in this study might have been due to strain differences or a difference in mouse age. However, the percentage of immunoglobulin and theta antigen positive cells from normal Balb/c mouse spleen cells indicated that there was a general correlation between cells classified by scanning electron microscopy and cells bearing globulin or theta antigen surface markers, similar to the study by Polliack *et al.* (*7*).

Within 5 days after infection with FLV a marked change in the percentage of the smooth and villous cell types was evident by scanning electron microscopy (Fig. 1B). An increasing number of cells appeared with smooth surfaces; these were generally larger than the typical smooth cells observed in normal mice. This type of cell increased rapidly during the next few days, so that by days 7 to 10 after infection 45 percent of the lymphoid cells were of this type (Fig. 1, C and D, and Table 1). By day 17 most splenocytes had a smooth topography (Fig. 1E and Table 1). Many of these smooth cells appeared to have "holes" in their surface; these cells first became apparent on days 5 and 10 and increased in number so that by day 30 numerous cells of this type were prominent (Fig. 2, A and B). These changes in cell type preceded the development of overt splenomegaly, which first became evident on days 7 to 10 and reached a maximum by 25 to 30 days after infection (Table 1). By this time most of the spleen cells were of the large, smooth-surfaced type, with few normal lymphocytes evident.

Immunosuppression preceded development of overt splenomegaly and the marked alteration of cell types shown by scanning electron microscopy (Table 1). However, FLV-associated antigen became evident in the spleen as early as 2 days after infection, and then increased rapidly so that a majority of the spleen cells showed FLV surface antigen by the tenth day after infection (Table 1). Similarly, the percentage of splenocytes containing surface immunoglobulin decreased as a function of time after infection. This decrease paralleled the decrease in number and percentage of highly villous cells in the spleen and was consistent with the likelihood that many villous cells represented B cells bearing surface immunoglobulin. There was a somewhat slower but still consistent decrease in the number and percentage of cells staining with antitheta serum during the course of infection (Table 1). It should be noted that these changes were not due merely to splenomegaly and dilution of "normal" spleen cells by tumor cells (Table 1).

The results of these studies are consistent with and extend earlier findings concerning histologic and ultrastructural features of lymphoid tissue from FLV-infected mice (*5, 6*). In those studies the marked immunodepression induced by FLV infection seemed temporally related to the morphologic alteration in the spleen, the organ where more than 95 percent of the antibody-forming cells appear in normal mice after a single intravenous or intraperitoneal injection of sheep red blood cells. The examinations with scanning electron microscopy in the present study further revealed rapid changes of cell types which are difficult to ascertain by direct histologic studies or transmission electron microscopy (*5, 6*).

The earlier studies had shown that cells rich in ribosomal particles and endoplasmic reticulum appeared within the first week after infection and, moreover, that many intracellular and surface particles characteristic of C-type virus were present in such cells. In the present study numerous large, smooth cells with a "spongy" surface punctuated by many holes were observed at the same time intervals after virus infection. It was not possible, however, to identify viruslike particles budding from these cells, even at higher magnifications (× 20,000). Nevertheless, the earlier transmission electron microscopy studies (*5, 6*), as well as fluorescent antibody studies (*4*), showed that a large percentage of cells in the spleen of FLV-infected mice contain virus-associated antigen. Thus the "holes" in the surface of the spleen cells might represent changes due to either virus replication or, alternatively, to immunologic injury due to antigen-antibody reactions on the surface of infected lymphocytes following a possible immune response of the host to the FLV antigen. However, further studies are necessary to determine whether an antivirus immune response early after infection induces these changes. Similarly, the relation between topographic changes revealed by scanning electron microscopy to other changes induced by leukemia virus infection, especially immunosuppression, require investigation.

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28 April 1975

Differentiation of Red Blood Cells in vitro

Abstract. *Differentiation of red blood cells occurs in organ cultures of both liver and kidney tissue from tadpoles of the bullfrog* Rana catesbeiana. *Our evidence indicates that different red blood cell lines are produced by the two tissues and that these different cell lines contain different tadpole hemoglobins.*

Using organ cultures of kidney and liver tissue from tadpoles of the bullfrog *Rana catesbeiana*, we have evidence that these two erythropoietic tissues simultaneously produce different red blood cell lines containing different tadpole hemoglobins.

The larval stages of the bullfrog last for 1 to 2½ years, depending on how many winter seasons the animal must endure before reaching metamorphosis. During the larval period, the most obvious morphological changes are the overall growth of the animal and the development of hind legs. Three major and one or more minor types of hemoglobin are found in the circulating red blood cells (RBC's) during this period (*1*). Both the liver and the kidneys have been suggested as possible erythropoietic sites in the tadpole (*2*).

