of CO_2 exchange and transpiration were similar at leaf temperatures up to 37.5°C. At 37.5°C the peak of the curve representing CO_2 exchange became flattened, and at 40°C a sharply decreased rate of exchange occurred over a period of 4 minutes when the rate of transpiration was near its maximum (Fig. 2). The decrease was still more marked at 42.5°C, the exchange being positive during an interval of about 2 minutes. The behavior of the plant at any given temperature did not exhibit hysteresis, the phenomena observed being independent of the direction of the preceding change of temperature. The depressions were noted in ten oscillations.

These surprising fluctuations in CO_2 exchange by the leaf when the level of light, concentration of CO_2, and leaf temperature were constant, and the leaf diffusive resistance minimum, could be due to partial inhibition of photosynthesis, a stimulation of respiration, or both. Respiration is known to increase with an increase in temperature (*3*) but leaf temperature was constant during any one oscillation. The anomalous fluctuations occurred only at the higher temperatures. While the influence of temperature on the rate of respiration could account for a general reduction in the rate of CO_2 exchange, it cannot be invoked to explain the sudden efflux of CO_2, unless this efflux was under stomatal control.

Stomatal control of CO_2 diffusion offered the main control of CO_2 exchange at leaf temperatures up to 37.5°C and during part of each oscillation at higher temperatures. It is clear, however, that another mechanism limited CO_2 exchange at higher temperatures, as this exchange was depressed when the leaf diffusive resistance was minimum (Fig. 3). Other resistances to CO_2 transfer are involved in CO_2 exchange (*4*), and it may be that they are temperature-dependent. If the rate of photosynthesis was limited by another resistance, then a leveling rather than a periodic depression would be expected.

The influence of leaf temperature on the rate of CO_2 exchange was also observed under conditions when the leaf diffusive resistance was constant. In these experiments the stomatal diffusive resistance was the major component of the leaf diffusive resistance. Under steady-state conditions CO_2 exchange at both 30° and 42.5°C was virtually the same at the same leaf diffusive resistance (Fig. 3). Furthermore, in one experiment leaf temperature was maintained at 42.5°C for 6 hours with a low leaf diffusive resistance without a significant change in CO_2 exchange. In contrast, there was a significant reduction in the rate of CO_2 exchange at the lowest leaf resistance during oscillations in stomata at 42.5°C.

The only parameters, beside leaf temperature, we could link to this depression were a lowered level of plant water and high rate of transpiration. Carbon dioxide exchange was closely related to transpiration up to a rate of 6×10^{-6} g cm^{-2} sec^{-1}, but at higher rates of transpiration the rate of exchange declined (Fig. 4). The rate of transpiration may have influenced photosynthesis through lowered water content of the leaf, but the depressions of CO_2 exchange often occurred without any consistent reduction in leaf water. If the depressions are directly related to the water relations of the plant, it would appear that the rate of flow of water rather than the content of water in the leaf has influenced the photosynthetic process. This suggestion cannot be valid unless we suppose that the effect is dependent on temperature, as high rates of transpiration occurred without influencing the rate of CO_2 exchange at lower temperatures. Under steady-state conditions, the leaf responds to increasing water stress mainly by increasing stomatal diffusive resistance, so that the special condition, noted during the oscillations, of a high transpiration rate with a low content of plant water is unlikely to occur under steady states.

The effect is difficult to explain but may be due to variation in water content between organelles within the leaf, encouraged by temperature-dependent permeability changes, high transpiration rates, and low content of plant water. This could alter the volume, structure, or the capacity for ion exchange of the chloroplasts, and thus influence the photosynthetic capacity of the leaves. A particular explanation could be the effect of water content of chloroplasts on the reversible uncoupling of photophosphorylation from electron transport of the photosynthetic process (see *5*).

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Limb Regeneration: Induction in the Newborn Opossum

Abstract. *The marsupial* Didelphys virginiana *(the North American opossum) is uniquely suited for studies of mammalian limb replacement. By transplanting nervous tissue to the limb, regeneration has been successfully induced in this mammal.*

In lower vertebrates at least two factors, nervous (*1*) and hormonal (*2*), are decisive in determining whether or not regeneration will occur after limb amputation. Investigators have used this knowledge to induce regeneration in typically nonregenerating appendages of adult anurans (frogs) (*3*) and reptiles (lizards) (*4*). However, previous attempts to induce mammalian limb regeneration have been disappointing (*5*).

