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Studies of Cellular Toxicity of Unconjugated Bilirubin in Kernicteric Brain*

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Intracerebral deposition of unconjugated bilirubin is believed to be primarily responsible for the neurological manifestations of kernicterus[1] (1). Numerous *in vitro* studies have been carried out on the effect of unconjugated bilirubin on metabolic activity of liver and brain mitochondria (2), brain homogenates (3), cells grown in tissue culture (4, 5), and unicellular organisms (6). The majority of these investigations have shown that the pigment depresses oxygen consumption and, to a greater extent, the phosphorylation of these preparations (7). Accordingly, it has been postulated that unconjugated bilirubin exerts its cytotoxic effect in kernicteric brain by uncoupling phosphorylation from oxidation (7), with resultant decrease in synthesis of adenosine triphosphate (ATP) and subsequent impairment of energy-dependent cerebral metabolism.

An *in vivo* assessment of this concept has been carried out in the present investigation. Oxygen consumption and ATP concentration have been measured in the brain of kernicteric Gunn rats, and the results of these studies are the basis for this report.

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[1] "Bilirubin encephalopathy" is probably a more accurate term, inasmuch as pigmentation may affect any part of the brain. The word "kernicterus" has been used in this report because of its brevity and common usage.

Methods

Experimental design

Two-week-old Gunn rats, a Wistar strain with hereditary unconjugated hyperbilirubinemia due to a deficiency of glucuronyl transferase, were injected with sodium sulfisoxazole (Gantrisin),[2] 250 mg per kg subcutaneously daily, to achieve a uniform time of onset of kernicterus. The sulfonamide, prepared for injection as a 4% solution in distilled water, is believed to displace unconjugated bilirubin from its attachment to serum albumin (8) and enhance transfer of the pigment into brain (9). Previous studies have shown that Gantrisin, at these and higher doses, has no deleterious effect in nonicteric newborn or icteric adult Gunn rats (10). The age of the animals was selected for three reasons: *a*) serum bilirubin levels reach a peak at about 14 to 21 days in these rats (9), *b*) the size of the brain is adequate for regional ATP and oxygen consumption measurements in individual animals, and *c*) locomotor function at this age is sufficiently well developed to allow assessment of neurological signs. Rats with spontaneous kernicterus, variable as to time of onset, were not used. The controls consisted of nonicteric (heterozygous) Gunn rat littermates injected with weight-adjusted quantities of Gantrisin and normal Wistar rats, matched for age, uninjected or given 0.85% saline equal in volume to the Gantrisin solution. Brain ATP concentration or oxygen consumption was measured concurrently in the control and kernicteric animals at various stages of neurological impairment. As an additional control, the above studies were also carried out in another group of nonicteric Gunn rats and their icteric, but asymptomatic, littermates, which had not received sulfonamides.

Methods of analysis

ATP measurement. Unanesthetized animals were rapidly frozen by submersion in a container with dry ice and acetone. The frozen brains were then chiseled out on dry ice, sectioned, and inspected for yellow pigmenta-

[2] Hoffman La Roche, Nutley, N. J.

tion. Frozen sections (20 to 80 mg) of cerebral cortex, cerebellum, and subcortex comprising the area of the basal ganglia were then assayed for ATP by luciferin-luciferase luminescence as described in detail previously (11). Where present, pigmented tissue was sought for ATP assay. The results were expressed as micromoles of ATP per gram brain wet weight. ATP concentration was also calculated on the basis of brain protein concentration (12) and dry weight, values for the latter having been measured in six sets each of kernicteric, icteric asymptomatic, and anicteric control animals. Cerebral ATP concentration was almost identical in littermates submerged either in dry ice-acetone or in the more generally employed liquid nitrogen, indicating that the freezing procedure used in the present studies adequately "fixed" the labile nucleotide. Addition *in vitro* of unconjugated bilirubin to frozen rat brain did not affect the cerebral ATP concentration as compared with adjacent brain tissue assayed without added pigment. Duplicate brain sections from comparable areas in the same animal agreed within 1.82%. Kernicteric animals weighed less than the controls. Internal organs of smaller animals may freeze faster, resulting in a decreased decay of their ATP concentration. However, in control studies of normal rats with this weight difference, comparable cerebral ATP levels were obtained.

