nism of the *Paramecium* Ca channel (*21*) is not only voltage-sensitive but also ion-dependent. The site or sites at which these ions exert their effect must be accessible from the outside, because activation of the Ca channel must precede the ion flux and is altered by external ions in our experiments.

It is not clear whether ions, permeant or impermeant, are involved in the gating of voltage-sensitive channels in general, although recent studies of K channels suggest this (*5*). The gating of the Na channel seems to be affected by Zn^{2+}, and Ni^{2+} and possibly Ca^{2+} or Mg^{2+} (*22*). Whether the presence of Ca^{2+} or another divalent cation affects gating currents of the Ca channel (*23*) has not been tested directly. Changes in the rate of Ca channel activation or deactivation have been noted in an insect muscle, *Helix* neuron, and frog muscle (*4, 19*).

If the gating process is viewed as involving conformational changes of the channel that is accessible to ions, it is not surprising that there is an interaction between the gating charges and the ions. The situation may be analogous to the substrate- or cofactor-enzyme interaction involved in changes of enzyme conformation.

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7. A reference electrode was placed near the cell, except when replaced with the iontophoresis electrode. An inverted microscope was used to allow electrode access from above with a short submersion length. The time constant of the *I-V* converter was < 100 μsec; the open-loop gain of the feedback circuit ≃ ×250; and the series resistance to the cell < 90 kilohms with and < 200 kilohms without the reference electrode; electrodes were filled with 3*M* KCl with resistance < 20 megohms.
8. None or less than 10 percent of the Ca conductance was inactivated at this holding level.
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14. Y. Saimi, in preparation. The 5 m*M* TEA suppresses the delayed K current up to 75 percent in the Ca solution. The remaining outward current appears to be larger in the Sr and Ba solutions, which should have hastened rather than delayed the apparent fall of the inward current.
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17. The current change after 0.5 msec was measured and taken as the tail current because of the capacitative surge (see text).
18. The differences in the τ's of the tail currents in our three solutions cannot be accounted for by the possible differences in surface charge. First, comparison of the τ's in the Ca solution at −35 mV and in the Sr or Ba solution at −45 mV [the maximum possible difference attributable to surface charge is ~ 10 mV as judged by the *I-V* relations of Ca versus Sr or Ba inward currents (Fig. 1C)] reveals that the τ's of Sr or Ba tails remain larger than that of the Ca tail. Second, the *I-V* relations in the Sr and Ba solutions are identical, while the τ for the Ba tail is larger than that for the Sr tail (see text).
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21. We estimated that four or five gating particles were involved in the Ba^{2+} current, assuming there is little inactivation of this current. Y. Naitoh (unpublished results) found five particles for the Ca^{2+} current. Therefore, the basic gating process appears to be the same in various solutions, but the rate constants seem to be different.
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Bilirubin-Induced Modulation of Cerebral Protein Phosphorylation in Neonate Rabbits in vivo

Abstract. *Protein phosphorylation in cerebral cell-free preparations from neonate rabbits was inhibited by bilirubin and promoted by aminophylline when these substances had been administered intravenously. In animals given both compounds, the bilirubin-induced inhibition of phosphorylation was partly reversed by aminophylline. Adenosine 3′,5′-monophosphate added in vitro during the assays also increased protein phosphorylation. These data introduce new concepts in the pathogenesis of kernicterus.*

Kernicterus is a complication of severe unconjugated hyperbilirubinemia confined almost entirely to the neonatal period. Even though the disease has been studied at the molecular level, the major biochemical defect underlying bilirubin encephalopathy has yet to be determined. Bilirubin inhibits oxidative processes in isolated mitochondria, suggesting that it exerts its cytotoxic effect in the kernicteric brain by diminishing local adenosine triphosphate (ATP) levels, thus leading eventually to impairment of energy-dependent cerebral metabolism (*1*).

The report that bilirubin in vitro inhibits its adenosine 3′,5′-monophosphate (cyclic AMP)–dependent protein kinase activity with purified histone as the substrate has introduced new concepts in the mechanism of pathogenesis of kernicterus (*2*). Yet, unequivocal linkage of this action of bilirubin to the pathogenesis of bilirubin-induced encephalopathy requires demonstration of a similar effect in vivo.

