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9. In this reaction, the incubation mixture was identical to that of the "isotope exchange" method (*8*), except that 2 m*M* palmityl-CoA was present instead of 5 m*M* palmitylcarnitine, and no CoA was added. Incubation was at 37°C for 5 and 10 minutes. Isolation of palmityl[$^{14}$C]carnitine was performed as in the "isotope exchange" method.
10. Muscle samples (1 to 3 g), frozen and stored in liquid nitrogen, were thawed in nine volumes of ice-cold 0.15*M* KCl, 50 m*M* tris-HCl, *p*H 7.5, finely minced, and homogenized in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 1,000*g* for 15 minutes, and the supernatant was passed through two layers of cheesecloth and centrifuged at 14,000*g* for 20 minutes. The mitochondrial pellet was rinsed gently with medium to remove the superficial fluffy layer, and resuspended in 0.5 to 1.0 ml of extraction medium. As judged by ultrastructural examination (E. Bonilla) these fractions appeared remarkably pure, and the mitochondria were well preserved.
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## Ontogeny and Peripheralization of Thymic Lymphocytes

Abstract. *Reciprocal transplantation of undifferentiated thymic primordia between diploid and triploid chromosomally marked frog embryos has revealed that thymic lymphocytes are ontogenically derived from elements of the thymic primordium rather than from blood-borne stem cells that migrated into the developing organ. Virtually all the lymphocytes in the spleen, kidneys, and bone marrow of adult frogs are descendants of these original thymic stem lymphocytes.*

The embryonic derivation of thymic lymphocytes and of lymphocytes in the peripheral lymphoid tissues has been controversial (*1*). Studies of 12-day-old mouse thymic rudiments cultured in vitro (*2*) suggested that some epithelial cells of the rudiment itself transformed into lymphocytes. However, the argument that lymphocyte precursor cells had already migrated into the thymic epithelium from the blood islets of the yolk sac by 12 days of gestation (*3*) is consonant with the theory currently in vogue that during thymus differentiation, thymic lymphocytes are derived from invading hemocytoblast-like cells rather than from epithelial cells. This hypothesis has received substantial support from a variety of experimental protocols (*4*), most notably those in which vascular migration pathways of chromosomally labeled cells have been determined in 7- to 8-day-old chick embryos (*1, 5*).

We have reinvestigated the question of the thymic versus the extrathymic origin of lymphocytes in the developing thymus and peripheral lymphoid organs in an amphibian system. The embryo of the leopard frog, *Rana pipiens*, has provided us with a model in which we have been able to identify and chromosomally label the presumptive thymic tissue, remove it at a very early embryonic stage (even before it can be called a rudiment) from an embryo whose vascular system and blood islands are still undifferentiated, and transplant it orthotopically (either unilaterally or bilaterally) to an unlabeled recipient of the same age. We have been able to rear these chimeric embryos through metamorphosis and then quantitatively determine the relative percent of donor and host cells that populate in the differentiated transplant and the lymphoid organs of the host. Such procedures, which are necessary to resolve this question, are technically impossible with murine embryos.

The following experiments reveal that, at least in the frog, thymic lymphocytes arise in situ from components in the presumptive thymus area of very early embryos. After thymic differentiation, these lymphocytes leave the thymus and seed the peripheral lymphoid organs.

To verify the suspected location of the presumptive thymus tissue, we microsurgically extirpated the gill buds from avascular 72-hour (3-mm) tail bud