Measurement of cerebral oxygen consumption. Unanesthetized rats were decapitated, and the whole brain was enucleated, weighed, and finely homogenized with 5 vol of 0.04 M KH_2PO_4 buffer, previously adjusted to pH 7.6 with 0.1 N NaOH in a precooled glass homogenizer submerged in crushed ice. The above procedure was completed in exactly 6 minutes. Into prechilled Warburg flasks were placed 1.4 ml of homogenate, 0.4 ml 0.04 M phosphate buffer, and 0.2 ml of 10 mg per ml glucose. A strip of fluted filter paper saturated with 0.2 ml of 10% KOH was placed in the center well to absorb liberated carbon dioxide. The flasks, placed on ice, were gassed for 5 minutes with pure oxygen, and after 10 minutes of equilibration oxygen consumption was measured by standard Warburg manometry (13) at 10-minute intervals for 1 hour. All incubations were carried out in triplicate at 37° C and 50 strokes per minute; the results were averaged and expressed in microliters of oxygen consumed per milligram tissue (wet weight) per hour. In some instances the protein concentration of the experimental and control homogenates was determined, and the results were also calculated per milligram protein. Demineralized distilled water was used for preparation of all solutions and the washing of glassware.

To permit estimation in triplicate of the respiration of individual cerebellums (200 to 300 mg), we adopted an enhanced incubation system based on one previously used for obtaining maximal P:O ratios in rat liver mitochondria (13). Each cerebellum was homogenized in 15 vol (per weight of tissue) of a buffer mixture, pH 7.4, containing 0.25 M sucrose, 0.1 M sodium succinate, and 0.1 M potassium phosphate (monobasic) in the ratio 1.0: 0.3: 0.4, respectively. One and four-tenths ml of the homogenate was added to a prechilled Warburg flask containing 0.5 ml of the phosphate-succinate-sucrose mixture in its main compartment, 0.2 ml 10% KOH in its center well, and 0.2 ml 0.25 M glucose plus 2.5 mg hexokinase[3] in its side arm. We added 0.1 ml of 0.01 M magnesium sulfate last to the reaction mixture in the main compartment. The whole system was approximately 0.38 mole per L. After gassing, the chilled flasks were brought to temperature equilibrium in the Warburg water bath, zero reading was taken after 10 minutes, and the content of the side arm was tipped into the reaction chamber. Three pairs of experiments with whole kernicteric and normal brain were also carried out in this incubation medium for purpose of comparison.

ATPase assay. Unconjugated bilirubin *in vitro* has been shown previously to increase the ATPase activity of rat hepatic mitochondria, an effect potentiated by magnesium ions and optimal at 0.2 mM pigment concentration (7). To assess this for brain, we prepared mitochondria as described previously (14) from whole brain of female Sprague-Dawley rats (60 to 80 g in weight). Two-tenths ml of the fresh mitochondrial suspension in 0.2 M sucrose (contained 0.21 mg mitochondrial protein N) and ATP in 0.3 M Tris, pH 7.6, were incubated in quintuplicate at 37° C for 10 minutes, with and without added Mg^{++} or unconjugated bilirubin (0.2 mmole per L). The exact composition of each of the four reaction mediums is shown in Table II. The incubation was stopped by the addition of 8% perchloric acid, and phosphorus (P) was determined in the supernate (16). The P concentration present in each reaction mixture after incubating the homogenate alone (without ATP) and the ATP alone (without homogenate) were subtracted from the results obtained with the whole mitochondrial incubate. Control studies indicated that bilirubin did not interfere with the P measurement and that neither substrate, Mg^{++}, nor mitochondrial ATPase was exhausted during the assay. Reliability of the method employed for the ATPase assay was confirmed by reproducing the results of Skou with Na-K ATPase of submicroscopic brain particles (17).