This report represents an important step in a series of studies that have progressed from in vivo to in vitro evaluation of sites of hemoglobin synthesis and erythropoiesis in bullfrog tadpoles. We first found that

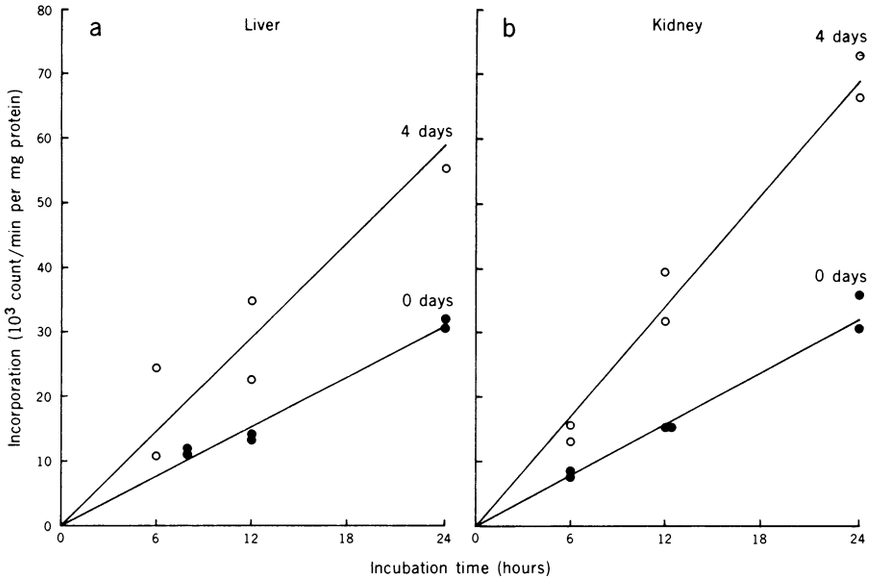

Fig. 1. Incorporation of ^{14}C-labeled amino acids into protein by organ cultures. Organ pieces were dissected from tadpoles and either used immediately for in vitro protein synthesis (*3*) or cultured as described in the text. (a) Liver tissue pieces incubated 0 to 24 hours at 22.5°C in Wolf-Quimby medium in a fashion similar to Shambough, Balinsky, and Cohen (*10*), with 1.8 μc of ^{14}C-labeled amino acid mixture (New England Nuclear) added. Protein was purified and counted by standard procedures (*11*). (b) Similar data for kidney tissue pieces for 0 and 4 days of culture.

liver and kidneys from freshly bled tadpoles contained different proportions of three major hemoglobins, as judged by electrophoresis (*3*). Next, pieces of these two organs were incubated in the presence of radioactive precursors of hemoglobin; in kidney tissue the isotopes were incorporated mainly into the hemoglobin migrating most slowly (here designated Td-3), while in liver the isotopes were incorporated almost solely into the two faster migrating hemoglobin types (Td-1 and Td-2) (*3*). These latter results were consistent with the hemoglobin patterns obtained from freshly dissected tissues.

Although these studies showed that both the liver and the kidneys were involved in differentiation of red blood cells in tadpoles, there still remained at least two hypotheses that could explain the results: (i) Red blood cells are initially produced in one of the organs and then differentiate further (mature) in the second. Or (ii), both organs carry out erythropoiesis and elaborate different red blood cell lines containing different tadpole hemoglobins. We report here two lines of evidence that support the latter hypothesis.

First, when tadpoles are made progressively anemic by phenylhydrazine treatment (*4*), the different hemoglobins disappear from the circulation at different rates. Hemoglobins Td-1 and Td-2 (made predominately in the liver) decline in relative amounts faster than Td-3 (made only in the kidneys). These results suggest the presence of different red cell lines which are differentially susceptible to phenylhydrazine lysis (*5*).

Second, the main results were obtained with organ cultures of tadpole kidneys and liver maintained in vitro from 4 to 7 days. In these experiments kidneys and liver were dissected from stage X to XII tadpoles (*6*) under semisterile conditions, the organs were washed briefly in sterile Ringers solution (*7*) containing in each 250 ml: 32 mg of penicillin G, 50 mg of streptomycin sulfate, 25 mg of neomycin sulfate, and 25 mg of nystatin. The organs were passed to a second set of dishes containing antibiotic Ringers and cut into pieces no more than 1 mm in the largest dimension. These pieces were washed by a third passage through antibiotic Ringers and then cultured by a procedure similar to that described by Balls *et al.* (*8*). The culture dishes were made from 60-mm petri dishes fitted with supports made from Nichrome wire mesh. The organs pieces rested on sterilized lens tissue paper which in turn rested on the wire mesh; the tissue paper acted as a wick to the culture medium in the bottom of the dish below the wire mesh. The culture medium has the following components for each 150 ml: 100 ml of Leibovitz L-15 medium, 12 ml of fetal calf serum, 6 ml of calf serum, 3 ml of tryptose phosphate broth (all from Grand Island Biological Co.), 29 ml of glass-distilled water, 10 mg of penicillin G, 15 mg of streptomycin sulfate, and 1.5 mg of Fungizone (Grand Island; other antibiotics from Sigma Chemical Co.). The cultures were maintained at 20°C, and the medium was changed every 3 days.

