could be found in this case (fig. 2). This compound interferes with the unchanged drug when the colorimetric assay is used. But its quantity is small and should be negligible in most cases.

Very different results between the 2 methods were obtained when analyzing urines of subjects with insufficient renal function, to whom multiple oral doses of SMZ had been administered. Here, the results found by the colorimetric assay have in part been much higher than those found by TLC (table).

TLC showed that these urine samples contained a very high quantity of Bratton-Marshall positive metabolites (zones II, III and IV in fig. 2). By TLC the separation of the unchanged drug from these metabolites is possible, but in the colorimetric assay they interfere strongly. Identification of the compounds of zone II was tried by mass-spectrometry. The main compound in this zone could be identified as sulfanilamide, which is reported to be a urinary metabolite[3]. Furthermore, mass-spectrometry gave a hint of the presence of the metabolites 5-hydroxymethyl-sulfamethoxazole[5] and N4-acetyl-5-hydroxymethyl-sulfamethoxazole[3]. The compounds of zones III and IV have not yet been identified.

Our results show that the colorimetric assay for SMZ in urine may lead in special cases to too high a level of sulfonamide being recorded. Therefore a specific chromatographic method is preferable for the determination of sulfonamide concentrations in urine.

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The serum levels of unbound bilirubin that induce changes in some brain mitochondrial reactions in newborn guinea-pigs

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Summary. Bilirubin in different concentrations was injected in newborn guinea-pigs and the following parameters were determined: serum total and unbound bilirubin, whole brain bilirubin content and oxygen consumption, NADH-cytochrome c reductase and ATPase activities in brain mitochondria. The results showed a significant correlation between decreased rates of brain metabolism and the elevation of serum total and unbound bilirubin.

It has long been known that bilirubin can produce kernicterus by interfering with brain metabolism[3]. Electron microscopy has shown that mitochondrial alterations occur early during the development of bilirubin encephalopathy[4,5], although it has not been clearly demonstrated which specific metabolic steps are directly responsible for the chemical pathology of the disease. The specific enzymes or enzyme systems affected by bilirubin have been extensively reviewed[6].

On the clinical side, a point that has puzzled pediatricians is the choice of the blood parameter to follow in handling infants at risk[7]. Up to now, one has had to rely upon either the serum binding capacity or the unbound bilirubin values as determined mainly by the peroxidase method. The indirect bilirubin value alone is viewed as having the most serious drawbacks of all the parameters available.

On the other hand, these parameters could be useful if it were possible to establish a secure relationship between their serum values and early signs of brain damage. In looking for such a correlation, we used an experimental model in which guinea-pigs were made hyperbilirubinemic by i.v. injections of bilirubin solutions. The serum values of unbound bilirubin were compared to the amount of bilirubin entering the brain tissue, as well as to the activities of some mitochondrial systems.

Material and methods. Experimental hyperbilirubinemia. All experiments were carried out on spontaneously (at term) delivered newborn guinea-pigs, weighing 80 (SD ± 16) g, at birth. A PE-10 polyethylene catheter attached to a continuous dropping system was inserted in the superficial vein along the antero-lateral aspect of an upper foreleg for infusion of bilirubin and isotonic saline solutions. The animals were anesthetized with Nembutal® (Abbott Laboratories), 40 mg/kg b.wt i.p. The desired amount of crystalline bilirubin, $\varepsilon_{452\,nm}^{CHCl_3} = 60{,}100$ (Merck), was dissolved in 1 ml of 0.1 N NaOH in the dark, rapidly mixed with 1 ml of adult guinea-pig serum, and the pH adjusted to 8.0 with 1 N acetic acid as described elsewhere[8]. Samples of about 1 ml of blood were drawn from the jugular vein opposite to the site of infusion, after an infusion time of approximately 30 min.

Spectrophotometric determinations of bilirubin in whole brain. At the end of the experiment the animals were decapitated and the whole brain was removed. The brain

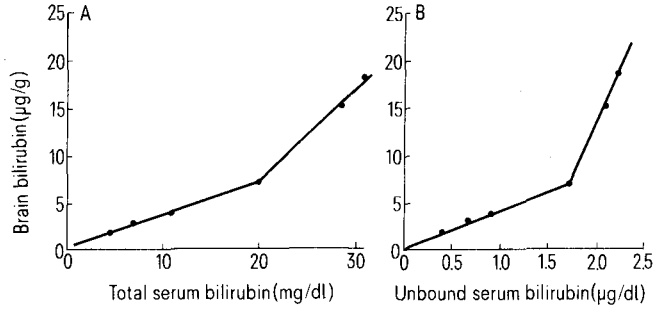

Figure 1 A and B. Relationship of serum bilirubin and brain bilirubin content in hyperbilirubinemic newborn guinea-pigs. *A* Total serum bilirubin vs brain bilirubin; lower slope line: $y = 0.160 + 0.358\ x$; higher slope line: $y = -12.5 + 0.98\ x$. *B* Unbound bilirubin vs brain bilirubin; lower slope line: $y = 1.144 + 4.19\ x$; higher slope line: $y = -30.7 + 22.4\ x$.

