Summary. The effects of the suckling stimulus upon release and subsequent repletion of anterior pituitary (AP) lactogen were investigated in hooded Norway rats on the third or fourth day of lactation. Two hr of suckling resulted in an 88.5% reduction in lactogen content. The AP lactogen content gradually increased during the postsuckling interval with restoration to presuckling levels after 8 hr. To determine whether lactogen repletion would occur *in vitro*, AP were obtained from rats immediately following 2 hr of suckling, and cultured for 2 hr. In contrast to the results obtained *in vivo*, lactogen repletion did not occur *in vitro*.

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Received Feb. 3, 1969. P.S.E.B.M., 1969, Vol. 131.

The Interaction of a Hydrophobic Probe with Human Blood Cells* (33922)

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Many biologic properties of mammalian cells are dependent upon the conformational features of macromolecular components of the cell membranes and cell contents. Such biologic properties include contact inhibition (1), viral adsorption (2), and antigen recognition (3). Since hydrophobic interactions play a key role in the maintenance of specific macromolecular conformations on and within cells, a study of the interaction between hydrophobic probes and mammalian cells can provide direct information on the nature of cell receptors and recognition sites. Arylaminonaphthalenesulfonates are useful as probes for hydrophobic regions on macromolecules (4–6). Members of this class of compounds show enhanced fluorescence quantum yield when located in a hydrophobic environment. We here report an extension of the use of an arylaminonaphthalenesulfonate to the study of hydrophobic regions of mammalian cells.

Materials and Methods. The interaction between 1,8-anilinonaphthalenesulfonate (ANS) and human peripheral blood cells was investigated. Leukocytes and erythrocytes were prepared from heparinized blood by sedimentation in 1% gelatin (7). The leukocytes were washed four times by centrifugation in 0.05 *M* phosphate–0.15 *M* NaCl, pH 7.2 (PBS) and resuspended in PBS or in tissue culture medium F-12 (Grand Island Biological Co.) at a concentration of 1×10^6

* Supported in part by Grant 5-RO1-AI-07754 from the National Institutes of Health.

TABLE I. Photomultiplier Output in the Presence of Human White Blood Cells.[a]

Subject	ANS (M) ($\times 10^{-3}$)	E	n	SE
Leukocyte	0	2.8	89	0.2
Background	0	0	23	0.0
Leukocyte	2.5	28.0	107	0.7
Background	2.5	2.7	18	0.4

[a] E, mean photomultiplier output; n, number of measurements; SE, standard error of the mean; E per leukocyte was measured with leukocyte image focused on the phototube. Background measurements were obtained with cells positioned off the phototube input.

cells/ml. The ANS (K&K Laboratories, Plainview, N..Y) was used as the NH_4^+ salt. The fluorescence of individual cells was measured with a quantitative fluorescence microscope equipped with a photodetector as described by Rotman (8) with the following modification: the excitation filter was a Corning 5113 and the barrier filter was a combination Wratten 2A plus Wratten 8. Photomicrography was performed with a Polaroid camera using type 46-L film, a BG-12 excitation filter and a Wratten 15 barrier filter. Cell counts and trypan blue exclusion viability studies (9) were performed with a hemacytometer and standard microscope.

Results and Discussion. The photomultiplier output of the quantitative fluorescence microscope is given in Table I. The output per leukocyte in the presence of 2.5×10^{-3} ANS was 10 times greater than the output per cell in the absence of ANS. That the increased photomultiplier output in the presence of ANS was associated with cellular fluorescence is confirmed by photomicrography as shown in Fig. 1a, b, d, and e. The cellular images can be seen with both the excitation and barrier filters in place only in the presence of ANS (Fig. 1a and d). The background in the absence of ANS was virtually nil (Fig. 1e) in agreement with the data given in Table I. Accordingly, fluorescence intensity per cell (I) was defined as follows:

$$I = E_{C,ANS} - E_{B,ANS} - E_{C,PBS},$$

where E is the photomultiplier output of the fluorescence microscope. The double subscripts refer to E with a cell positioned beneath the phototube (C), to background (B), to phosphate buffered saline (PBS) and to ANS.