ATPase activity was also measured as described above in homogenates of cortex and cerebellum removed from five sets each of kernicteric and icteric asymptomatic Gunn rat littermates. The exact compositions of the reaction mediums are shown in Table III. Protein measurements were carried out on the mitochondrial suspension and brain homogenates.

Ancillary studies. Rats with advanced kernicterus differed generally from the control littermates in that they

[3] Due to unavailability of a single large source, different hexokinase preparations were used for measurement of cerebellar respiration in each of the three groups of animals listed in Table I. The enzymes used for the three groups, respectively, were Calbiochem (Los Angeles, Calif.) hexokinase, lot 52834; Sigma (St. Louis, Mo.) hexokinase type IV, lot 123 B-7050; and Sigma hexokinase in ammonium sulfate, lot 104 B-1240. The quantity of hexokinase used, calculated from the activity given for each batch of the enzyme, was that just sufficient to give an optimal rate of P transfer.

suckled poorly and were, on the average, 25% underweight, whereas a few were stuporous and exhibited periodic twitching movements, though not outright convulsions. Since state of consciousness (18*a*) and psychomotor activity (18*b*) per se may influence the ATP concentration of cerebral cortex and possibly of other parts of the brain, the condition of each animal was carefully recorded, and those with the above abnormalities were separately assessed statistically. As additional controls, several separate series of normal 2-week-old Wistar rats (20 to 25 g) were *a*) subjected to 48 to 72 hours of complete fast, *b*) anesthetized with ether with loss of consciousness for 30 minutes, *c*) given pentylenetetrazol (0.3 mg per animal ip in 0.2 ml saline) with production of periodic clonic-tonic convulsions for 15 minutes, or *d*) injected with ammonium acetate (11) with a resulting stuporous-tremulous state for 30 minutes. Cerebral cortical and cerebellar ATP concentrations in these groups were then compared with those of normal littermates. Whole brain oxygen consumption was measured in the fasted and etherized groups and in normal controls.

Results

Neurologic findings. Neurologic signs generally appeared in the following sequential pattern in the icteric 2-week-old Gunn rats given sulfisoxazole: incoordination, ataxia (especially of hind limbs), progressive impairment of righting, drowsiness, neck extension, stupor, periodic twitching movements progressing to convulsions, and death. Incoordination, ataxia, and slight disturbance of righting occurred within 1 to 3 days of sulfonamide injection, and these animals were arbitrarily assigned to a group with "early kernicterus." Subsequent neurologic signs up to but not including outright convulsions developed after 2 to 5 days of Gantrisin administration and were deemed evidence of "advanced kernicterus." All animals were thus classified before knowledge of degree of brain pigmentation or of cerebral ATP concentration and oxygen consumption. Control nonicteric littermates given sulfisoxazole and those icteric but not injected with sulfonamides were asymptomatic.

There was virtually no yellow pigmentation of the cerebral cortex in either kernicteric group. Animals with advanced kernicterus generally showed pinpoint pigmentation of the subcortex in the area of the basal ganglia, whereas only occasional staining was present in rats with mild neurological signs. The cerebellum was most uniformly pigmented, progressing from spotty pigmentation in early kernicterus to a more diffuse and more intense staining with advanced disease. No pigmentation was observed in the brain of asymptomatic rats.