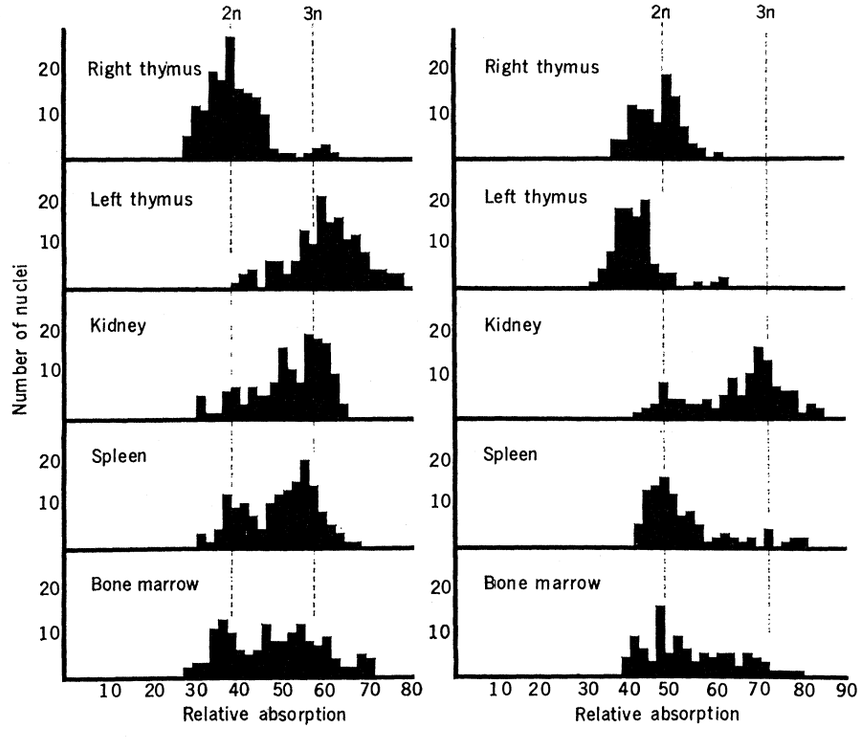


Fig. 1. Frequency distribution of absorption measurements of Feulgen-stained nuclei of tissues from two postmetamorphic triploid frog hosts. One recipient (left) had been grafted in embryonic life with one orthotopic diploid gill (thymic) primordium from the right side of a donor (unilateral exchange). Histograms at right are derived from a triploid host whose paired thymic primordia had been replaced in embryonic life with the left and right donor (diploid) gill buds (bilateral exchange). Dashed vertical lines indicate absorption maxima for each ploidy class (*12*).

stage-17 embryos (*6*) and transplanted them heterotopically to the midventral surfaces of other stage-17 embryos. At this stage of development, which follows closure of the neural folds by only a few hours, the gill bud area is histologically recognizable only as three embryonic germ layers. Nine days after transplantation, the donors and hosts were histologically evaluated. In the abdominal region of the hosts, each transplanted gill bud had self-differentiated into a typical array of gill-associated structures including a histologically perfect thymus. In the region of the excised gill buds of the donors, there were no thymus or other gill-arch structures.

In our next series of experiments, we orthotopically and unilaterally exchanged one gill bud containing its presumptive thymus rudiment between six pairs of stage-17 embryos. One embryo of each pair was triploid ($3n = 39$); the other was diploid ($2n = 26$). Triploidy was induced by subjecting freshly fertilized eggs to hydrostatic pressure (*7*). After we reared each embryo beyond metamorphosis, we determined the ploidy (donor or host) of the cells in its thymuses, kidneys, spleen, and bone marrow (femur).

This was accomplished by making cell suspensions from these organs, staining them for DNA with the Feulgen reagent, and then determining the relative absorption of individually stained nuclei with a scanning integrating type GN-2 Barr and Stroud microdensitometer (*8*). Since the amount of stain absorbed by any given nucleus is proportional to its DNA content, measurements of the light absorbed by the stain reflected the DNA content (that is, ploidy) of that nucleus. The histograms at the left in Fig. 1 are derived from nuclear measurements of the lymphocytes from various tissues of a representative adult triploid frog that had received a diploid gill bud transplant early in its embryonic life. Since virtually all of the cells (95 percent) in the adult thymus that had differentiated from the transplant were diploid, while those in the contralateral host's own thymus gland were triploid, the conclusion that thymic lymphocytes in an adult frog thymus are derived from cells of the gill bud area (that is, the thymus primordium) appears inescapable. Moreover, this observation indicates that each thymus gland of an adult frog is almost exclusively populated by an apparently self-perpetuating population of cells that originally self-differentiated in that gland. Those triploid (host) cells in the predominantly $2n$ transplanted thymus constituted only a minor subpopulation (5 percent). Whether these cells originated from an extrathymic source, or whether they indicate a minimal recirculation of host thymus-derived cells to the transplanted organ during larval or adult life, is unclear.