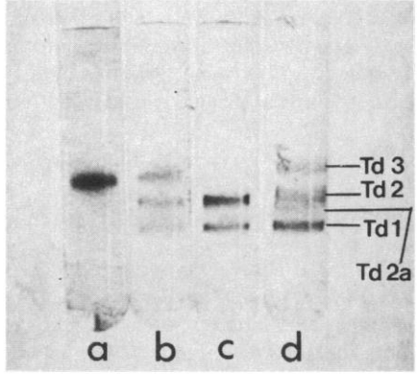

Fig. 2. Electrophoretic patterns of hemoglobins from organ cultures after 7 days. Organ pieces were cultured as described in the text. The red blood cell lysate and organ extracts were prepared, subjected to electrophoresis, and stained with benzidene-H_2O_2 as previously described (*3*): (a) hemoglobin pattern from cultured kidney tissue; (b) hemoglobin pattern of a combined culture of liver and kidney; (c) hemoglobin pattern from cultured liver tissue; (d) hemoglobin pattern of circulating RBC's obtained from the tadpole before its organs were cultured. Migration of hemoglobins was from top to bottom (cathode to anode). These distinct hemoglobin types are here labeled Td-1, Td-2, and Td-3.

This technique has been used to maintain organ pieces of tadpole kidney, liver, spleen, lung, tail muscle, heart muscle, and pancreas up to 3 months with no visible necrosis in the tissues; gill tissue, however, seems to degenerate in culture. The organ pieces are so thin that they are translucent, and necrosis is easily detected even when it rarely occurs. The tissue pieces seem to increase in size during this time, although we have no data, such as mitotic indexes, to indicate the frequency of cell division.

The tissues used in our study (kidney and liver) appeared to be as healthy after 7 days in culture as when they were first dissected from the animals. The organ pieces contained some red blood cells from the circulation when first cultured, and these cells appeared to drain out of the pieces during the first few days of culture. These organ pieces incorporate radioactive amino acids into protein at a linear rate (Fig. 1) after 0 and 4 days of culture (*9*) and maintain the same specific activity for the enzyme glucose-6-phosphate dehydrogenase as freshly dissected tissue. We have also observed that new islands of red blood cells appear on the surface of the tissue pieces (especially kidney) after 4 to 6 days in culture. We think that we are actually observing red blood cell differentiation in vitro in these cultures.

The data in Fig. 2 also support our contention that red blood cell differentiation occurs in vitro in these organ pieces. After 7 days in culture, kidney tissue contains only the slowest moving tadpole hemoglobin band (Td-3), as shown in Fig. 2a. In contrast, the liver cultures contain none of this hemoglobin, but they do contain two

other tadpole hemoglobins, Td-1 and Td-2 (Fig. 2c). The origin of hemoglobin Td-2a is open to question, since we have not found it in any of the organ culture extracts.

These results provide evidence that two different erythropoietic sites are simultaneously active in tadpoles, and that the two erythropoietic organs promote differentiation of different red blood cell lines containing different tadpole hemoglobins. We recognize the possibility of a single circulating stem cell which could become lodged in the two organs and develop into the two red blood cell lines under the influence of the different environments in the two organs. Whether there are two types of stem cell or only one, it would be interesting to know what the different factors are in the two organs that promote differentiation of RBC's containing different hemoglobins. The culture system described in this paper should allow these important questions to be investigated.