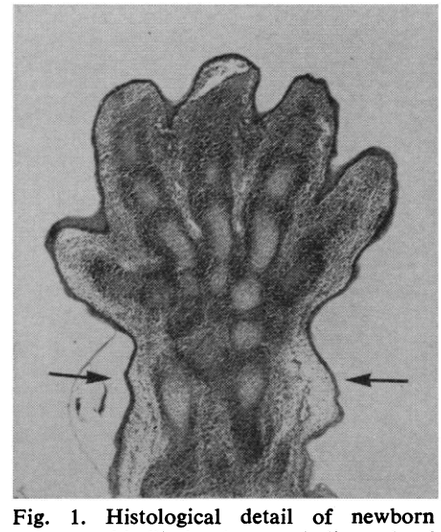

Fig. 1. Histological detail of newborn opossum hindlimb. Arrows indicate level of amputation (× 36).

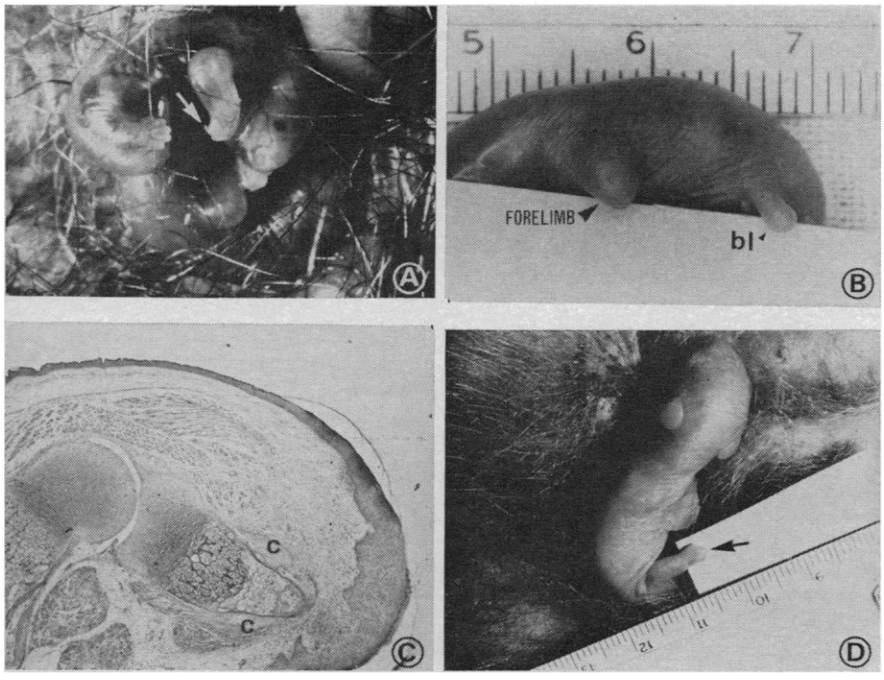

Fig. 2. Controls. (A) Newborn opossum (1 cm long) in pouch showing dichotomy of limb development: forelimbs well-developed with digits and claws (arrow); hindlimb with margins of the digits just becoming evident (× 7.8). (B) Twelve-day old opossum. Simple amputation of left forelimb and left hindlimb was performed 6 days after birth (thus, photo shows animal after 6 days "regeneration"). No external indication of regenerative response in forelimb, but note blastema-like appearance of hindlimb (*bl*) (scale in centimeters). (C) Photomicrograph of sagital section of forelimb in Fig. 2B. Note thickened apical epidermis, but also note the premature redifferentiation of subjacent tissues and the cartilagenous callus (*c*) forming around the radius (× 21). (D) Control hindlimb showing the best regenerative response after simple amputation. Note digit-like protuberance (arrow). Animal 42 days old, amputated above ankle 5 days after birth (therefore pictured after 35 days "regeneration") (scale in centimeters).