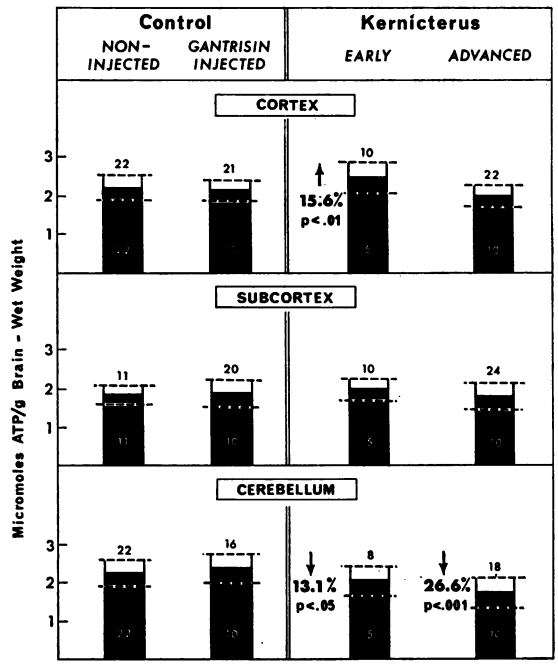

FIG. 1. EFFECT OF KERNICTERUS ON CEREBRAL ATP CONCENTRATION. The solid bars refer to means ± standard deviations (extended broken lines) of ATP in various parts of the brain. Results are expressed in terms of tissue wet weight. Similar results were obtained on the basis of tissue protein and dry weight. Numbers inside the bars refer to the total number of animals used in each group and those above the bars, to the number of brain sections assayed. Gantrisin-injected controls were asymptomatic nonicteric littermates of Gunn rats. Noninjected controls were asymptomatic Wistar rats of the same age.

ATP concentration in brain. ATP concentration in cerebral cortex, subcortex, and cerebellum of kernicteric and control rats is shown in Figure 1. Comparable nucleotide levels were found in each part of the brain studied in Gantrisin-injected anicteric control rats (littermates of the kernicteric animals) and in the normal noninjected Wistar rats, matched for age. The means ± standard deviations for cerebral cortex, subcortex, and cerebellum in these two groups, respectively, were 2.18 ± 0.26 and 2.25 ± 0.32; 1.86 ± 0.33 and 1.83 ± 0.21; 2.37 ± 0.35 and 2.23 ± 0.34 μmoles per g brain (wet weight). In the group with early kernicterus, cortical ATP concentration was somewhat higher (2.52 ± 0.38 μmoles per g—p

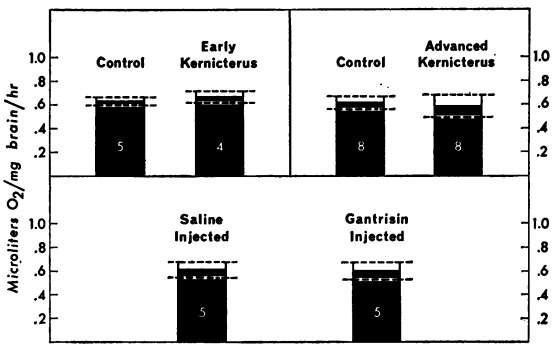

FIG. 2. EFFECT OF KERNICTERUS ON OXYGEN CONSUMPTION OF WHOLE BRAIN. The solid bars refer to mean levels ± standard deviations (extended broken lines). Number of animals is indicated inside the bars. Controls in upper panel are asymptomatic nonicteric Gunn rat littermates given Gantrisin. The Gantrisin- and saline-injected groups in the lower panel are asymptomatic 2-week-old normal Wistar rat littermates. Composition of incubation medium is described in Methods. Results are expressed in terms of tissue wet weight. Similar results were obtained on basis of tissue protein.

