berg (*5*) were unable to demonstrate conversion of [1-^{14}C]galactose to $^{14}CO_2$ by galactosemic fibroblasts under conditions where normal cells performed this conversion. However, the incubation period in their system was only 90 minutes, and there was an excess of glucose present (*4*) which would have effectively inhibited any galactose utilization.

Recent studies (*15*) have shown that the rate and total amount of $^{14}CO_2$ produced by cells in culture is a function of the extracellular concentration of [1-^{14}C]galactose, and that the difference in the extent of conversion of galactose to CO_2 between galactosemic and normal cells disappears as the concentration of galactose is reduced to those used in our experiments. This suggests that alternate metabolic pathways for galactose which lead to CO_2 production are present in galactosemic cells, but such alternate pathways become saturated with galactose at much lower levels than occur in the normal system. Of the three alternate pathways for galactose utilization that have been proposed and investigated (*3, 16*), the only route leading to CO_2 production for which there is substantial evidence is through the pyrophosphorylase reaction. Support for an alternate metabolic pathway beyond the galactokinase reaction which would lead to CO_2 production comes from an experiment in which we incubated human galactokinase-deficient cells (KIN) (*17*) with [1-^{14}C]galactose and in which we could not detect any $^{14}CO_2$ production. This finding is consistent with the absence of [1-^{14}C]galactose decomposition products giving rise to the $^{14}CO_2$ results in the transferase-deficient cells; it also suggests that the pyrophosphorylase reaction is the only significant one of the three proposed alternate pathways for galactose utilization.

The demonstration of significant galactose metabolism by galactosemic cells in culture indicates that effective alternate metabolic pathways that do not involve dead-end products are operative in vitro and may be functional in this disease in vivo as well. The inhibitory effect of glucose on galactose utilization in vitro suggests that glucose may play a protective role in galactosemia in vivo and is consistent with observations that galactosemic cells have nearly normal growth characteristics in medium containing 95 mg of galactose and 5 mg of glucose per 100 ml of cell culture medium (*5, 6*).

In addition to the activation or stabilization of a malfunctioning enzyme (*2*), two additional indirect approaches to therapy in genetic diseases might be considered: (i) to activate or make more efficient any alternate metabolic pathways that exist; and (ii) to inhibit utilization of the defective pathway if it leads to the accumulation of cytotoxic products. On the basis of the findings reported here, it should now be possible to explore each of these alternatives in galactosemia.

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17. Kindly provided by Dr. Robert S. Krooth.

1 November 1971

Axons: Isolation from Mammalian Central Nervous System

Abstract. *Centrifugation of a homogenate of white matter, in a solution of buffered sucrose containing salt, produces a floating layer of myelinated axons. When these are suspended in hypotonic buffer, the myelin swells and strips away from the axon. Axons are then separated from the myelin by centrifugation. The resulting preparation consists of a variable population of processes with lengths up to 200 micrometers and diameters between 0.3 and 5.0 micrometers. The axons contain neurofilaments and mitochondria, although no axolemma or neurotubules are evident. The preparation contains cerebroside and sulfatide, yet is essentially free of myelin.*

Biochemical characterization of axons has been limited to large, and easily dissected, fibers (*1*). Fractions that are enriched in nonmyelinated axons have been isolated but they have been studied morphologically only (*2*). We now report the details of our method for the isolation of axons from the mammalian central nervous system in quantities that are sufficient for biochemical characterization (*3*).

All operations were carried out at 0° to 4°C. Brainstem, or centrum semiovale, from fresh bovine brain was finely minced and homogenized in 100 volumes of medium A (0.85*M* sucrose, 0.10*M* NaCl, and 0.05*M* potassium phosphate buffer; *p*H 6.0) with six strokes of a loose pestle and four strokes of a tight pestle in a Dounce homogenizer (Kontes). Centrifugation of this homogenate at 7000 rev/min (9500*g*) for 30 minutes in the 150-ml tubes of the Sorvall HS-4 rotor produces a white floating layer and a pellet; the pellet is discarded. The floating layer from each tube is suspended, with the use of the homogenizer, in 60 ml of medium A, and centrifuged at 25,000 rev/min (75,500*g*) for 15 minutes (Spinco SW 25.2 rotor). The floating layer obtained by this procedure is again suspended in medium A and centrifuged as before to ensure more complete removal of any trapped nuclei and capillaries.