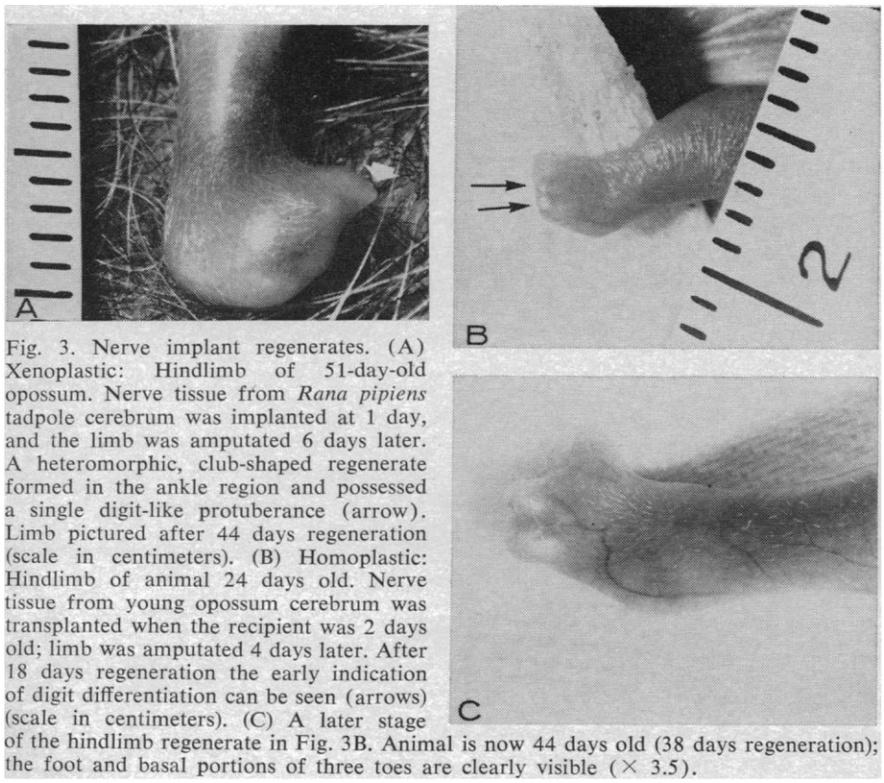

Fig. 3. Nerve implant regenerates. (A) Xenoplastic: Hindlimb of 51-day-old opossum. Nerve tissue from *Rana pipiens* tadpole cerebrum was implanted at 1 day, and the limb was amputated 6 days later. A heteromorphic, club-shaped regenerate formed in the ankle region and possessed a single digit-like protuberance (arrow). Limb pictured after 44 days regeneration (scale in centimeters). (B) Homoplastic: Hindlimb of animal 24 days old. Nerve tissue from young opossum cerebrum was transplanted when the recipient was 2 days old; limb was amputated 4 days later. After 18 days regeneration the early indication of digit differentiation can be seen (arrows) (scale in centimeters). (C) A later stage of the hindlimb regenerate in Fig. 3B. Animal is now 44 days old (38 days regeneration); the foot and basal portions of three toes are clearly visible (× 3.5).

With increasing age the rate of regeneration and even the ability to regenerate diminish; for example, the developing hindlimbs of metamorphosing frogs can regenerate during early stages of development but lose this ability before the adult condition is attained (*6*). (This does not mean that adults undergo irreversible modifications in limb tissue which preclude regeneration, for the successful induction of adult anuran and reptilian limb regeneration indicates that this is not the case. But, as a general rule, "younger" tissues are more responsive.) Some developing mammalian tissue can adjust to environmental changes and insults. Nevertheless, during mammalian ontogeny this ability wanes, and at birth mammals are incapable of regenerating limbs.

Nicholas had shown that amputated forelimbs of 14-day-old, or older, rat embryos *in utero* do not regenerate (*7*). And pilot experiments with mouse digits indicated that our present knowledge was still too rudimentary for induction of regeneration in newborn rodents to be attempted. Marsupials are unique in that they are born at a very early state of development. The newborn opossum thus presents a rare opportunity to perform chronic experiments on extremely young mammalian limbs.