< 0.01), and in advanced disease similar to that of the control littermates. The nucleotide levels in the subcortex in both kernicteric groups were comparable to those of the controls. The need to process samples in excess of 20 mg (to keep brain frozen during weighing) rendered difficult the removal of small icteric sections from subcortex of both kernicteric groups and cerebellum of mildly affected animals without including unstained tissue. Nonetheless, in the seven most severely kernicteric rats with drowsiness and neck extension, the mean nucleotide level in subcortex was 2.04 ± 0.34 μmoles per g, 8.5% [though not significantly (19)] below the values for control littermates. Cerebellar ATP concentrations in animals with early and advanced kernicterus were, respectively, 2.06 ± 0.36 and 1.74 ± 0.38 μmoles per g. These values are 13.1% ($p < 0.05$) and 26.6% ($p < 0.001$) lower than those of asymptomatic littermate controls (Figure 1). Separation of the advanced group into three less and seven more severely affected animals revealed cerebellar ATP levels of 2.00 ± 0.21 and 1.60 ± 0.42 μmoles per g. The latter two values are, respectively, 15.6% ($p < 0.025$) and 32.5% ($p < 0.001$) lower than those of control cerebellum. In the three kernicteric rats with twitching movements, the cortical, but not subcortical or cerebellar, ATP levels were lower than in the remaining seven animals with advanced disease. Inclusion or exclusion of these data, however, for comparison with the control group had no statistical bearing on the results, and these values have not been separately plotted.

The ATP concentrations, mean ± standard deviation, for cortex, subcortex, and cerebellum of 8 to 10 sets of nonicteric Gunn rats and their icteric but asymptomatic littermates were, respectively, 2.25 ± 0.13 and 2.39 ± 0.13; 2.13 ± 0.11 and 2.14 ± 0.22; and 2.28 ± 0.21 and 2.31 ± 0.16 μmoles per g wet weight. The cortical ATP value is slightly higher in the icteric group ($p = 0.04$). This somewhat elevated cortical ATP value noted in both the asymptomatic icteric and mildly kernicteric animals (apparently unrelated to brain pigmentation in the latter) is unexplained but may be due to some subtle functional alteration in cortical activity. Prolonged fasting, brief periods of ether-induced coma, periodic pentylenetetrazol convulsions, and ammonia-induced stupor failed to affect cortical or cerebellar ATP levels in 2-

TABLE I

Effect of kernicterus on oxygen consumption of cerebellum

Group*	Asymptomatic controls†		Kernicterus
	Heterozygous	Homozygous	
	Oxygen consumption‡		
		μl/mg tissue/hour	
	1.03	1.14	0.63
	1.18	1.21	0.92
	1.20	1.24	1.00
	1.21	1.28	1.13
1	1.28	1.51	1.14
	1.45		
	1.51		
	1.54		
	1.55		
Mean ± SD	1.33 ± 0.19	1.28 ± 0.14	0.96 ± 0.21§
	1.40		1.22
2	1.43		1.26
	1.65		15.8
3	1.85		1.69
	2.07		

* Different preparations of hexokinase were used for each experiment. All experimental animals had "advanced" kernicterus.

† Heterozygous controls were nonicteric littermates given Gantrisin, whereas homozygous controls were icteric asymptomatic Gunn rats not injected with sulfonamide.

‡ Cerebellar oxygen consumption is expressed in terms of tissue wet weight. Protein determination on tissue homogenates and calculation of data on that basis yielded similar results. Composition of incubation medium is described in Methods. Each number refers to an average of triplicate determinations carried out on a single cerebellum.

§ Significantly lower than heterozygous controls ($p < 0.01$) and the homozygous controls ($p < 0.01$).

TABLE II

Effect of unconjugated bilirubin on cerebral mitochondrial ATPase activity

Incubation system	Mitochondria*	ATP†	Tris†	$MgCl_2$†	Unconjugated† bilirubin	Results‡
	ml	*ml*	*ml*	*ml*	*ml*	
1	0.2	0.2	0.9			2.51 ± 0.28
2	0.2	0.2	0.8		0.1	2.82 ± 0.55
3	0.2	0.2	0.8	0.1		23.74 ± 0.25
4	0.2	0.2	0.7	0.1	0.1	22.53 ± 0.95§

* 0.2 ml of mitochondrial suspension was equal to 0.21 mg mitochondrial protein N.