The measurement of fluorescence intensity per cell (I) was used to estimate the binding of ANS to hydrophobic regions of peripheral human blood cells. If the interactions between ANS and cells were indeed analogous with ANS binding to individual macromolecules, the bond should be noncovalent and reversible. Leukocytes were exposed to 2.5×10^{-3} M ANS in PBS for 20 min at room temperature. A sample was removed and washed three times by centrifugation in PBS. Values of I were obtained for the individual cells and are given in Table II. After three washes I fell to 14% of its original value. The difference between the mean values of I before and after washing is highly significant according to the t test ($p(t) < 0.001$). That the binding of ANS to leukocytes is indeed reversible was confirmed by photomicrography (Fig. 1c and f). Thus it appears that the bonds between ANS and leukocytes are noncovalent.

Blood cells were morphologically classified as granulocytes, lymphocytes, and erythrocytes and I was measured for each type. The results are given in Table III. The ANS concentration was 2.5×10^{-3} M. Cells were obtained from three normal subjects (Expts. 1–3) and from a patient with chronic lymphocytic leukemia (Expt. 4). In each case the mean value of I for granulocytes was indistinguishable from that for lymphocytes. Furthermore, the mean I for leukemic lymphocytes was not distinguishable from that for normal lymphocytes. Thus the leukemic cells from this patient do not differ in hydro-

TABLE II. Effect of Washing on ANS Binding to Human Leukocytes.[a]

Leukocytes	I	n	SE
ANS (2.5×10^{-3} M)	22.0	7	2.5
ANS (2.5×10^{-3} M) followed by PBS (3 washes)	3.0	9	1.2

[a] I, mean fluorescence intensity; n, number of cells; SE, standard error.

phobicity from the cells of normal subjects. Variances of the data in Table III were calculated and then compared by means of the F test. In each experiment the variance of I for granulocytes was significantly greater than that for lymphocytes (Expt. 1, $p(F) < 0.05$; Expt. 2, $p(F) < 0.005$; Expt. 3, $p(F) < 0.005$; Expt. 4, $p(F) < 0.01$). These results correlate with the work of Van Dilla *et al.* (10) who found that lymphocytes and granulocytes are very similar in mean cell volume but that granulocytes show greater variation. By way of comparison the value of I for erythrocytes was one fiftieth of that for granulocytes (Table III, Expt 1, $p(t) < 0.0005$, $p(F) < 0.005$) and for lymphocytes ($p(t) < 0.05$, $p(F) < 0.005$. These values for $p(t)$ were calculated for two independent samples with different variances.) Leukocyte volume is only two to four times greater than erythrocyte volume (10) and the ratio of leukocyte surface area or radius to the respective parameter for the erythrocyte is even closer to unity (11). Thus differences in cell size alone cannot explain the fiftyfold difference in I that was observed. It thus may be concluded that leukocytes possess many more hydrophobic regions of low dielectric constant which bind ANS than do erythrocytes.

