began before or shortly after dusk, which coincides with the peak activity of both *C. fumiferana* and *C. pinus* males (*5*). Therefore, the timing of the sex pheromone release is unlikely to be an important factor. Possibly the actual quantities of pheromone released by the females differ with the species, but it is also possible that secondary chemicals are involved that increase the specificity of the sex pheromones of each species as has been suggested by Roelofs and Tette (*6*).

The apparent affinity between *C. orae* and *C. pinus* is surprising, for *C. orae* is more similar in polymorphic characters to the group of *C. fumiferana, C. occidentalis,* and *C. biennis.* Furthermore, *C. orae* feeds on spruce and fir, whereas *C. pinus* feeds on pine, a dichotomy that has been accepted as a natural taxonomic division in the genus *Choristoneura.* The morphological differences between *C. orae* and *C. pinus* and the difference in sex pheromone between *C. orae* and the other western species confirm that *C. orae* is a valid species. Conceivably *C. orae, C. pinus,* and, possibly, *C. viridis* arose from a common ancestor and diverged through allopatric evolution; but the similarity of the sex pheromones among eastern and western species may be an example of parallel evolution, *C. orae, C. biennis,* and *C. occidentalis* evolving as sibling species in the west, *C. pinus* and *C. fumiferana* as sibling species in the east.

The fact that *C. fumiferana, C. occidentalis,* and *C. biennis* all have the same or very similar pheromones raises questions concerning their relationship and possible isolating mechanisms. The range of the three species is not clearly defined at present, and there may be areas of contact. However, the potential for hybridization is reduced by ecological and temporal isolation and possibly also by the presence of secondary chemicals in the sex pheromones. The true relationship of these species will have to await detailed studies in the areas of potential contact.

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Density Gradient Separation of Marrow Cells Restricted for Antibody Class

Abstract. *Primitive cells of (C3H × C57BL/10)F_1 mouse bone marrow, participating with thymocytes in immune responses to sheep erythrocytes, are already committed to the immunoglobulin M or immunoglobulin G antibody class. By equilibrium centrifugation in discontinuous gradients of bovine serum albumin, cells responsible for production of IgM immunocytes migrate to the denser regions, whereas those responsible for IgG immunocytes remain in the lower density regions.*

Although it is generally accepted that individual immunocytes synthesize antibody of a single molecular class (*1*), it is still debated at which stage of differentiation precursor cells become class restricted (*2, 3*). In the mouse, splenic precursors of hemolytic plaque-forming cells (PFC) induced by sheep erythrocytes (SRBC) are not pluripotent. Results of cell transfer experiments indicated (i) that splenic antigen-sensitive units generate direct (IgM) and indirect (IgG) PFC independently of each other (*2*); and (ii) that in the course of immunization, splenic memory units for different subclasses of indirect (IgG) PFC develop independently (*3*). If, however, splenic antigen-sensitive units were pluripotent, as proposed by Nossal *et al.*, by Papermaster, and by Sterzl (*4*), "genetic or phenotypic shifts" within a clone must be postulated to account for the sequential production of different classes of antibody during immune responses. Attempts to identify cells simultaneously producing two classes of antibody were not successful, even during the critical times of transitions from IgM to IgG, or from γG_1 to γG_2 responses (*1*). In addition, the frequent occurrence of splenic focal areas containing direct and indirect PFC, reported by Papermaster (*4*) in support of "genetic or phenotypic shifts," became meaningless once it was realized that splenic precursor units for direct PFC are several times more frequent than the units for indirect PFC (*2*), and bound to overlap.

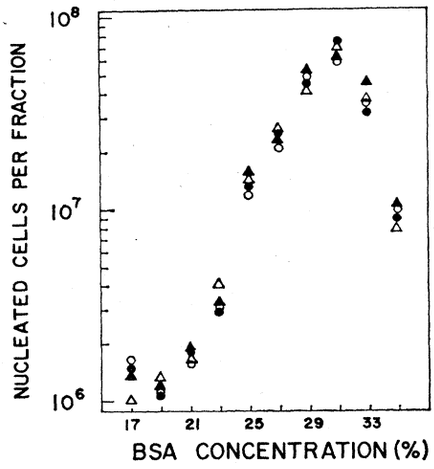

Fig. 1. Density distribution profile of nucleated cells of (C3H × C57BL/10)F_1 mouse bone marrow. 2.5×10^8 cells were layered on the top of the gradient. Symbols indicate individual separations.

