pigment in the cytoplasm of only a few neurons. Many microglial phagocytes. Few astrocytes.
II. Kittens injected with bilirubin intracerebrally	1	5	3	20 hrs.	A focal area of golden yellow pigmentation approximately 3 by 2 by 2 mm. at injection site in frontal lobe. Microscopic examination showed yellow pigment in interstitial tissues but not within the neurons, which were intact. Few leukocytes.
	2	5	3	22 "	A linear area of golden yellow pigmentation 4 mm. long and 2 mm. across in white matter of frontal lobe. Yellow pigment present in interstitial tissues but not in neurons.
	3	5	3	2 days	No pigmentation visible to naked eye or in histological preparations.
	4	4	2	2 "	" "
	5	4	2	3 "	" "
	6	3	3	3 "	" "

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were killed 8 to 15 days after the injections had been made. The cerebral pigment was always canary-yellow in color, and it did not turn green in the brains of 12 kittens, which were stored in 10 per cent formalin for periods up to 3 weeks, but on the contrary gradually faded, as does the pigment of naturally occurring kernicterus in human beings, as has already been stated. Microscopic examinations were made of the cerebral tissues of each of the 16 kittens; these showed that the yellow pigment was regularly present within the neurons and in the intersitial tissues of the brains of each of the animals of this experimental group. The microscopic findings will be described in detail in the next section.

In the 2 kittens killed 20 and 22 hours after 3 mg. of bilirubin had been injected intracerebrally small areas of golden yellow pigmentation 2 to 3 mm. across were found at the sites where the bilirubin had been deposited. When the pigmented cerebral tissues were stored in 10 per cent formalin the pigment did not fade but took on a light yellowish green hue and remained stable (Table I). The cerebral tissues of the 4 kittens killed 36 to 72 hours after the intracerebral injection of 2 or 3 mg. of bilirubin contained no pigment that was visible to the naked eye. Detailed cytological examinations of the cerebral tissues from the injection sites of all animals, prepared by frozen section and by embedding in celloidin, failed to disclose intraneuronal pigment. Yellow pigment was present, however, in the interstitial tissues of the brains of the 2 kittens killed 20 and 22 hours after the injection of bilirubin, though the nerve cells appeared intact and there were few leucocytes in the interstitial tissues.

Effects of Mesobilirubin on Neurons of the Central Nervous System under Experimental Conditions

The pigment of naturally occurring kernicterus is best demonstrated in tissues prepared by frozen section, since the dehydrating agents employed for embedding by other methods often extract much of the kernicteric pigment, as is well known (5). It soon became clear in the present studies that the pigment of experimental kernicterus is likewise more abundant in tissues prepared by frozen section. These were stained by hematoxylin and eosin or by Hortega's silver carbonate method for astrocytes, while other portions of pigmented tissue were embedded in celloidin and sections of them were stained by Nissl's method, by Masson's trichrome method, and by Loyez's method for myelin.

The frozen sections showed an abundance of light yellow pigment within neurons and also within microglial phagocytes and free in the interstitial tissues throughout the areas of gross pigmentation in the brains of the 16 kittens that had received intracerebral injections of mesobilirubin, as already described. While the pigment-containing neurons were found in the brains of all animals given mesobilirubin, they were generally more numerous in those killed within 7 days after the injection. Not all the nerve cells within an area contained visible pigment; indeed only rarely were more than a third of them so involved. Under high magnification the intracellular pigment appeared particulate, the particles being distributed more or less at random throughout the cytoplasm of the cell (Fig. 2). It seemed noteworthy that the nuclei, nucleoli, and cell mem-

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branes of the pigmented cells, as studied in the frozen sections, were not perceptibly altered.

To learn more about these structures, and also about the Nissl substance in the pigmented cells, tissues were taken from the brains of all 16 kittens in areas immediately adjoining those used in the preparation of the frozen sections. These blocks were embedded in celloidin and stained by Nissl's method. The neurons were as numerous as were those in the brains of normal kittens examined for control purposes; furthermore they all appeared wholly normal, with intact nuclei having conspicuous nucleoli and deeply stained chromatin, and with finely granular Nissl substance distributed evenly throughout the cytoplasm. The tissues contained few or no leukocytes, and the astrocytic proliferation, which became manifest in the brains of kittens 5 or more days after the injection, was always minimal in degree.