Each series of experiments began in January; by early summer, work with the living pouch young was completed, and the parents were released. The opossums were trapped in areas adjoining New Orleans during the January to May breeding season. Some of the captured females contained pouch litters young enough to be used in the following experiments [developmental charts prepared by earlier investigators were used to determine the approximate age of the pups (*8*, *9*)]. However, most animals were bred in captivity, and newborns were obtained from females that gave birth in the laboratory.

Females were anesthetized with an inhalant anesthetic, Penthrane (Abbott Laboratories), and all amputations were performed under a dissecting scope with iridectomy scissors while the young remained attached to the teats of the anesthetized mother. Limbs were amputated directly above the wrist or ankle. Small loss of blood made the use of ligatures unnecessary. Although aseptic techniques were employed, no elaborate sterilization procedures were utilized, nor was it necessary to employ antibiotics, for in over 220 amputations infection was never noted.

Wound healing and all changes in the limb stumps after amputation were followed by repeated gross observations; at various intervals, when noteworthy changes occurred, the limbs were photographed. Periodically some limbs were fixed for histological examination; limbs were embedded in paraffin and sectioned at 8 μm. Serial sections were stained with hematoxylin and eosin or Mallory's polychromatic stain.

Throughout the course of these experiments, the animals remained attached to the mother within the protective and nourishing environment of the marsupial pouch. Adult opossums had free access to water and Gaines Meal (General Foods Corp.).

Although McCrady (*10*) had shown that newborn opossum limbs (Figs. 1 and 2A) were not capable of regenerating after simple amputation, our first experiments were performed to determine the nature of their response to amputation. At birth, the embryo-like opossums display a striking dichotomy in limb development (Fig. 2A). Forelimbs have well-developed digits complete with claws, which permit the newborn animal to crawl from the birth canal to the pouch; but hindlimbs are rather rudimentary structures, with merely the early external indications of digits. However, histological examination has shown that cartilagenous models of all the phalanges are already present in the hindlimb of the newborn opossum (Fig. 1).

Gross observations verified that forelimbs were unable to regenerate after simple amputation (Fig. 2B). But histological examination disclosed that even the newborn forelimb exhibited a limited regenerative response (Fig. 2C); the epidermis underwent apical thickening, and the underlying tissues did undergo some dedifferentiation. However, although an appreciable amount of dedifferentiation did occur in the stump tissues, this was immediately followed by premature redifferentiation; the typical pattern of callus formation can be seen in Fig. 2C.

In contrast to the forelimb, the less differentiated tissues of the hindlimb displayed a rather remarkable regenerative response and in one case even gave rise to a blastema-like structure (Fig. 2B). Nevertheless, this structure also underwent premature redifferentiation and resulted in very limited replacement of the amputated portion of the limb. Simple amputation of the hindlimb does not lead to regeneration, and our results confirm McCrady's (*10*) finding that newborn opossums cannot regenerate hindlimbs after simple amputation. Of 94 hindlimb controls, Fig. 2D depicts the best regenerative response obtained after simple amputation. In this case, a flattened structure developed, and 37 days after amputation the very earliest indication of a digit-like protuberance was visible (Fig. 2D). This remarkable response of the hindlimb, albeit insufficient to produce a regenerate, lent encouragement for future attempts at inducing limb regeneration in the hindlimb.