The second major finding from these unilateral transplantation experiments is that those lymphocytes (or their direct descendants) which differentiated within the transplanted thymus primordium constituted a significant percentage of the total cell population of the kidney (15 percent), spleen (30 percent), and bone marrow (43 percent) of the adult (Fig. 1, left). Differential counts of adult frog kidney cells indicate that approximately 27 percent of the total number of cells examined are lymphocytes. Thus, the percent of lymphocytes derived from a single transplanted thymus primordium approaches 50 percent of the total lymphocyte population of each peripheral lymphoid organ we studied.

Our next step was to determine whether the donor-derived lymphocytes in the periphery initially originated from the thymus primordium or from other tissues which had also self-differentiated from the gill bud transplant. We first unilaterally exchanged chromosomally labeled gill buds between pairs of stage-17 embryos. When the recipients developed to larval stage I (*9*), we removed the differentiated thymus gland that had developed from the gill bud. Others have demonstrated that, prior to stage I, lymphocytes are not found in the peripheral lymphoid organs of the frog (*10*). After the unilaterally thymectomized larvae were reared beyond metamorphosis, their lymphocytes were analyzed cytophotometrically. The observation that all lymphoid tissues of these unilaterally thymectomized frogs contained only cells with the chromosomal complement of the host clearly indicates that the lymphocytes in these organs are derived from no component of the gill bud other than the thymus.

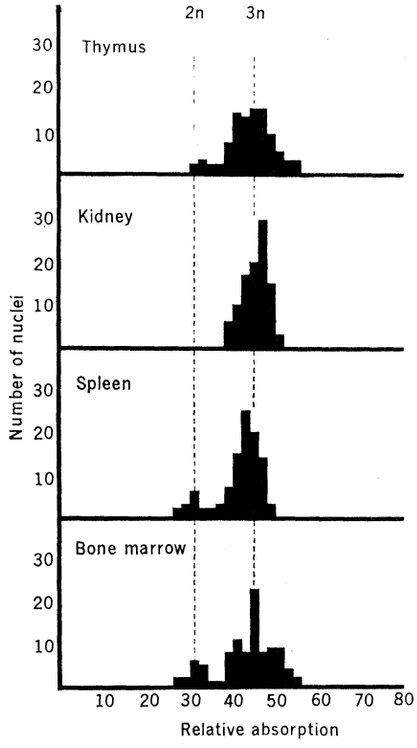


Fig. 2. Frequency distribution of absorption measurements of Feulgen-stained nuclei of tissues from a postmetamorphic triploid frog which had been grafted in embryonic life with presumptive blood islands from a diploid embryo. Dashed vertical lines indicate absorption maxima from each ploidy class (*12*).

We performed our final two experimental series to determine the numerical extent to which the cells in the peripheral lymphoid organs are seeded from the developing thymus. Our previous experiments involved the reciprocal exchange of either the left or the right gill bud. We reasoned that if we could orthotopically substitute both gill buds of a triploid embryo with the gill buds from a diploid animal, an appreciable if not twofold increase of donor-derived lymphocytes in the periphery should occur. Indeed, the distribution histograms shown at the right in Fig. 1 point out that following such a bilateral exchange, approximately 85 percent of the cells of the spleen and bone marrow of the adult triploid frog were diploid. Although donor cells constituted only 22 percent of the cell population of the kidney, it must be recalled that approximately 27 percent of the cells of a normal suspension of kidney cells are lymphocytes. As expected, virtually all the cells in each thymus were donor-derived.

Since bilateral thymus transplantation still failed to account for *all* the cells in the bone marrow, spleen, and kidney, we searched for another embryonic source of hemopoietic elements. The most likely candidate was the network of presumptive blood-forming elements on the ventral surface of the embryo. To determine the contribution of these ventral blood islands to the cells of the periphery, presumptive blood islands were removed from stage-17 triploid embryos and transplanted orthotopically

to diploid hosts. In postmetamorphic life (Fig. 2), the contributions from these ventral blood islands was evidenced as 11 to 19 percent of the total population of spleen and bone marrow cells. The small number of cells in the thymus (about 7 percent) that were derived from the ventral blood islands supports our earlier conclusion that, unlike those of birds, anuran thymic lymphocytes are derived almost exclusively from the area of the presumptive thymus gland. Furthermore, since the bone marrow and spleens of adult frogs are myeloid and erythroid as well as lymphoid, it seems most likely that the primary contribution of the ventral blood islands is to the granulocyte and nucleated erythrocyte populations of these peripheral tissues. Detailed differential cell counts, however, are needed to clarify this possibility.