In this report, new direct evidence is presented in support of class restriction of precursor units. Bone marrow cells capable of generating either direct or indirect PFC were separated by equilibrium density-gradient centrifugation. Cells were harvested from the long bones of 12-week-old (C3H/He×C57-BL/10)F_1 female mice, suspended in Eagle's medium, and subjected to equilibrium centrifugation in a discontinuous gradient of bovine serum albumin (BSA), according to Dicke *et al.* (*5, 6*). Five to ten cell fractions, designated 1 to 10, were collected from the interphases, beginning at the top, between 17 and 19 percent BSA. The fractions were diluted in 4.7 ml of Ca- and Mg-free phosphate-buffered (*p*H 7.2) saline (PBS), centrifuged at 1000 rev/min for 10 minutes at 10°C, and resuspended in PBS. Nucleated cells were counted with an electronic particle counter (*7*). Density distributions of cells are presented in Fig. 1. The profiles were reproducible and displayed one major peak in the denser region of the gradient. The proportion of cells recovered per fraction was 0.5 to 2 percent in the lower density region (17 to 23 percent BSA), 5 to 10 percent in the intermediate region (25 to 27 percent BSA), and

5 to 30 percent in the high density region (29 to 35 percent BSA). The nucleated cells that were recovered after centrifugation ranged from 78 percent to 86 percent of input.

To test for potentially immunocompetent cells, each marrow fraction was added to an equal volume of thymocyte suspension, and the mixtures were transplanted via the tail vein into each mouse in several groups of syngeneic female mice that were heavily irradiated (*8*). Each group contained five to ten animals, and each inoculum contained a limiting number of 2.5×10^5 bone marrow cells (fractionated or not) and an excess number of 5×10^7 thymocytes (*9*). Eighteen hours after transplantation, recipients received an intravenous injection of 5×10^8 washed SRBC. Direct (IgM) and indirect (IgG) PFC of recipient spleens were enumerated 9 days after transplantation by the agarose-slide technique (*10*). Animals were classified as "positive" or "negative," depending on whether their spleens contained PFC in numbers greater than or equal to those of controls. Negative controls were irradiated mice inoculated with marrow cells and thymocytes but not with SRBC, and mice inoculated with marrow cells (fractionated or not) and SRBC but not with thymocytes. The numbers of PFC in spleens of these controls did not exceed 200 direct and 100 indirect. In positive spleens, the numbers of PFC ranged from 200 to 5000 direct and from 100 to 3000 indirect. The pooled results of three experiments with marrow from non-immune donors are presented in Fig. 2, along with the results of a fourth experiment with marrow of immune donors (*11*).

Table 1. Frequencies of antibody responses to SRBC in irradiated recipients of fractionated marrow cells, 5×10^7 thymocytes, and 5×10^8 SRBC. NF, nonfractionated marrow cells; +, positive; G.M., geometric mean PFC/ positive spleen.

Fraction	Direct PFC		Indirect PFC	
	+ Spleen/ No. grafted	G.M.	+ Spleen/ No. grafted	G.M.
	1.25×10^5 Marrow cells			
1–2	1/9	985	3/8	274
3	1/9	800	4/9	429
4	3/10	619	8/10	649
5	7/9	641	1/9	175
6	9/10	723	0/10	
NF	5/8	492	2/8	205
	5×10^5 Marrow cells			
1–2	1/5	765	4/5	263
3	0/5		4/5	364
4	0/5		3/5	325
5	3/5	572	2/5	650
6	5/5	459	0/5	
10	4/5	780	0/5	
NF	5/5	700	2/5	242

In each experiment, a large proportion of mice injected with marrow cells of the low density region (fractions 1 to 4) had spleens positive for indirect PFC, but negative for direct PFC. An opposite pattern was seen in spleens of mice injected with the same number of nonfractionated marrow cells: 83 percent of the spleens were positive for direct PFC, while only 39 percent were positive for indirect PFC. Considering that the low density region of the gradient contained only a few percent of all the cells subjected to separation, fractions 1 to 3 of nonprimed, and 3 to 4 of primed bone marrow, were considerably enriched with precursor units of PFC. However, most of such precursors were capable of generating indirect but not direct PFC, upon interaction with thymocytes and antigen. Since thymic antigen-reactive cells are not subject to class differentiation, while marrow-derived cells are (*9*), the precursor units concentrated in fractions 1 to 4 were presumably restricted for the molecular class of antibody to be secreted by descendant PFC.