Protein phosphorylation was studied in the cerebrum of neonate rabbits after they were given bilirubin or aminophylline, or both, intravenously at various doses in 1 ml of solvent. An attempt was made to evaluate the effect of bilirubin on the overall estimation of protein phosphorylation. In addition, the individual contributions from cyclic AMP–dependent as well as cyclic AMP–independent routes of phosphorylation were examined.

The experiments were carried out in three groups of animals: those in group A were treated with bilirubin (Sigma); those in group B received aminophylline (theophylline and ethylenediamine; Cooper); and those in group C were injected with aminophylline (2 mg per 100 g of body weight, intravenously) 30 minutes after the administration of various doses of bilirubin. Rabbits were of both sexes, 3 to 4 days old, and weighed 65 to 90 g each; they were decapitated 30 minutes after bilirubin or aminophylline administration. Cerebrum homogenates were centrifuged (30,000*g* for 20 minutes), and supernatants in phosphate buffer (0.05*M*, *p*H 7) were used as the source of the enzyme. Protein phosphorylation determined (*3*) with purified histone (fraction II-A, Sigma) as the substrate, was expressed as picomoles of ^{32}P-labeled inorganic phosphate ($^{32}P_i$) from [γ-^{32}P]ATP

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(Amersham) incorporated per milligram of protein (enzyme preparation) per minute. Statistical evaluation was performed with Student's *t*-test.

Bilirubin levels in the serum and brain tissue were determined in group A at the time of death (*4*) and depended, in a nearly linear fashion, on the dose administered. The ratio of serum to brain bilirubin remained approximately 20. Thus, at the conditions employed, total brain bilirubin levels ranged from 0.8 to 2.92 mg per 100 g of brain tissue and from 18.2 to 60.6 mg per 100 ml of serum.

At these concentrations, bilirubin inhibited protein phosphorylation (group A in Table 1). In comparison with the solvent-treated controls, this inhibition was statistically highly significant ($P < .001$), even at the dose of 5 mg of bilirubin. A few minutes after receiving the injection, all animals treated with bilirubin developed the sluggishness and sleepiness characteristic of the effect of this dye; the effect was proportional to the dose administered.

Addition of cyclic AMP (2.5 μmole) to the incubation mixture during the in vitro assay of protein phosphorylation increased the incorporation of $^{32}P_i$ into histone by 14.6 to 35.1 percent, indicating the presence of an active cyclic AMP–dependent phosphorylating enzyme in the tested preparations (Table 1). The effect of cyclic AMP was highly significant in the controls ($P < .001$) and significant in bilirubin-treated animals ($.001 < P < .01$).

Injection of aminophylline, a potential cyclic AMP–phosphodiesterase inhibitor (*5*), in various doses 30 minutes before the animals were killed, increased cerebral protein phosphorylation (group B in Table 1). A few minutes after being given aminophylline the animals developed the irritability and hyperactivity characteristic of the effect of this drug, which, as with bilirubin, was dose-dependent. The increase in phosphorylating activity by aminophylline was insignificant ($.05 < P < .1$) at the dose of 0.5 mg per 100 g of body weight but highly significant ($P < .001$) at the dose of 2 mg per 100 g of body weight, in comparison with the solvent-treated controls.

In bilirubin-treated animals, injection of 2 mg of aminophylline per 100 g of body weight (group C) partially restored protein phosphorylation, but an overall reduction of activity prevailed at doses of bilirubin higher than 10 mg (Table 1). In particular, at the dose of 20 mg the overall inhibition of phosphorylation was highly significant compared with the control level ($P < .001$) (Table 1).

Figure 1 shows the percent changes in control protein phosphorylation effected by bilirubin, aminophylline, or both, as estimated from the results of Table 1 (in the absence of cyclic AMP). The fraction of cyclic AMP–dependent stimulation of phosphorylation induced by aminophylline in the bilirubin-treated animals maintained a nearly constant magnitude throughout the range of bilirubin doses administered, indicating an effect of the dye specifically on the protein kinases that were not cyclic AMP–dependent. However, the possibility cannot be excluded that higher levels of brain bilirubin concentrations may also affect cyclic AMP–dependent protein kinase activity in vivo.