The final floating layer, containing myelinated axons and myelin, is then suspended in 0.05*M* potassium phosphate buffer, *p*H 6.0 (5 ml of buffer

per 150 ml of original homogenate), with three to four strokes of the loose pestle in the homogenizer. When the myelin has been removed from a majority of axons, as is determined by phase microscopy (usually 1 to 2 hours), the axon suspension is diluted with seven parts of medium A and centrifuged at 25,000 rev/min (81,500*g*) for 10 minutes in a SW 27 rotor; the axons form a pellet and the myelin floats to the surface. The axon pellets are suspended and centrifuged as before (usually three to four times) until no myelin is observed floating above the axonal pellet. The dry weight yield of axons is about 1.5 mg per gram of fresh white matter.

Lipid extractions and analyses were carried out as described previously (*4*). Assays of the myelin-specific enzyme 2′,3′-cyclic nucleotide-3′-phosphohydrolase were performed as described by Olafson and Drummond (*5*); however deoxycholate was added to stimulate the enzyme activity (*6*). Disc-gel electrophoresis with 15 percent polyacrylamide gels was performed according to Maizel and Laemmli (*7*), and electron microscopy was performed as described (*8*).

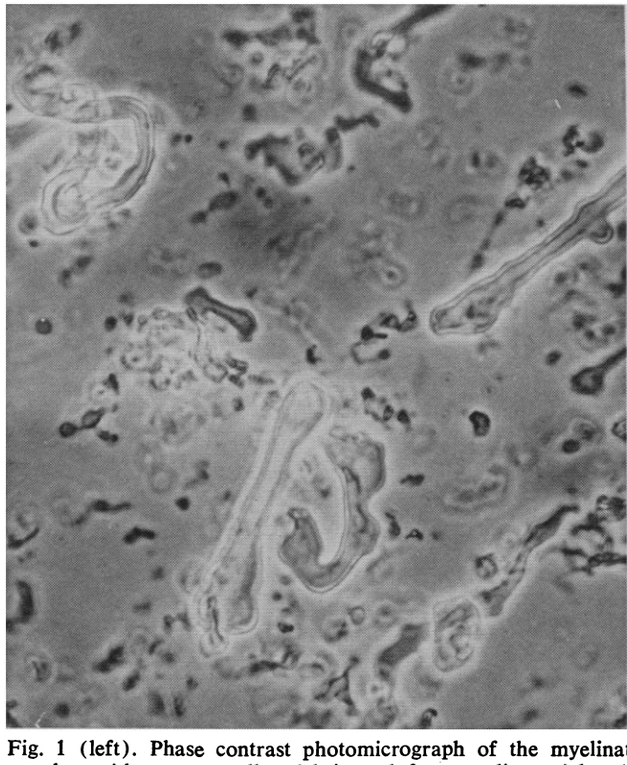
The fact that the pellet obtained in the first step of a myelin isolation procedure (*9*) contained intact axons indicated the possibility that myelin-free axons from brain could be isolated. Many attempts to separate these axon fragments from the other components of the pellet were unsuccessful, and therefore we developed the alternative approach described here.

The compact myelin structure is preserved in isotonic solutions of salt (*10*); in hypotonic saline or in sucrose solutions, even of high osmolality, the myelin sheath swells. It is essential to maintain the integrity of the axon-myelin relation in the first step of our procedure. This is accomplished by the inclusion of 0.10*M* NaCl and 0.05*M* potassium phosphate buffer in the medium. This solution is also sufficiently dense to allow the myelinated axons to float to the surface when centrifuged. Since excessive mechanical stress may cause disruption of the axon, and separation of myelin from the axon, the relatively mild homogenization procedure is utilized. Once the myelinated axons are purified, incubation of the preparation in the hypotonic medium allows the myelin to swell. Following this vesicles form which peel off from the axon (*11*).