A great proportion of recipients of the intermediate density fractions 5 and 6 had spleens that were positive for direct but not for indirect PFC. In view of the yield of nucleated cells in these fractions (Fig. 1) and of the high percentage of positive spleens for direct PFC in recipients of nonfractionated marrow, enrichment of precursor units was less pronounced in fractions 5 and 6 than in fractions 1 to 4. The frequencies of positive spleens decreased among recipients of the high density fractions 7 to 10, although 70 percent of input cells were recovered in this region of the gradient. The few positive spleens contained direct but not indirect PFC. Thus, precursor units were depleted in the high density region and enriched in the low and intermediate regions. Depletion and enrichment for the two classes of precursor units were unequally distributed along the gradient.

As the number of 2.5×10^5 marrow cells was selected for convenience, it was desirable to verify that the results were not unduly affected by this choice.

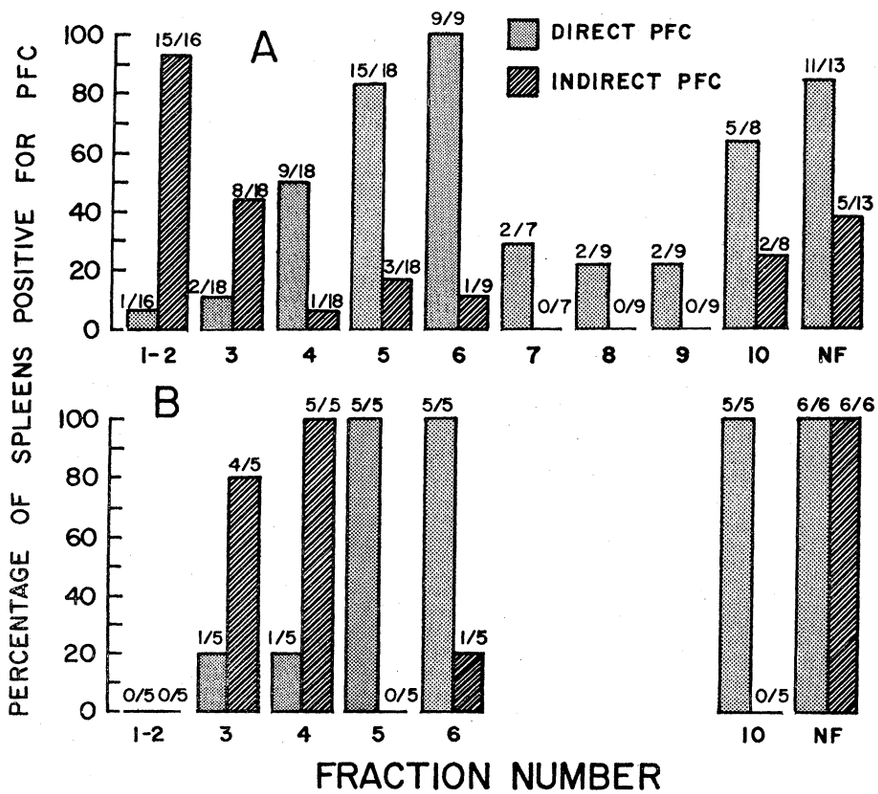

Fig. 2. Numbers and percentages of spleens positive for direct and indirect plaque-forming cells hemolytic for SRBC, 9 days after irradiation and transplantation of 5×10^7 thymocytes with 2.5×10^5 nonfractionated (*NF*, far right) or fractionated marrow cells subjected to equilibrium density gradient centrifugation. Fraction numbers correspond to different concentrations of bovine serum albumin (*6*). (A) Pooled data from three experiments with bone marrow of nonimmune donors. (B) Data from one experiment with marrow of immune donors (*11*).

In two additional experiments 1.25×10^5 and 5×10^5 nonprimed marrow cells of each fraction were transplanted into irradiated mice with 5×10^7 thymocytes (Table 1). Separation of precursor units for direct and indirect PFC was detected in both experiments. It was reassuring that the enrichment of indirect PFC precursors in the lower density region and of direct PFC precursors in the denser region was not missed because of a twofold dilution. Likewise, a twofold concentration did not provide enough marrow cells to compensate for the depletion of precursor units in any of the pertinent fractions.