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9. The data in Fig. 1 do not indicate rates of protein synthesis per se, since sizes of the amino acid pools were not determined for the cultures. However, we have determined rates of incorporation of amino acids into the trichloroacetic acid (TCA)-soluble fractions; those data show that the increases in rates of incorporation into protein after 4 days of culture (Fig. 1) are not due to changes in rates of incorporation into the TCA-soluble fractions.
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Parathyroid Hormone and 25-Hydroxy Vitamin D_3: Synergistic and Antagonistic Effects on Renal Phosphate Transport

Abstract. *The effect on phosphate excretion of graded doses of parathyroid hormone (PTH) and the biologically active vitamin D_3 metabolite, 25-hydroxycholecalciferol (25-HCC), administered singly and in combination, were studied in the nonexpanded, vitamin D–depleted thyroparathyroidectomized rat. Infusion of 1 unit of 25-HCC per hour for 6 hours induced an antiphosphaturia only when administered with 0.2 unit of PTH per hour, while neither agent alone changed phosphate excretion. A dose of 2.0 units of PTH per hour did not cause phosphaturia unless given with 1 unit of 25-HCC per hour. In pharmacologic dosage (5 units per hour), PTH produced phosphaturia in the absence of the metabolite.*

A number of observations recorded by clinicians as well as basic science investigators over the past 30 years suggest that under certain circumstances both vitamin D and parathyroid hormone (PTH) depend on the presence of the other for the full expression of the characteristic biological effects of either (*1, 2*). For this interdependence to be demonstrated, physiological, as compared to pharmacological, amounts of each agent must be used. Thus, for example, the capacity of PTH to mobilize bone mineral requires small "priming" doses of vitamin D, according to most investigators (*3–5*). Similarly, it is generally agreed that stimulation of the intestinal transport of calcium required physiological amounts of the vitamin (*6*). However, it is also generally recognized that pharmacological doses of the vitamin can act on both gut and bone, leading to an elevation of serum calcium in the absence of PTH (*2, 7, 8*), an observation that has provided the rationale for the therapy of hypoparathyroidism and resistant forms of rickets with massive doses of vitamin D (*9*).

The interdependence of PTH and vitamin D in the renal tubules has been much less clearly defined. Opinion is divided as to whether small amounts of vitamin D are necessary for PTH to bring about phosphaturia (*5, 10, 11*) or not (*3, 5, 12*). The opposing views on this matter seem related to two principal circumstances: (i) much less attention has been directed toward an evaluation of vitamin D effects on the kidney than on the other two target organs, bone and gut (*13*); and (ii) supraphysiological amounts of one or both agents may have been used in the pertinent studies. We now report the effects on the urinary excretion of phosphate of the single and combined administration of graded doses of the biologically active metabolite of vitamin D_3, 25-hydroxy vitamin D_3 [25-hydroxycholecalciferol (25-HCC)] (*14*), and of PTH.

Female weanling rats deficient in vitamin D (Holtzman Company, Madison, Wisconsin) were fed a diet containing 0.400 percent calcium and 0.426 percent phosphorus, supplemented with vitamins A, B, C, E, and K (Teklad Mills, Chagrin Falls, Ohio). Matched control rats were given exactly the same diet, except that approximately 25 international units (I.U.) of vitamin D per day was added. From each new shipment of 24 to 30 rats, 3 to 5 animals were selected at random and killed after 3 to 4 weeks on the D-deficient diet, so that serum calcium and phosphorus could be determined. Groups of rats in which the mean calcium did not fall below 6.5 mg per 100 ml of serum were not used. Thyroparathyroidectomy (TPTX) was performed by electrocautery under light anesthesia (ether), a bladder catheter was inserted, and perfusion of the tail vein was begun. The animals were then placed in a restraining cage and urines were obtained with a fraction collector at intervals of 1 to 2 hours. The technique was a modification of that originally described by Cotlove (*15*) as adapted by Rasmussen and his co-workers (*4, 5*).

The perfusion mixture contained 4 percent glucose, and 10 m*M* calcium, 20 m*M* sodium, 2.5 m*M* potassium, and 5m*M* magnesium, as the respective chloride salts. A priming dose of inulin (approximately 0.2 ml of a 10 percent solution) was given. The sustaining solution, containing inulin (5 mg/ml) was administered at 1.3 ml per hour with a constant infusion pump so that volume expansion of the animals would be avoided. After a 14- to 16-hour equilibration period and a 4- to 6-hour control period, either 25-HCC (*16*), suspended in propylene glycol, or a highly purified preparation of PTH (Wilson Laboratories, lot 156552), dissolved in normal saline solution, or both, was administered at a rate of 0.023 ml per hour. Control animals received only the vehicle used for the 25-HCC (propylene glycol). Groups of five to eight animals were given doses of PTH from 0.2 to 5 units per hour or 25-HCC, 1 to 100 units per hour, or a combination of these agents. The animals were killed after 6 hours of drug or hormone infusion (total perfusion period, 26 hours), or both, and blood was obtained by aortic puncture. Urinary inulin was stable in most animals after the equilibration period of 14 to 16 hours; the data from those rats in which wide fluctuations in urinary inulin excretion were observed were discarded. Urine