This increase in the potential for platelet aggregation and plug formation in normal, human PRP in response to bile salts is similar to that previously reported for low concentrations of adenosine diphosphate[3] and calcium ions[2].

Since PRP is always contaminated with variable numbers of red blood cells, erythrocyte counts were done on all PRPs and were found to be less than 1% of the platelet count. Thus a PRP containing 400,000 platelets/µl contained less than 4000 erythrocytes. If the average ADP content is assumed to be $4 \times 10^{-6} M/10^{11}$ red cells[4] and if all the contained ADP were released into the test system then the final concentration of ADP (contributed by erythrocytes) would be of the order of $10^{-8} M$. This amount of ADP does not cause rapid platelet aggregation and plug formation (Table). However, it might be argued that 'low density' red cells remaining in PRP might represent a select portion of the total red cell population with very high ADP content. For this reason it is not possible at this time to fully exclude the possibility that components released from red cells may contribute to platelet aggregation initiated by bile salts.

Extracts of freeze-dried human platelets have been examined by gas-liquid chromatography in the laboratory of Dr. D. KOWLESSAR. The preliminary findings indicated peaks with retention times similar to those obtained with standards of deoxycholic, chenodeoxycholic and cholic acid and in amounts greater than could be accounted for by contamination with plasma. Further studies will be necessary before final identification of these peaks can be achieved.

The data in this report indicate that exogenous bile salts can cause platelet aggregation and that bile salts may be present within or on platelets. An intensive search for bile salts or bile acids in platelets appears desirable.

Effect of bile salts[a] on platelet aggregation[b]

Substance tested		Seconds to produce First particles	Snow storm	Large aggregates	'Platelet plug'
Sodium deoxycholate		12	19	23	28
Sodium taurodeoxycholate		20	25	30	[c]
Sodium taurochenodeoxycholate		25	30	35	[c]
Sodium taurocholate		25	35	50	[c]
Controls	Taurine ($8 \times 10^{-3} M$)	35	[c]	[c]	[c]
	ADP ($1 \times 10^{-8} M$)[d]	50	[c]	[c]	[c]
	Buffered saline	[c]	[c]	[c]	[c]

[a] Each bile salt was tested at a final concentration of $7.5 \times 10^{-4} M$. [b] Details of the test system are given in reference[2]. In brief, 1 ml of PRP was added to a plastic loop. This was followed rapidly by 0.1 ml of buffered saline solution. The loop was closed and the contents mixed for 1 min by rotating at 12 rpm. Bile salt or control solution (0.1 ml) was added, the loop was closed and rotated again. A stop watch was started to time the occurrence of the aggregation parameters. The PRP was kept in siliconized glass tubes at 20 °C and all tests were run at this temperature. [c] The event did not occur. [d] Included as a control to show that ADP which could possibly be released from small numbers of erythrocytes cannot account for the potent effect of bile salts on platelet aggregation.

Zusammenfassung. Nachweis, dass der Zusatz von Gallensäure die Aggregation menschlicher Plättchen im Plasma in vitro herbeiführen kann und dass die Säure selbst in den Plättchen vorhanden sein dürfte.

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Survival of Embryonic Limb Bud Transplants in Snapping Turtles[1]

Embryologists have shown that allogeneic and xenogeneic tissue can be transplanted successfully between ectothermic vertebrate embryos[2]. The ultimate fate of such grafts received little attention until the discoveries of histocompatibility antigens and immunological tolerance. During the past decade there has been some clarification of the relationship between immunological tolerance and the degree of genetic disparity between amphibian donor-host combinations[3–6]. On the other hand, others reported wide ranges of reactions, from tolerance to rapid rejection[7], rejection of xenografts in one direction, tolerance in the other[8,9] and rejection or tolerance depending on graft dosage[10,11].

A recent article from our laboratory reported the fate of embryonic transplants in two xenogeneic turtle combinations. The rejection of *Amyda ferox* parts (pigment and carapace rudiments) by *Chelydra serpentina*, occurred a few months after hatching. However, partial or complete acceptance for up to 4 years of *Chysemys picta* parts by *Chelydra serpentina* was found in all cases[12]. The studies in the present report involve limb bud transplants, whose viability or destruction can be assessed more accurately than that of less conspicuous embryonic rudiments. These studies were designed to investigate the fate of allogeneic and xenogeneic embryonic transplants in the snapping turtle, *Chelydra serpentina*.

Materials and methods. Stocks of snapping turtles, *Chelydra serpentina*, and painted turtles, *Chrysemys picta*, with fertile eggs were obtained in early June from the Lemberger Company in Oshkosh, Wisconsin, USA, and from the Oak Orchard Conservation Area in central New York, USA. They were fed whitefish once a week and were maintained in a large tank containing 3–4 inches of water at about 20 °C until the procurement of their eggs.

The methods of procurement of turtle eggs, the general operative procedures, and the post-operative care used in this work were those described in a previous report[13].