The data can only be explained by class differentiation of marrow cells responsible for PFC production. Most likely this cell type is a precursor of PFC, dependent on cooperation with other cells and on antigen for further differentiation and maturation. However, the data do not exclude that "accessory" cells of marrow origin, with yet undefined but necessary immunological functions (*12*), are also class-differentiated, or instrumental in conferring class restriction to PFC precursors and to splenic antigen-sensitive units. The potential of transplanted marrow cells was tested for up to 9 days. It is unlikely that at later intervals precursor units would generate PFC of both classes, since in previous experiments restriction was verified at 25 and 35 days after transplantation (*2, 9*). Thus, the data are the first direct demonstration of commitment to antibody class in relatively undifferentiated and immature cells of the immune system, and of a biophysical counterpart, that is, variations of density possibly secondary to osmolality gradient (*13*). As class-differentiation of marrow cells appears to be under genetic control in mice (*14*), the combined use of genetic variants and physical separation of cells should provide a discriminating approach for elucidating one of the sources of antibody heterogeneity.

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6. A single lot of BSA (lot 119B-0250; fraction V, Sigma) was used to prepare a 35 percent stock solution in tris buffer, *p*H 7.2. The osmolarity was adjusted to 340 milliosmols by an indirect procedure (*5*). The gradient was prepared by layering 0.3 ml each of 35, 33, 31, 29, 27, 25, 23, 21, and 19 percent BSA in a glass tube (5 by 135 mm). The cells to be separated (2.5×10^8 per tube) were harvested from three donors and suspended in 0.5 ml of 17 percent BSA solution; the suspension was layered on the top of the gradient. The tubes were centrifuged (269 rotor of an International model PR-6) at 2000 rev/min for 30 minutes at 10°C. Equilibrium was presumably achieved since centrifugation at 2750 rev/min for 45 minutes did not change the yield of nucleated cells in each fraction.
7. Celloscope model 112 (Particle Data) equipped with a 100-μm aperture.
8. The concentration of each bone marrow fraction was adjusted to 5×10^5 cell/ml. Thymocytes from 9-week-old syngeneic female donors were suspended in Eagle's medium (10^8 cell/ml). Each mouse was injected with 1 ml of the cell mixture 2 to 4 hours after exposure to 900 to 950 rads of ^{137}Cs gamma radiation.
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Penicillin as an Epileptogenic Agent: Effect on an Isolated Synapse

Abstract. *Penicillin enhances the excitatory postsynaptic potential of the squid stellate ganglion. This effect suggests the hypothesis that the epileptic focus created by the topical application of penicillin to the mammalian cerebral cortex is produced by the facilitation of excitatory synaptic coupling within the preexisting positive recurrent feedback system.*

Penicillin is an extremely potent epileptogenic agent widely used to create experimental foci, but its cellular mode of action is poorly understood. Recent studies with the isolated crayfish stretch receptor (*1*) revealed that penicillin increases excitability in this neuron in two ways: (i) by augmenting membrane resistance so that comparable outward current pulses produce a greater reduction of the membrane potential, and (ii) by slowly depolarizing the cell and thus bringing it nearer the firing level. Further studies of a more complex neuronal aggregate, which contains at least one excitatory synapse, seemed required to clarify the way in which this agent induces experimental cortical epileptic foci. Specifically, it was deemed essential to determine if penicillin is capable of increasing the effectiveness of excitatory synaptic action since this is a crucial postulate of recent theories of penicillin epileptogenesis (*2*).

The isolated giant axon and stellate ganglion of the squid (*Loligo vulgaris*) were chosen for these studies. The direct effect of penicillin on the membrane of the giant axon itself was also examined in order to test the generality of the earlier findings of the crayfish stretch receptor.

The giant axon and stellate ganglion were isolated in the conventional manner (*3*) and mounted in a perfusion chamber of very small volume filled with oxygenated seawater. Both pre- and postsynaptic axons were stimulated by externally applied silver electrodes, and microelectrodes filled with KCl were used for intracellular recording from the postsynaptic axon, either at the ganglion or at some distance from it. Extracellular recording electrodes were blunt micropipettes filled with seawater. Conventional cathode follower and amplifying circuits were used. Only Na^+ penicillin (Upjohn) was employed in these experiments. The high content of Na^+ in the seawater made it unnecessary to compensate for the amount of Na^+ in the penicillin salt at the concentrations used [5,000 to 50,000 international units (I.U.) per milliliter]. The technique of allowing for the measured junction potentials was previously detailed (*1*).

In general, the effects of penicillin on the membrane potential of the giant