Table 1. Cerebral protein kinase activity in relation to bilirubin and aminophylline administration. The solvent was a solution of 0.1*N* NaOH (adjusted with HCl to *p*H 8.1) for bilirubin and 5 percent glucose for aminophylline. Values of protein kinase activity are means ± standard error, expressed in picomoles of P_i per milligram of protein per minute.

Number of animals	Drug administered (mg/100 g)	Protein kinase activity in vitro (pmole/mg-min)	
		In the absence of cyclic AMP	In the presence of cyclic AMP
		Control	
51	Solvent	2590 ± 270	3485 ± 447
		Group A: bilirubin	
10	5	1834 ± 180	2232 ± 272
11	10	1648 ± 205	2120 ± 357
10	15	1455 ± 91	1841 ± 176
10	20	1270 ± 198	1594 ± 221
		Group B: aminophylline	
10	0.5	2878 ± 365	3534 ± 435
10	1.0	3215 ± 340	3597 ± 464
10	2.0	3588 ± 393	3773 ± 537
		Group C: bilirubin plus 2 mg of aminophylline	
10	5	2836 ± 317	3002 ± 314
11	10	2515 ± 178	2645 ± 144
10	15	2292 ± 296	2413 ± 350
10	20	2164 ± 265	2275 ± 264

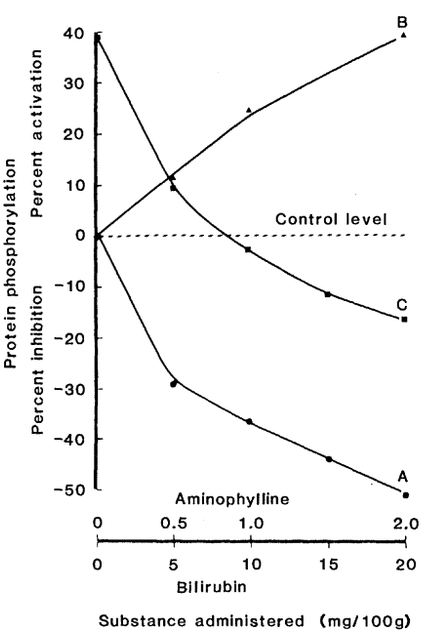

Fig. 1. Effect of bilirubin and aminophylline on rabbit cerebral protein phosphorylation in vivo. The points plotted were calculated from the values in Table 1 for protein kinase activity in the absence of cyclic AMP. *A*, *B*, and *C* refer to groups A, B, and C of Table 1.

The results of this study indicate a differential effect of bilirubin on protein phosphorylation. Bilirubin, even at a concentration in the brain as low as 0.8 mg per 100 g of tissue, appreciably reduces protein phosphorylation in vivo. The study of the individual effects of bilirubin and aminophylline, and the results obtained on the combined action of these substances in vivo, have resolved the overall measured phosphorylating activity—under the conditions specified—into cyclic AMP–dependent, bilirubin-insensitive and cyclic AMP–independent, bilirubin-sensitive routes of phosphorylation (Fig. 1).

This finding appears to be at variance with previously reported kinetic studies in vitro with commercially purified cyclic AMP–dependent protein kinase; these studies indicated an existing competition by bilirubin for the site of the enzyme binding cyclic AMP (*2*). However, the concentrations of bilirubin used in vitro (6 μg/100 μl) were considerably higher than those obtained in vivo (2.9 μg per 100 mg of tissue). Increased mortality of the animals limited the upper attainable bilirubin levels to approximately 3 μg per 100 mg of brain tissue. The possibility thus exists that cyclic AMP–dependent protein kinase in vivo in brain is sensitive to bilirubin only at higher concentrations than those obtained in this study. Autoradiographic studies of the phosphorylated proteins in high-resolution electropherograms (*6*) are necessary to establish or rule out this

possibility. Nevertheless, the results of studies both in vitro and in vivo strongly support the view that bilirubin toxicity in vivo starts with a selective action on protein phosphorylation, a pathway central to an array of biochemical events linking bilirubin to bilirubin-induced impairment of energy-dependent cerebral metabolism.

Cyclic AMP–dependent phosphorylation of target brain cell proteins is a central pathway to diverse cellular functions such as neurotransmitter-mediated transmission of the nervous impulse at the synaptic junction (*6–8*) and selective nuclear activation through histone phosphorylation (*8*). As these functions have different duration times (*8*), from a few milliseconds (neurotransmission) to years (memory), influence exerted on them externally may induce correspondingly short time effects or long-lasting modifications.