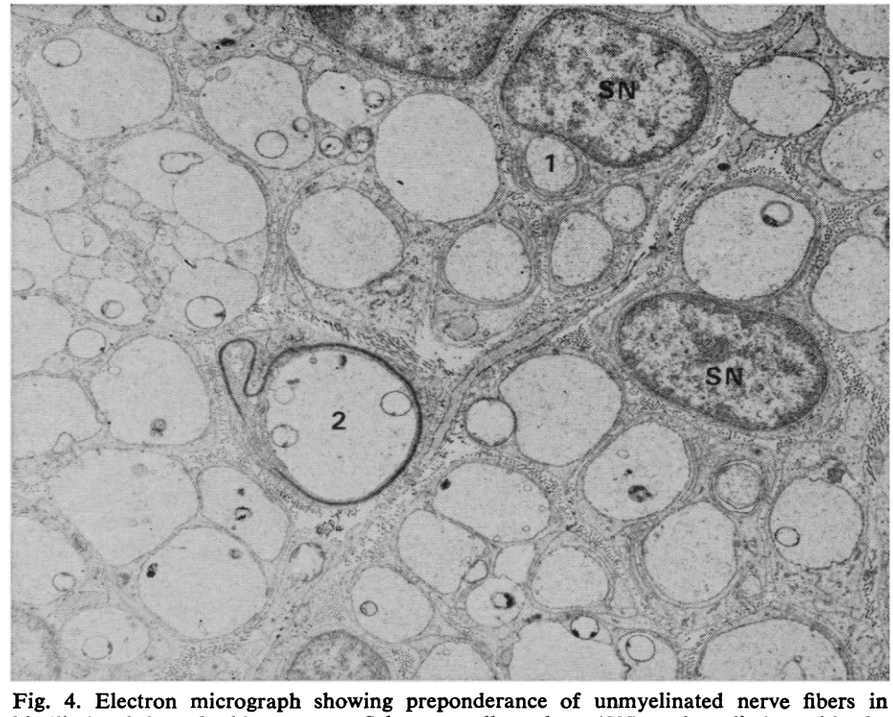

Fig. 4. Electron micrograph showing preponderance of unmyelinated nerve fibers in hindlimb of 3-week-old opossum. Schwann cell nucleus (*SN*); axis cylinder with the earliest indications of myelin envelopment (1); slightly later stage in myelinization (2); all other nerve fibers are essentially naked ($\times$ 9,000).

Since preliminary experiments indicated that hindlimbs amputated later than 1 week after birth exhibited a pattern of healing similar to that of the forelimb, subsequent experiments were performed on hindlimbs of animals less than 1 week old. Earlier investigations have shown that during the first week of life, the opossum's immune mechanism is inoperative (*11*). In fact, at birth the thymus itself is merely an epithelial anlage (*9*). This lack of an immune mechanism permitted homologous and even heterologous transplants to persist in these young opossums.

Developing forebrain (cerebral cortex) was chosen as the source of nervous tissue for the transplantation experiments. The brain was removed from the donor and placed in chilled normal saline. The cerebrum was cut into several small pieces which were then picked up by a previously prepared fine-drawn glass pipette (inner diameter approximately 0.5 mm) whose surfaces had been lightly coated with silicone (Siliclad, Clay-Adams, Inc.) to facilitate transfer of the tissue. The slender point of a watchmaker's forceps was inserted into the proximal thigh region of the recipient hindlimb and extended distad, thereby creating a channel in the 1- or 2-day-old opossum limb. The pipette was inserted into this channel, and the small cylinder of nervous tissue (approximately 0.5 by 1.5 mm) was transferred to the hindlimb so that the long axis of the implant was parallel to the long axis of the limb.

Two to four days later the limb was amputated so that the amputation plane transected the implant. All amputations were attempted through the distal portions of the tibia and fibula (Fig. 1); but in some cases, because of the small size, amputation was inadvertently made through the ankle. When the operation was successful, a regenerative response ensued.

When nervous tissue from forebrain of young *Rana pipiens* tadpoles (Taylor-Kollros stage VII) was used, a positive response was noted in 3 of 14 cases. The

best regenerate resulting from these heterologous nerve transplants was a curiously shaped outgrowth (Fig. 3A) consisting of a distal club-shaped structure which emerged from the ankle region and possessed a single digit-like protuberance on its medial surface. This response surpassed that of controls with simple amputations, but did not approach the extent of development achieved by the homologous nerve transplants.

Opossum nerve tissue evoked a positive response in 8 of 30 cases. The best regenerate resulting from homologous transplants of young opossum cerebrum is shown in Fig. 3, B and C. Figure 3B shows the animal at 24 days of age (nerve tissue implanted at 2 days and limb amputated 4 days later); after 18 days of regeneration a recognizable foot-like structure possessing the first indications of the fourth and fifth toes (Fig. 3B) could be seen. Development continued, and 20 days later (38 days regeneration) a heteromorphic foot containing the basal portions of three toes was evident (Fig. 3C). Since hindlimb development at birth (6 days before amputation) had already reached a state where all of the bones were present as cartilagenous models, the replacement of the foot and three toes must be interpreted as regeneration and not embryonic regulation.