Examination of the "floating layer" of myelinated axons (Fig. 1) by light microscopy shows fibers that are 5 to 10 μm in diameter and several hundred micrometers in length, together with some free myelin vesicles. The myelin sheath is seen as a highly refractile coating on the darker axon.

The final axon preparation consists of both low-contrast large fibers, in which dark specks (presumably mitochondria) can be seen, as well as other, very small fibers. Occasional nuclei and capillaries are present, but essentially no myelin vesicles are detected.

Electron microscopy of the axon preparations (Fig. 2) reveals two distinct populations of processes. Most of these are 1.5 to 5.0 μm in diameter and contain abundant, loosely packed neurofilaments and some mitochondria. The remainder of the processes have diameters between 0.3 and 0.6 μm and are composed of densely packed filaments only. While the former are clearly axonal, the latter do not resemble any normal in situ component. These small dense processes may represent axons in which the packing density of the filaments has been altered, but they bear more resemblance to bundles of astroglial filaments. The occurrence of glial filaments in the present fraction is



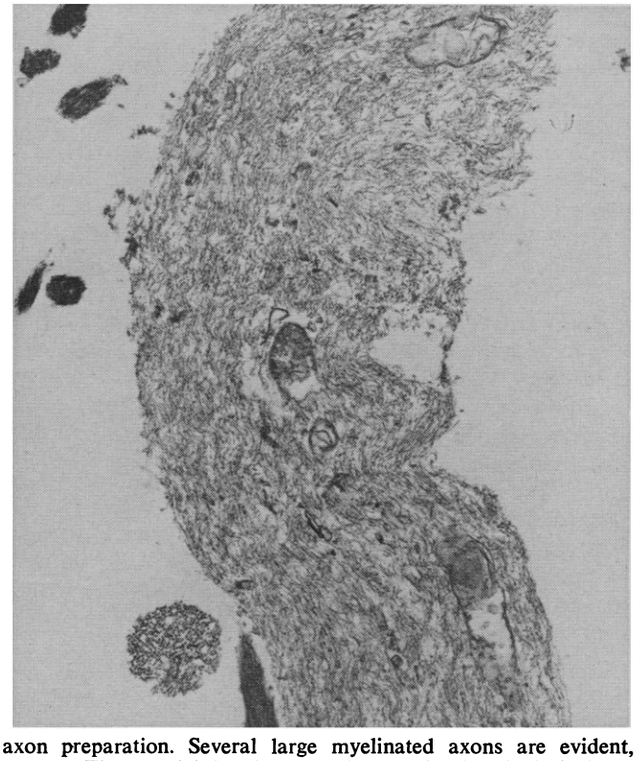


Fig. 1 (left). Phase contrast photomicrograph of the myelinated axon preparation. Several large myelinated axons are evident, together with some smaller debris and free myelin vesicles (× 600). Fig. 2. (right). Electron micrograph of a single isolated axon of the final preparation. Fixation by glutaraldehyde was followed by osmium tetroxide (*8*). Mitochondria are evident in the neurofilament-rich axoplasm. Also in the field are several small fibers with densely packed filaments (see text), and a small axon in cross section (× 20,000).

unexpected because they should have been removed during the preparation of myelinated axons. Thus, the origin of these dense profiles is still open to question. No axolemma or neurotubules are evident in either type of fiber. The absence of an axolemma may be an artifact of the preparation procedure for electron microscopy (*8*). Alternatively, this membrane may be lost through mechanical disruption, or it may separate with the myelin when the axons are suspended in the hypotonic medium. The absence of neurotubules is not unexpected since these are known to be labile structures and may depolymerize under the isolation conditions (*12*).