DISCUSSION

The positive diazo reactions and the findings of the light absorption studies made it plain that the yellow pigment isolated in the present study from the brains of the 3 infants with kernicterus was probably mesobilirubin. How the pigment is laid down in such cases, that is to say, whether it is formed *in situ* by the reduction of bilirubin or produced elsewhere in the body and secondarily deposited in the cerebral tissues, is not known. In this relation, the fact has interest though that brain tissue can reduce bilirubin to mesobilirubin *in vitro*, as Baumgartel has shown (11). For he observed that when alkaline solutions of biliverdin are incubated at 37°C. with sterile emulsions of brain tissue, the biliverdin is reduced to bilirubin within 24 hours, and the bilirubin is further reduced to mesobilirubin when the incubation is continued for an additional 48 hours, while still longer periods of incubation bring about a conversion of the mesobilirubin to urobilinogen. These observations make it seem probable that in some infants with hyperbilirubinemia, bilirubin crosses the blood-brain barrier and is then converted to mesobilirubin, thus giving rise to the yellow pigmentation that characterizes kernicterus. The blood-brain barrier, however, is normally impervious to bilirubin, as is well known, deeply jaundiced individuals having no bilirubin in their cerebral tissues as a rule (12), while only a small proportion of jaundiced infants exhibit kernicterus (1). If the above assumptions be true, it follows that the permeability of the blood-brain barrier must somehow have been increased in the exceptional infants with hyperbilirubinemia who develop kernicterus. Anemia (13), anoxia (13, 14), immaturity (4, 14), hepatic toxins (15), and the toxic effects of bile pigments (16) have all been suggested as factors that may influence the permeability of the blood-brain barrier to a degree that would allow bilirubin to pass from the blood stream into the cerebral tissues.

When small quantities of bilirubin were injected into the brains of kittens in the present work, focal areas of golden yellow pigmentation promptly appeared about the injection sites; but the pigment disappeared rapidly, presumably being carried off either by the spinal fluid or by the blood so that it was no

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longer visible in the brains of animals killed 36 hours later. Why was the bilirubin not converted to mesobilirubin under the conditions of the experiment? The findings do not provide an answer, though it seems probable that the factor of dilution by blood or spinal fluid, as already mentioned, may have importance in this relation, as perhaps also the fact that the reduction of bilirubin to mesobilirubin by brain tissues *in vitro*, as Baumgartel observed, requires approximately 48 hours.

From the studies here reported it is plain that intact neurons readily take up abundant quantities of mesobilirubin under experimental conditions and furthermore that the neurons in experimental kernicterus, like those in the naturally occurring disease of human beings (3), can be heavily pigmented with mesobilirubin without exhibiting other cytological changes. These facts provide evidence that neuronal damage is not necessarily present before the kernicteric pigment is deposited within the nerve cells in the naturally occurring disease, and furthermore that the pigment itself probably does not induce significant cytological changes in clinical instances. Hence the cause of the degenerative changes that are sometimes seen in the neurons in cases of naturally occurring kernicterus in human beings remains obscure.

SUMMARY

Kernicteric pigment was extracted by means of chloroform from the brains of 3 infants. Solutions of it gave a positive diazo reaction, and, as determined electrophotometrically, gave maximum absorption of light having a wave length of 425 mμ, being identical in these properties with chloroform solutions of crystalline mesobilirubin.

Experimental kernicterus was regularly induced by injecting crystalline mesobilirubin intracerebrally in newborn kittens, the pigment staining the cerebral tissues a bright canary-yellow and being deposited abundantly in the nerve cells, as microscopic examinations showed, although these latter were otherwise intact. Bilirubin, likewise injected intracerebrally in newborn kittens, had no such effects.

The possibility is discussed that the blood-brain barrier is altered in some infants with hyperbilirubinemia in such a way that bilirubin crosses it and is then reduced within the brain to mesobilirubin thus giving rise to the cerebral pigmentation of kernicterus. The fact that the pigment itself does not seem to damage the neurons, as the present studies show, makes it necessary to seek some other cause for the neuronal damage that is sometimes seen, in association with the pigmentation, in the naturally occurring disease.