† 0.3 M Tris, pH 7.6, was used. ATP and MgCl were dissolved in the Tris to give 7.5 and 5.0 mmoles per L, respectively, in the final solution. A weighed quantity of unconjugated bilirubin (Eastman Kodak, Rochester, N. Y.) was freshly dissolved in 1.0 ml N NaOH and then diluted appropriately in Tris to give a 0.2 mM concentration in the incubation medium. The actual bilirubin concentration was confirmed by diazo technique (15).

‡ Mean ± standard deviation of five separate assays in each system. Measured in micromoles P per milligram mitochondrial protein N per 10 minutes.

§ Statistically significantly lower than results in corresponding system 3 ($p < 0.05$).

week-old nonicteric Wistar rats, as compared with those in control littermates.

Oxygen consumption of brain. The effects of kernicterus and of Gantrisin administration per se on the oxygen consumption of whole rat brain are shown in Figure 2. In no instance was cerebral respiration in the experimental groups significantly different from that of the controls. Separation of rats with advanced kernicterus into those with moderate (four rats) and those with severe disease (four rats) did not alter these results. Comparable cerebral respiration, although at higher levels, was also noted in three sets of kernicteric animals and littermate controls using the succinate incubation medium. Fasting and ether-induced coma, as described in Methods, failed to significantly influence cerebral oxygen uptake of normal rats.

TABLE III

*Effect of kernicterus on cortical and cerebellar ATPase activity**

	Asymptomatic icteric controls (Mean ± SD)	Kernicterus (Mean ± SD)
	μmoles P/mg protein/10 min	
Cortex	1.45 ± 0.14 (11)†	1.39 ± 0.16 (8)
Cerebellum	1.73 ± 0.12 (9)	1.55 ± 0.20‡ (9)

* Tissue was homogenized in 30 vol of 0.3 M Tris, pH 7.6. Each incubation vessel contained 0.9 ml of homogenate, ATP, 7.5, and $MgCl_2$, 5 mmoles per L, the latter having been added in 0.4 ml of 0.3 M Tris. The final volume of each flask was 1.3 ml. Incubation temperature was 37° C; the final pH was 7.6.

† Number of assays.

‡ Significantly lower than that of corresponding control cerebellum ($p < 0.03$).

The effect of kernicterus on oxygen consumption of cerebellum is indicated in Table I. As shown in group 1, cerebellar oxygen consumption of nonicteric and icteric asymptomatic controls was comparable. By contrast, respiration of the kernicteric cerebellum was on the average 27.8% lower than for the heterozygous controls ($p < 0.01$) and 25% below that of the icteric asymptomatic controls ($p < 0.01$). Groups 2 and 3 are not susceptible to statistical analysis due to the small number of determinations and use of different hexokinase preparations for each experiment. The mean respiration of kernicteric cerebellum, however, was 12.4 and 11.4% lower for groups 2 and 3, respectively, as compared with their control littermates. The decrease in cerebellar O_2 consumption was noted in both stuporous and alert kernicteric animals.

ATPase activity. As shown in Table II, unconjugated bilirubin added to cerebral mitochondria *in vitro* had virtually no effect on the tissue ATPase activity. The ATPase activity of cortex removed from kernicteric and icteric but asymptomatic Gunn rats was comparable, whereas that of kernicteric cerebellum was slightly lower (Table III).

Miscellaneous observations. In the kernicteric animals the protein concentrations, mean ± standard deviation, of cortex, subcortex, and cerebellum were 127.5 ± 14.5, 126.3 ± 3.5, and 114.4 ± 14.8 mg per g wet weight, respectively; in the asymptomatic icteric rats they were 121.0 ± 9.3, 131.0 ± 14.3, and 114.0 ± 10.4 mg per g wet weight, and in the nonicteric controls, 128.7 ± 11.1, 125.0 ± 9.3, and 113.3 ± 10.8 mg per g wet weight. The

water content of the above tissues was also comparable in all the animal groups and averaged 79.10% of wet weight.