Our findings provide strong and direct evidence that most cells of the frog thymus are not derived from circulating embryonic mesenchymal cells. Rather, they differentiate from elements within developing thymus itself. These cells or their progeny then migrate out and colonize the kidney, spleen, and bone marrow. Circulating cells do not make a significant contribution to the thymus of postmetamorphic frogs. In short, it appears that during frog ontogeny, the thymus gland is the stem cell source of diverse populations of lymphocytes (for example, T and B cells). These data reinforce the fact that amphibians have provided and will continue to provide a powerful model with which to study the cellular basis of immunity (*11*).

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## Oscillation of Cyclic Adenosine Monophosphate Concentration during the Myocardial Contraction Cycle

Abstract. *The concentration of adenosine 3′,5′-monophosphate (cyclic AMP) rises and falls during each myocardial contraction cycle. Peak concentrations of cyclic AMP precede peak development of systolic tension. Epinephrine alters the normal oscillation in myocardial cyclic AMP and increases both diastolic and systolic concentrations of the cyclic nucleotide. These transient changes in myocardial cyclic AMP indicate a potential role for cyclic AMP as a beat-to-beat regulator of myocardial contractility.*

Adenosine 3′,5′-monophosphate (cyclic AMP) was originally proposed to mediate the inotropic action of catecholamines because they activated particulate membrane bound adenylate cyclase in a dose-related manner similar to their in vivo inotropic potency (*1*). Catecholamines increase myocardial cyclic AMP levels, and the increase in cyclic AMP precedes the peak developed tension by several seconds (*2*). It is now rather clear that an elevation of myocardial cyclic AMP is well correlated with the inotropic action of catecholamines (*3*). The exact mechanism by which cyclic AMP mediates these inotropic events is unknown. However, it has been suggested that cyclic AMP mediates the many physiological processes it controls by activation of cyclic AMP–dependent protein phosphorylation (*4*). Calcium ion is an important regulator of myocardial contractility. Calcium is thought to directly activate the contractile proteins by dissociating the troponin-tropomyosin inhibitory complex (*5*). Cyclic AMP–dependent protein kinase mediated phosphorylation of troponin has now been demonstrated (*6*). In addition, phosphorylase kinase will also phosphorylate troponin (*7*). The physiological significance of these phosphorylations remains to be demonstrated. The sarcoplasmic reticulum is believed to be the site from which calcium ion is released to initiate contraction. Relaxation is thought to begin by resequestration of the calcium (which activated the contractile proteins) back into the sarcoplasmic reticulum. Cyclic AMP increases calcium uptake in a cardiac microsomal fraction containing fragments of sarcoplasmic reticulum (*8*) and the cyclic AMP–dependent protein kinase increases calcium uptake into fragments of cardiac sarcoplasmic reticulum (*9*).

Evidence is now presented that myocardial cyclic AMP concentrations oscillate during each myocardial contraction (*10*) and that this oscillation is altered in the presence of epinephrine. These findings suggest that cyclic AMP could regulate normal and hormone-induced changes in myocardial contractility by regulating myocardial calcium metabolism.

Frog ventricular strips from *Rana pipiens* were suspended horizontally between a strain gauge and binding post. They were electrically stimulated with a 4-volt square wave pulse of 10-msec duration (12 $min^{-1}$) and maintained at 22° to 25°C. Contraction duration was about 2 seconds, and the strips were superfused with physiological salt solution containing 1 m*M* $CaCl_2$ (*3*). The experimental data in Fig. 1 was obtained on whole ventricle strips with a 1-g resting tension. The strips in Fig. 2 had an 0.5-g resting tension, and two strips from the same ventricle were simultaneously superfused and electrically stimulated so that their contractions were out of phase with each other. Thus simultaneous freezing of the strips between spring-loaded blocks cooled in liquid nitrogen stopped one strip in systole and the other in diastole. The contractions were monitored on a memory oscilloscope to confirm the exact time of tissue fixation during the contrac-