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EXPLANATION OF PLATE 46

The photographs were made by Mr. Julius Mesiar.

FIG. 1. Experimental kernicterus: A coronal section of the brain of a newborn kitten injected intracerebrally with a solution containing 3 mg. of crystalline mesobilirubin in 0.1 cc. of normal rabbit serum and killed 2 days later. There is an area of canary-yellow pigmentation, 5 mm. across, about the site of injection, in the regions of the caudate nucleus and subcortical white matter. When the tissues were exposed to air the pigment did not undergo a yellow to green color change; on the contrary, when the tissues were stored in 10 per cent formalin the pigment gradually faded and after 3 weeks was no longer visible. Fresh specimen. × 5.

FIG. 2. A nerve cell in the pigmented area of the brain of the kitten of Fig. 1. There are abundant yellow pigment granules within the cytoplasm of the neuron and also in the interstitial tissues below the plasma membrane of the cell shown. The cell is otherwise unaltered, however, and there is no leukocytic infiltration about it. Frozen section with hematoxylin and eosin stain. × 2000.

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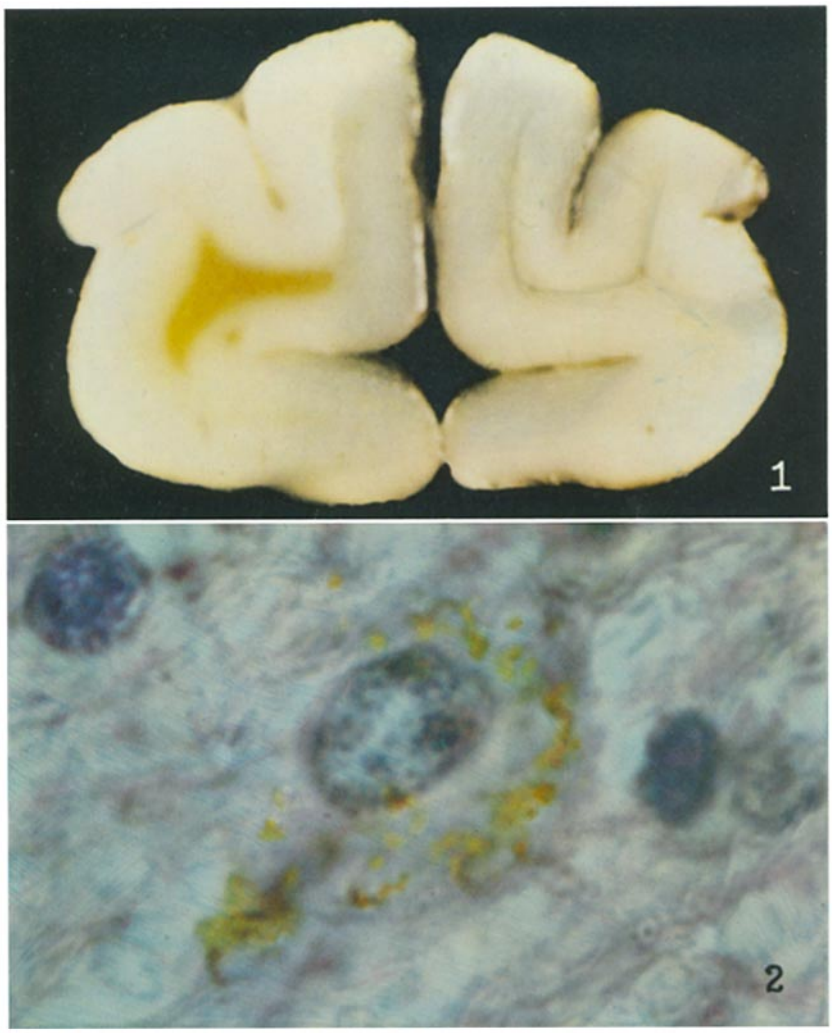

(Vogel: Pathogenesis of kernicterus)

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