Operative procedure. Limb bud transplantation was performed on embryos between stages 12 and 13. These stages were attained by 30 or 35 days incubation at 20 °C or about 9 days at 30 °C[14]. Some eggs were opened shortly after procurement. Others were opened immediately prior to operation. Both 20 °C and 30 °C constant temperature boxes were used in order to retard or speed up development so that the embryos to be used would reach the proper stage for operation at the same time.

Routine sterile technique was used throughout the operations. With the aid of a dissection microscope, the vitelline and amniotic membranes were cut with a glass needle to attain access to the embryo itself. A sharper glass needle was then used to cut off the limb as proximally as possible. The host's limb was removed in this manner and was discarded. A donor limb bud was cut off in the same manner and was carried over to the host in a drop of fluid yolk between the points of a pair of fine forceps. The transplant was pushed into an orthotopic position on the host embryo with a blunt glass needle. A small piece of tantalum foil was placed on the transplant with fine forceps to hold the grafted limb in place as it healed. Sulfadiazine powder was sprinkled over the open area of the egg as a precaution against infection. Bleeding usually stopped in 15–20 min. The eggs were kept in a covered, dry sterile bowl overnight. The following day the tantalum foil was removed with fine forceps. The eggs were put in separated finger bowls containing a few ml of water sprinkled with sulfadiazine powder. The bowls were sealed in plastic bags (Baggies) and kept in a room at approximately 25 °C. The animals hatched in early September after 9 to 10 weeks of incubation.

Operations were performed on forelimbs in some cases and on hindlimbs in others. Host embryos were always snapping turtles. Donors were siblings, non-siblings and painted turtles.

Results. Growth and development of limb bud transplants. Embryonic limb transplantation operations resulted in varying degrees of technical success. Those considered most successful were animals that maintained functional and morphologically normal transplants at hatching. These limb grafts developed at about the same rate as the normal limbs of the host. Allografts attained the size and morphological detail of normal host limbs. Xenografts also grew normally but attained the size and morphological distinction of the donor species, the painted turtle.

Others maintained limb transplants that were somewhat atypical in both form and function. Some grafts were simply undifferentiated masses of cells possessing only the pigmentation characteristics of the donor species. This was undoubtedly due to technical trauma. The grafts that were undifferentiated were not included in the data since the state of such transplants was difficult to assess.

Successfully transplanted limbs continued to develop after hatching of the recipient turtles.

Incidence of transplant rejection. Continued observations of grafted animals revealed the following incidence of transplant rejection:

Sibling allografts. There were no instances of rejection of sibling limb allografts up to one year after hatching. In the second and third years 5 instances of rejection were observed. One animal is now 5 years old and still maintains a healthy sibling limb allograft.

Non-sibling allografts. There were no rejections of limb allografts up to 6 months after hatching. In the following 6 month period, rejection was initiated in 2 allografts. Two more limbs underwent rejection in animals over one year of age.

Xenografts. In 1 limb xenograft rejection started before the host was 3 months old, in 2 between 6–12 months and in 2 between 1–2 years. Two xenografts survived into the 2–3 year interval, with no evidence of incompatibility. See Figure 1 for summary of results gathered thus far.

The rejection process. Each of the above instances of rejection involved a chronic resorption process which lasted for several months. Incompatibility was evidenced by sloughing of epidermis, change of pigmentation and loss of claws, followed by a gradual loss of muscle tone and bone structure (Figure 2). There was no wound at any time during the rejection process. Although only a flaccid, functionless stump remained, it is impossible to state with certainty that the transplant was completely rejected. It must be pointed out that the graft-host junction is obliterated as the transplant 'takes' so that the limits of the graft are no longer evident. In chronic rejection processes, remnants of transplants often remain indefinitely. Thus, we can only state that there is gross evidence of tissue incompatibility in such allografts and xenografts at various times after hatching.

Discussion. Limb buds can be successfully transplanted between turtle embryos. The differentiation and growth patterns of both allogeneic and xenogeneic transplants are normal and host animals hatch with functional limb

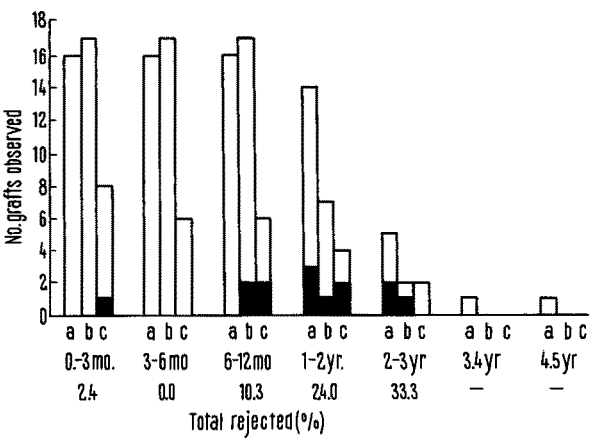

Fig. 1. The chart indicates the number of rejections (shaded) per total number of transplants observed during a given age interval. a) Sibling allografts; b) non-sibling allografts; c) xenografts (donors were painted turtles).