The axonal lipid content is 13.4 ± 1.3 percent of the dry weight and contains 60.0 ± 2.7 percent phospholipid, 20.1 ± 1.1 percent cholesterol and 20.1 ± 2.6 percent galactolipid. The galactolipid is composed of both cerebrosides and sulfatides in a molar ratio of approximately 2 to 1. The finding of significant amounts of cerebroside and sulfatide made it essential to ascertain that these lipids were not the result of myelin contamination since we calculated that 14 percent myelin contamination could account for all of the galactolipid.

Myelin fragments were seldom seen in the electron micrographs. The specific activity of the enzyme 2′,3′-cyclic nucleotide-3′-phosphohydrolase in the axon preparation was 0.1 unit per milligram of protein—less than 2 percent of the specific activity of 7.70 units per milligram of protein determined for bovine myelin. Disc-gel electrophoresis of 200 μg of total axonal protein indicated mostly proteins of high molecular weight with a prominent band corresponding to neurofilament protein (*13*). Electrophoresis of myelin proteins mixed with axonal proteins showed that we could detect a 2 percent contamination of myelin protein, but no specific myelin proteins were detected.

This indicates that myelin contamination of the axon fraction is negligible. We therefore conclude that these galactolipids are intrinsic axonal constituents, a finding consistent with previous reports showing an extramyelin compartment for these compounds (*4, 14*).

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17 November 1971; revised 17 December 1971 ■

Oxygen Affinity in Red Cells: Changes Induced in vivo by Propranolol

Abstract. *Propranolol, a blocking agent for the beta adrenergic receptor, produces a redistribution of 2,3-diphosphoglycerate in the red cell. At concentrations of 3.3 × 10⁻⁵M, 2,3-diphosphoglycerate in the red cell membrane becomes unbound in vitro. The administration of propranolol to humans produces similar changes and results in a decrease in the affinity of hemoglobin for oxygen.*

The concentration of 2,3-diphosphoglycerate (DPG) in the human erythrocyte plays a central role in regulating the affinity of hemoglobin for oxygen (*1*). As the concentration of DPG increases, the affinity of hemoglobin for oxygen decreases. In a wide variety of clinical disorders, the position of the oxygen-hemoglobin dissociation curve, as reflected in the P_{50} (the partial pressure of oxygen at which the hemoglobin is 50 percent saturated), bears a direct relation to the concentration of DPG in the red cell (*2*); however, exceptions to this rule have been described (*3*). Recently, Pendleton and his co-workers (*4*) observed that when propranolol, a blocking agent for the beta adrenergic receptor, was added to a suspension of intact human erythrocytes, it produced a shift to the right in the position of the oxygen-hemoglobin dissociation curve without producing any change in the concentration of DPG in the red cell. This effect was not observed when propranolol was added to either hemoglobin in solution or to suspensions of erythrocytes depleted of DPG. We have found that propranolol produces a release of DPG that is bound to the red cell membrane, both in vitro and in vivo, and that its administration to humans results in a decrease in the affinity of hemoglobin for oxygen.

Freshly drawn blood was obtained from healthy adult volunteers, was mixed with heparin, and was divided into portions to which was added varying concentrations of propranolol hydrochloride. The whole blood was then allowed to remain at room temperature for 10 minutes and a portion was re-

Table 1. The effects of propranolol ($3.3 \times 10^{-5}M$) in vitro on distribution of 2,3-diphosphoglycerate (DPG) in the red cell, and the effects of epinephrine ($3.3 \times 10^{-5}M$) on the interaction. Results are expressed as mean ± 1 S.D., for 20 experiments.

Incubation condition	DPG in red cell (μmole per gram of hemoglobin) in:		"Unbound" (%)
	Whole lysate	Stroma-free supernatant	
Whole blood	15.7 ± 1.4	10.9 ± 1.1	69.7 ± 4.6
Whole blood and propranolol	15.6 ± 1.2	15.8 ± 1.3	100 ± 0
Whole blood with propranolol and epinephrine	15.8 ± 1.3	10.0 ± 0.7	64.2 ± 5.3