Discussion

In this study of kernicteric Gunn rats, a statistically significant depletion of cerebellar ATP was demonstrated at all stages of neurological impairment (Figure 1). The validity of this finding was further attested to by the following observations: *1*) the decreased ATP concentration in kernicteric cerebellum was noted on the basis of wet weight as well as protein concentration and dry weight of the tissue; *2*) no substantial increase in cerebral microglial cells, which could alter the reference basis for the nucleotide determination, developed in experimental kernicterus of this brief duration (10); *3*) the control studies, here presented, indicated that the homozygous state per se (without neurologic impairment), fasting and weight loss, and Gantrisin administration do not account for the lower cerebellar ATP concentration of the experimental animals; *4*) the normal cortical ATP levels in the animals with advanced kernicterus served as an internal control and argued against the possibility of a nonspecific generalized depression of the nucleotide in the brain of the sick animals by such factors as hypoxia and other possible metabolic disturbances. In addition, many of the experimental animals demonstrated the diminished ATP level without evidence of stupor or hypoventilation, whereas the controls with brief but more severe disturbances of consciousness (ether- and ammonia-induced coma, pentylenetetrazol convulsions) failed to manifest a lower cerebellar ATP concentration. A rapid fall in cellular ATP to values one-third normal in 1 hour has also been demonstrated recently in a protein-free cell culture incubated with 2.5×10^{-5} M unconjugated bilirubin (5).

The decreased ATP concentration in kernicteric cerebellum may be the result of either impaired formation, increased utilization of the nucleotide, or a combination of both effects. The present approach does not delineate the precise mechanism of the ATP depletion, though the data, as discussed below, are more consistent with impaired synthesis of the nucleotide. Accelerated consumption of ATP may be due to increased metabolism or augmented ATPase activity of the brain. The selective decrease in cerebellar, but not whole brain, oxygen consumption in the kernicteric rats (Table I) argues against increased cerebellar metabolism in these animals while providing additional evidence of localization of bilirubin effect to that site. Furthermore, there was no bilirubin-induced stimulation of brain ATPase activity on incubating brain mitochondria with unconjugated bilirubin (Table II) or on assaying cerebellum removed from kernicteric as compared with control rats (Table III). These results are in agreement with those obtained with purified beef heart mitochondrial ATPase (20) and are contrary to those noted under somewhat different experimental conditions with rat liver mitochondria (7). Although it is not known to what extent *in vitro* measurements of oxygen consumption and ATPase activity reflect these processes *in vivo*, the above results point away from an effect of the bilirubin on increased ATP utilization and thus suggest an impairment of ATP synthesis. An alteration of cerebellar mitochondria recently demonstrated by electron and phase microscopy in kernicteric Gunn rats (21) may represent the structural counterpart of the above presented biochemical changes.

It is difficult to assess the functional importance of the degree of ATP depletion here noted in kernicteric cerebellum. First, there are only scanty data correlating ATP levels and selective impairment of cerebral function. A one-third decrease in brain nucleotide in acutely anoxic rats is associated with mortality (22), and a 27% decrease in medullary and pontine ATP concentration develops in rats with acute ammonium acetate-induced stupor (23). Contrariwise, chronic exposure of rats to 7% carbon dioxide leads to a two-thirds decline of the nucleotide in forebrain and medulla (as well as in liver and muscle) without overt signs of neurologic impairment (24). The very low control ATP values reported in the last study, however, raise questions concerning the methodology employed. Such different "toxins," acting over a variable time, may evoke different adaptive cerebral mechanisms and thus may not be comparable to each other or to the effect of bilirubin. Second, the ATP levels observed in the present investigation may not be representative of those actually present in various critical brain sites,

too small to assay by current methods. This is certainly true in these instances (subcortex, cerebellum in rats with early kernicterus) where the icteric tissue removed for ATP assay was necessarily "diluted" by inclusion of contiguous nonpigmented brain.