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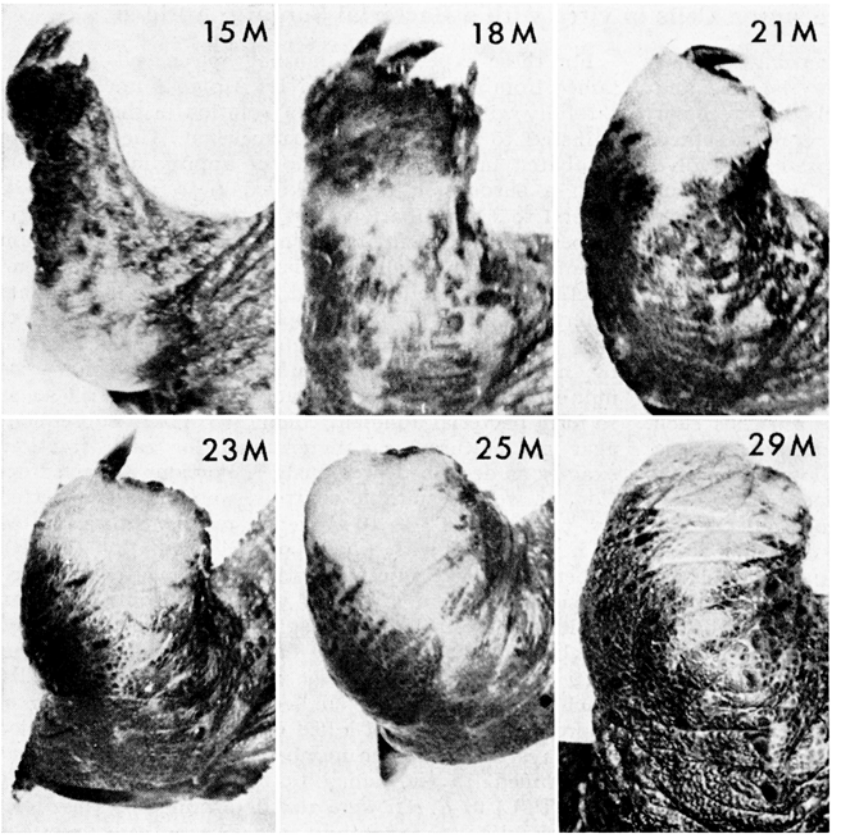

Fig. 2. Series of photographs showing the resorption of a sibling limb allograft beginning 15 months after hatching.

grafts in most instances. The xenografts maintain the morphological characteristics of the donor species.

The snapping turtle's full capacity to reject skin allografts and xenografts is attained several months after hatching[15]. Embryonic limb transplants usually do not show any signs of incompatibility well past the age of this immunological maturity. This can be partly explained by the fact that the limb transplants were performed in embryonic stages, when immunological tolerance is more easily and completely established.

Another factor which may influence graft survival is that limb transplants include bone, muscle, connective tissue and integument rudiments. All of these tissue types may not be equally immunogenic or one tissue type may enhance the survival of the rest and thereby prolong the survival of the total graft. In fact, the chronic rejection process itself may be due partly to the graft's size and the factors involved in destruction of a transplant which includes muscle and bone.

The variation of the age at which onset of limb rejection occurs is very large. The wide range of survival times may be explained by the additive effects of many weak histocompatibility loci. Chronic rejection of skin allografts and xenografts is the rule for snapping turtles as well as for many other ectothermic vertebrates[16]. There is substantial evidence that weak transplantation antigens bring on these chronic rejection responses and that the degree of sharing of these antigens results in the wide ranges of chronic rejection times[17]. The manifestations of such histocompatibility relationships in embryonic transplantation is still unknown.

The relationship between onset of rejection and phylogenetic diversity between host and donor can be seen in the fact that 3/8 xenografts underwent rejection before one year, whereas 2/17 allografts and 0/16 sibling allografts underwent rejection during the same period. The implication of this data is that onset of rejection of the limb transplant to some extent depends on the genetic disparity between individuals in the donor-host combination. In light of this evidence, the consistent prolonged survival of *Chrysemys* pigment and carapace rudiments on *Chelydra* is difficult to explain[12]. Perhaps limb bud transplants require separate assessment due to possible antigenicity variables such as origin of tissue rudiments and dosage.

Although closer parallels cannot be drawn at the present time, it is now evident that transplantation between embryos, in one reptilian species of ectothermic animals, does not assure permanent survival of the transplants.

Résumé. Des membres furent transplantés chez des tortues embryonnaires de *Chelydra serpentina* en combinaisons allogénique et xénogénique. Ces greffes survivaient longtemps, mais étaient usuellement rejettées de façon chronique.

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