Even though this report does not provide direct evidence for an effect of bilirubin on synaptic membrane–associated protein kinase, such an effect may not be excluded in view of the symptoms developed in the bilirubin-treated animals. The immediate toxic effect of bilirubin, induced by moderate hyperbilirubinemia, usually reversible and expressed by sleepiness, sluggishness, and disturbances of respiratory and cardiac function, may be due to impairment of synaptic protein phosphorylation. The clinically opposite symptoms arising from the intravenous administration of aminophylline (alertness, irritability, and hyperactivity) support this view. The long-lasting toxicity related to high and lasting hyperbilirubinemia may be due to impairment of phosphorylation on nuclear histones in the brain cell.

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Calanoid Copepods, Feeding Currents, and the Role of Gravity

Abstract. *Feeding currents of free-swimming calanoid copepods, observed through an expanded krypton laser beam and a back-focus dark-field optical system, show that these planktonic animals generate a double shear field to help in detecting food. The interrelation between flow field, perception of food items, and body orientation explains why these animals are generally negatively buoyant.*

Calanoid copepods are an integral part of the plankton of seas, estuaries, and lakes. Herbivorous calanoids (Fig. 1) graze on algae; carnivorous ones prey upon fellow zooplankters. Algae are sized from 2 to 100 μm and are dispersed at low concentrations in open pelagic waters. The planktonic world is therefore best described as a nutritionally dilute environment (*1*). Because of the pivotal role of the algae-zooplankton interface in the aquatic food web (*2*), this link has received considerable attention. Most research and textbook descriptions (*3*), however, have followed Cannon's (*4*) early interpretations about the copepod filtering mechanism and the feeding currents. Observations with high-speed microcinematography (*5*) does not support the idea that these animals are filter-feeders (*6*). Calanoids set up flow fields whose properties enhance the chance of detecting food items. The forces which determine the animal's velocity through the water column while feeding have to be balanced to provide a uniform flow field. One of these forces is gravity. The question then arises as to why these animals are not neutrally buoyant so that they can remain at the same depth without having to swim constantly.

Pelagic suspension feeders such as calanoids, salps, and appendicularians must scan large amounts of water for their daily rations because only one part per 10^5 to 10^7 parts of water is of nutritional value. Herbivorous calanoids generate a feeding current with their mouthparts (see cover). These currents were thought to pass through a mesh of setae and setules on the second maxillae. Algae were thought to be retained passively on this filter so that the spacing of setules and setae on this appendage determined the size of particles captured (*7*). However, observations of tethered calanoids (*5*), placed in a dye stream to visualize the flow (*8*), showed that they use second maxillae to capture and separate algae from the feeding current. Just before capture by second maxillae, other mouthparts direct algae into the capturing area through the "clap and fling" mechanism (*9*) and changes in shear fields (*10*). These observations suggested that calanoids perceive the approximate locations of nearby algae, and chemoreception probably assists in this recognition (*11*). Koehl and I observed that the feeding current is governed by viscous forces (low Reynolds number) and therefore has laminar flow (*8*). Any chemical diffusing from an alga sets up an active space (*12*) which will be deformed in the flow field in a predictable way. To take advantage of this deformation the animal must generate a feeding current with a stable flow structure.

Cannon (*4*) observed the feeding current in a drop of water under a microscope and described a relatively powerful pumping mechanism which circulated water against the walls of the drop, thereby creating eddies, counter-eddies, and an unnatural flow. To observe a natural flow field, I filmed feeding behavior with a collimated red light beam of low power (*13*). All light which passed through a 5-liter vessel of seawater undisturbed by particles was blocked in the back focus of the collector lens by a black dot on a glass surface (*14*). Algae and free-swimming calanoids scattered some light (Fig. 1), allowing filming at a speed of 100 frames per second (exposure time, 0.5 msec per frame) (*15*). Except for the laser light, the room was dark. The animals and their food items were at natural densities.

Fifteen films were evaluated, and only the paths of algae which were affected by the presence of the calanoid were drawn (Fig. 2). The structure of the feeding current (Fig. 2A) of *Eucalanus crassus* was constant during feeding bouts of 10 to 30 seconds. The flow field in front of the mouthparts shows a double shear field, one extending laterally from the

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