NADH-Cytochrome c reductase and ATPase activities and inorganic phosphorus content in brain mitochondria from hyperbilirubinemic newborn guinea-pigs

	Unbound serum bilirubin (μg/dl)	NADH-Cytochrome c reductase activity (nmoles/min x mg of protein)	ATPase activity (μgP_i released/mg of protein) Incubation time 10 min	20 min	Inorganic phosphorus content ($\mu g\ P_i$/mg of protein)
Controls	0.020±0.006	10.48±0.28	20.4±1.5	27.2±2.5	16.7±0.58
Experimentals	0.36±0.07	9.31±0.73	20.3±2.4	26.5±1.2	18.1±0.98
	0.78±0.07	8.02±0.43*	19.5±1.9	27.7±2.9	13.6±1.5**
	1.4 ±0.06	7.78±0.48*	24.4±1.0	34.4±1.9	14.1±1.5**
	2.3 ±0.08	6.43±0.46*	21.5±1.6	28.7±2.4	14.0±1.4**

Note: The figures above are the mean results (±SE) of at least 5 experiments.
* Statistically different from controls (Student's t-test) $p < 0.001$.
** Statistically different from controls (Student's t-test) $p < 0.05$.

was thoroughly perfused with isotonic saline and the choroid plexuses were wiped off. The brain was weighed and homogenized with 8 ml of chloroform per g of wet weight, in a Potter-Elvehjem tissue grinder. The chloroform phase was separated by mild centrifugation and this bilirubin-containing phase was used directly for spectrophotometric measurement at 452 nm with a Beckman 25 spectrophotometer. For recovery experiments known amounts of bilirubin were added directly to homogenized brain from non-injected guinea-pigs, and extracted as described above. The blank for spectrophotometric readings consisted of chloroform extracts of brain from non-injected guinea-pigs.

Analytical procedures for serum bilirubin determinations. Direct and total serum bilirubin were determined by a modified microdiazo reaction[9]. Serum unbound bilirubin was determined by the peroxidase method as described previously[10], using horseradish peroxidase (EC 1.11.1.7) type I, purchased from Sigma Chemical Co.

Enzymatic assays and oxygen consumption in mitochondrial preparations. Whole brains from decapitated jaundiced and control animals were quickly removed, washed in isotonic saline and homogenized in an ice bath with 0.25 M sucrose. Mitochondria were isolated according to a procedure described previously[11]. All assays were carried out in a medium containing heavy and light mitochondrial fractions. All protein determinations were done according to Lowry et al.[12]. Mitochondrial oxygen uptake was measured polarographically in a GME oxygraph, KM Model (Gilson Instruments) using a Clark electrode. The results were expressed in μl of oxygen consumed/h × mg of protein[13]. ATPase assay was done by a method described elsewhere[13] for liver mitochondria; inorganic phosphorus (P_i) was determined by colorimetry[14]. The results were expressed in μg of P_i released/mg of protein. NADH cytochrome c reductase activity was determined following the reduction of cytochrome c (from horse heart, type VI; Sigma Chemical Co.) spectrophotometrically at 37 °C at 550 nm[15]. The results were expressed in nmoles of reduced cytochrome c/min × mg of protein.

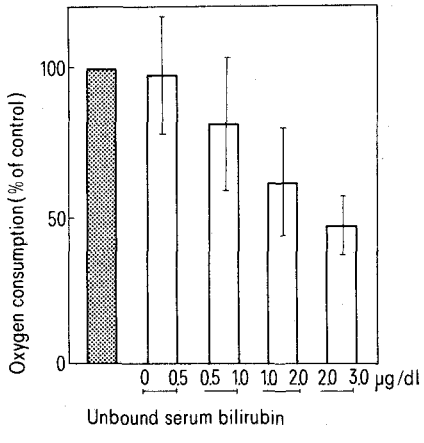

Figure 2. Oxygen consumption (μl O_2/h × mg of protein) in brain mitochondria and unbound serum bilirubin (μg/dl) (average and range) in newborn guinea-pigs, ▨, controls; □, experimentals.

Results. The i.v. infusion of bilirubin solutions led to increased serum levels which were accompanied by increasing amounts of this pigment being deposited in brain tissue. Figure 1 shows the results of experiments done in guinea-pigs divided in to 6 groups that received 1.0, 1.5, 2.5, 5.0, 10.0 and 12.5 mg of bilirubin, respectively. Each point represents the mean values for 12 animals. Figure 1A shows a linear relationship between serum and whole brain bilirubin concentrations, with two intersecting straight lines being obtained. The slope change occurred when total serum bilirubin concentration was around 20 mg/dl. As shown in figure 1B, the same kind of relationship holds for unbound bilirubin, with a slope change in the region of serum levels of 1.7 μg/dl.

The serum direct bilirubin values for all animals, controls and experimentals, were 0.55 (SD±0.28) mg/dl.