Notwithstanding this, in these kernicteric Gunn rat studies there was a strong suggestion of a correlation among the type and severity of neurologic signs, the site and intensity of tissue pigmentation, and the degree of ATP depletion in the brain. Thus the cortex, which was not stained, and the subcortex, which showed only spotty pigmentation, manifested, respectively, either no lowering or only a suggestion of a decrease in the nucleotide. By contrast, there was a progressive depletion of cerebellar ATP with increasing cerebellar signs and pigmentation (Figure 1). Although firm conclusions are unwarranted, these data and the observation that ATP level in cerebellum of animals with severe kernicterus fell to 73.5% of control, a degree of depletion comparable to that cited above for the symptomatic anoxic and ammonium-injected rats, suggest that the ATP drop here reported is of functional significance.

Two comments are pertinent to a comparison of kernicterus precipitated in the present experimental animals and that developing spontaneously in infants. *1*) Kernicteric Gunn rats and newborn infants resemble each other in that both manifest neurologic signs, grossly visible pigmentation of cerebral nuclear masses that persist in formalin, and degeneration of pigment-containing nerve cells (25). The pigment in human kernicteric brain has been conclusively identified as unconjugated bilirubin (26), whereas the absorption spectrum of icteric Gunn rat brain is different (maximum at 410 mμ vs. 440 mμ in infants) (25). It is not known whether these different spectral characteristics are due to methodologic difficulties in studying small quantities of pigmented Gunn rat brain or to the presence of a bilirubin metabolite in the brain of these animals. *2*) Involvement of the cerebellum was especially prominent in the present study, whereas pigmentation in the area of basal ganglia was next most common. Comparison of the neurological findings in rats with those in man is difficult since locomotion, which was especially followed in the animals, cannot be assessed in infants. It is of interest, however, that in a series of 35 cases of human kernicterus, the cerebellum was pigmented second most often (25 instances), preceded only by the basal ganglia (32 instances) (27). Furthermore, the extensive communications between the basal ganglia and cerebellum and lack of definite understanding of their respective functions suggest that the neurological signs attributed in infants to lesions of basal ganglia may be due, at least partly, to cerebellar impairment. Because of the above considerations, extrapolation from the present findings to man must be cautious. These data, however, which are consistent with those obtained *in vitro* with bilirubin, are believed to represent the first *in vivo* evidence that impaired cerebral phosphorylation may be an important feature of kernicterus.

Summary

To investigate the hypothesis that unconjugated bilirubin exerts its cellular toxic effect in kernicteric brain by interfering with oxidative phosphorylation, adenosine triphosphate concentration (luciferin-luciferase method) and oxygen consumption (Warburg manometry) have been measured in brain of kernicteric and suitable control rats.

Two-week-old icteric Gunn rats were given sulfisoxazole (250 mg per kg) precipitating kernicterus characterized in sequence by incoordination, ataxia, drowsiness, convulsions, and death. Hyperbilirubinemia of subcortical areas and especially cerebellum was noted regularly, and the latter appeared to correlate with severity of cerebellar signs. ATP concentration was similar in cerebral cortex and subcortex of kernicteric and control littermates. By contrast, mean ATP levels for cerebellum of control animals and those with "early" and "advanced" kernicterus, respectively, were 2.37 ± 0.35, 2.06 ± 0.36, and 1.74 ± 0.38 μmoles per g wet weight. The latter two values are statistically significantly lower than those of control brain ($p = < 0.05$ and < 0.001). Control studies suggest that altered state of consciousness and decreased weight and presumably nutrition of kernicteric animals do not explain their low cerebellar nucleotide level. Whole brain oxygen consumption of kernicteric and control rats was similar, whereas the respiration of kernicteric cerebellum was on the average 19.3% lower.

The localization of ATP depletion and impaired respiration to the hyperpigmented kernicteric cerebellum and the presence of neurologic signs consistent with involvement of that site suggest that impaired phosphorylation may be an important feature of kernicterus.

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