The manner in which regeneration proceeds in the opossum hindlimb is very similar to regeneration of the metamorphosing frog hindlimb. As in hindlimb regeneration in the metamorphosing frog, histological landmarks which indicate the original plane of amputation are soon lost. Initially there exists a slight difference in tissue densities in the regenerating opossum limb, but it soon becomes impossible to detect the level of amputation. The histological aspects of regenerative phenomena in these two developing limb systems have been compared (*12*).

Two unique characteristics of the newborn opossum undoubtedly contributed to the development of regenerates: (i) absence of an immune mechanism, which prevented rejection of nerve implants; and (ii) short gestation period—a mere 12.75 days [at birth the opossum is equivalent in development to a 12-day rat embryo or a 2-month human fetus (*13*)]. Another feature of the opossum hindlimb which may participate in its ability to regenerate is the relatively "immature" state of the nerve fibers after they make their appearance in the limb. As late as 3 weeks after birth, nerves of the hindlimb are essentially in an unmyelinated condition (Fig. 4).

My studies demonstrate that young opossum limbs can regenerate when additional nervous tissue is supplied. Results of control experiments indicate that neither the trauma of simple amputation, the trauma of implantation, nor the implantation of other homoplastic tissues (for example, liver or kidney) can evoke the regenerative response which results after implantation of brain tissue. Although the opossum has afforded the opportunity to induce regeneration in young mammals, it should be pointed out that we are no closer to an understanding of the mechanism of nerve action in regeneration than before. However, we are now in a position to compare this mammalian limb regeneration with regeneration in lower vertebrates; as their similarities and differences become apparent, additional insight into the phenomenon of mammalian cellular differentiation should be gained. Hopefully, once these factors are ascertained in young opossum regenerates, the newly acquired knowledge can then be successfully applied to other mammals.

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8 May 1968

Feeding and Core Temperature in Albino Rats: Changes Induced by Preoptic Heating and Cooling

Abstract. *At an ambient temperature of 25°C, selective cooling of the area preoptica medialis to 24° ± 1°C produced a significant decrease in food intake together with hyperthermia. Heating the same area to 43° ± 1°C resulted in the opposite effects. At an ambient temperature of 35°C, heating the area preoptica medialis to 43°C resulted in a decrease in food intake despite concomitant hypothermia.*

There is ample evidence for multiple factors affecting feeding behavior (*1*). "Glucostats," "thermostats," energy flow, psychic factors, stomach distention, and specialized receptors for various catecholamines and other hormones (*2*) all may play a role, but the overall equation for the regulation and control of food intake is yet to be written.

Since ingestion of food adds calories, a hungry homeotherm in a hot environment must choose between ingestion of fuel for metabolism and maintenance of thermal stasis; in most instances, in all mammals observed, the demand for normothermia overrides the "hunger" signal (*3*).

Temperature-sensing receptors that relay information about ambient temperature and internal body or nonbrain "core" temperature to the central nervous system exist in the periphery. There are also the temperature-sensitive neurons in the preoptic area (PO) of the diencephalon (*4*). Which of these, if any, provides the prepotent signal that determines whether the animal will eat more or less? In other words, which piece of information or contribution of bits of information govern the animal's feeding behavior?

We used male Sprague-Dawley albino rats, caged individually. During feeding test periods, each animal was placed in a box (24 by 27 by 29 mm high) with transparent plastic walls, set on a floor of galvanized mesh (*5*). Rats had free access to water in the home cage, and the amount consumed daily between trials was measured. In all feeding experiments, dry Purina chow was used. Each animal was fed for a 2-hour period, at the same time each day, in a lighted room. Data for this report were collected after food intake became stabilized.

A liquid-cooled (or heated) thermode was devised, with an exposed gold-plated silver tip 0.35 mm in diam-