The oxygen consumption of isolated mitochondria was also modified by the increased serum levels of unbound bilirubin. Figure 2 shows the results of such experiments as compared to control groups. It can be seen that serum unbound bilirubin in the range of 0.5 to 1.0 μg/dl was accompanied by a significant decrease ($p < 0.001$), in the brain mitochondrial oxygen uptake. Higher values of serum unbound bilirubin were accompanied by a progressive inhibition of this mitochondrial system until it reached about 47% of the activity of the controls. The table shows that mitochondrial NADH-cytochrome c reductase activity was affected by bilirubin in a way similar to the oxygen consumption. The P_i content determined in brain mitochondria was significantly reduced when serum concentrations of unbound bilirubin were around 0.78 μg/dl, whereas no significant changes in ATPase activity could be detected in the entire range of unbound bilirubin levels tested.

Discussion. The data shown in this paper are in agreement with the idea that there is a relationship between serum unbound bilirubin levels and brain uptake followed by changes in mitochondrial processes. In general, the work done in this field has demonstrated the metabolic action of bilirubin on mitochondrial systems by incubating isolated mitochondria from several tissues with quite high concentrations of the pigment in vitro[16]. The present approach consists of searching for the possible clinical importance of the described actions of unbound bilirubin, by attempting a

direct correlation between its serum levels and the already described changes induced by it in brain tissue. A similar approach was tried before[17] in Gunn rat sucklings, but the authors reported a lack of relationship between cerebellar bilirubin content and serum concentrations of the unbound fraction. Among other possibilities, the different results reported here could be explained either by the fact that whole brain was used or by the possibility that suckling could affect the transport of bilirubin across the blood-brain barrier.

The brain bilirubin uptake, as shown in this paper, is a biphasic process. The amount of the pigment entering the brain per mg increase of total serum bilirubin is 2.75 times higher when the total serum levels are above 20 mg/dl. This can be seen by the slope change in the curve. When the serum unbound bilirubin concentrations are in the range of 1.7 µg/dl there is also an abrupt change in the slope of the line, enhancing the uptake by a factor of 5.35.

These data taken together suggest that the risk of bilirubin encephalopathy may be directly linked to serum levels of unbound bilirubin.

It is noteworthy that infants with serum indirect bilirubin at the levels of 11 and 22 mg/dl had serum unbound bilirubin of 0.77 and 1.87 µg/dl, respectively[18] which are very similar to the values reported in the present paper.

It has been reported that infants with early signs of kernicterus had serum unbound bilirubin of 1.75 µg/dl[19] and 1.17 µg/dl[20]. These values are very close to the figures shown in the present work, suggesting that they may be indeed the values of risk.

It should be pointed out that although the serum levels reported above are usually accompanied by early signs of kernicterus, lower values are responsible for biochemical changes which may not necessarily be accompanied by clinical signs. This would make them valuable as a guide for preventive therapy.

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A sensitive and inexpensive high-performance liquid chromatographic assay for tyrosine hydroxylase

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Summary. We describe a highly sensitive assay method for tyrosine hydroxylase (TH) using high-performance liquid chromatography with amperometric determination. This assay method could be applicable to any tissues with low enzyme activity, such as rat cerebellum. We also describe the kinetic properties of TH in rat cerebral cortex.

Tyrosine hydroxylase (L-tyrosine, tetrahydropteridine: oxygen oxidoreductase; E.C. 1.12.16.2) catalyzes the conversion of tyrosine to DOPA[1], the rate-limiting step in the biosynthesis of catecholamines. A highly sensitive assay method for tyrosine hydroxylase activity is frequently required for physiological and pathological studies.

The sensitive radiometric assays in current use for tyrosine hydroxylase activity measure the radioactive DOPA or water produced from labelled tyrosine. Recently, we developed a non-radiometric assay for tyrosine hydroxylase activity by high-performance liquid chromatography with an amperometric detector[2]. But the sensitivity is still not enough to measure the activity in the cerebral cortex.

In this communication, we describe an improved assay method for tyrosine hydroxylase activity which can be applied to any tissues containing low enzyme activity such as rat cerebellum. We also describe the kinetic properties of tyrosine hydroxylase in rat frontal cerebral cortex.

Materials. Male Sprague-Dawley rats weighing 200–250 g were killed by decapitation and the brains were rapidly removed. Rat frontal cerebral cortex, cerebellum, and hippocampus were collected, homogenized with 4 vol. of 0.32 moles/l sucrose, and the homogenates were frozen at −80 °C until use. Brain tissues from adult male SRJ:CD-1 (ICR) mice and New Zealand albino rabbits were also obtained by the method described above.

Experimental procedures. The standard incubation mixture consisted of the following components in a total volume of 500 µl (final concentrations in parentheses): 50 µl of 0.5 moles/l-potassium phosphate buffer, pH 6.64 (50 mmoles/l), 50 µl of 0.1 moles/l ascorbic acid (10 mmoles/l), 50 µl of 10 mmoles/l 6-methyl-5,6,7,8-