Cells were exposed to ANS in medium F-12 for 20 min and viability was determined

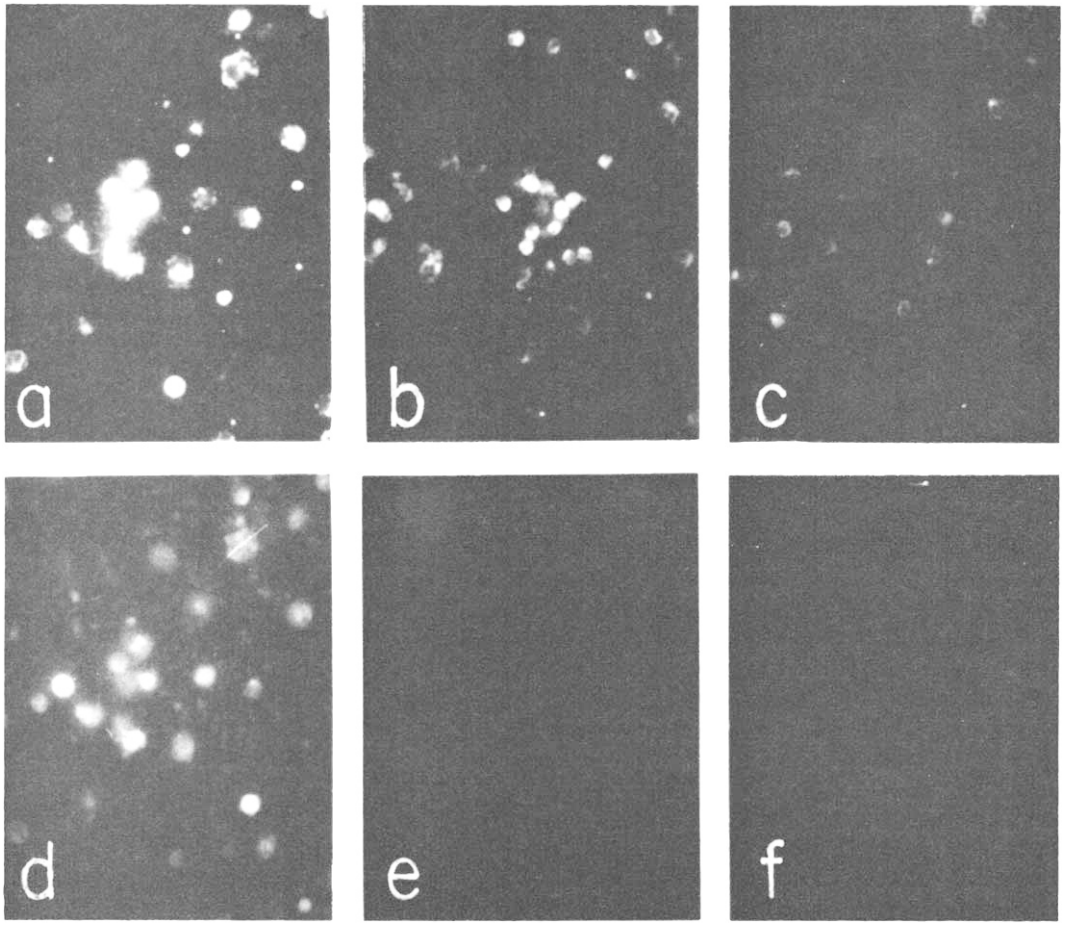

FIG. 1. Fluorescence of peripheral blood cells: (a–c), Wratten 15 barrier filter; (d–f), BG-12 excitation filter and Wratten 15 barrier filter. (a and d), 2.5×10^{-3} M ANS; (b and e), phosphate-buffered saline (PBS); (c and f), 2.5×10^{-3} M ANS for 20 min followed by 3 washes by centrifugation in PBS and final resuspension in PBS.

TABLE III. The Binding of 1,8-Anilinonaphthalenesulfonate to Different Types of Peripheral Blood Cells.[a]

Expt. no.	Subject	Granulocytes			Lymphocytes			Erythrocytes		
		I	SE	*n*	*I*	SE	*n*	*I*	SE	*n*
1	Normal	33.3	6.4	15	25.7	4.0	12	0.6	0.3	3
2	Normal	24.2	3.1	20	40.0	1.1	20	—	—	—
3	Normal	19.6	2.5	20	25.3	0.8	20	—	—	—
4	Chronic lymphatic leukemia	18.3	3.1	20	26.3	1.7	20	—	—	—

[a] *I*, mean fluorescence intensity per cell; SE, standard error of the mean; *n*, number of cells scored.

by trypan blue exclusion. The results are shown in Table IV. The ANS did not affect cell viability up to a concentration of 2.5×10^{-3} *M*. Additional studies showed viability at that ANS concentration to be independent of time up to at least 105 min. Cell viability of leukocytes treated with ANS was also tested by means of the fluorescein diacetate method of Rotman and Papermaster (12) and the results agreed with those shown in Table IV.

TABLE IV. Effect of ANS on Leukocyte Viability.

ANS conc (*M*)	Fraction viable	No. of cells scored
0	.98	968
5×10^{-4}	.98	1144
2.5×10^{-3}	.98	721
5×10^{-3}	.91	1838
5×10^{-2}	.00	(Cells were lysed)

Conclusion. We conclude that ANS binds to hydrophobic regions and forms fluorescent noncovalent complexes with human peripheral leukocytes. Furthermore, the fluorometric detection of the complexes is possible at an ANS concentration which does not kill the cells. Thus the use of ANS permits the physico-chemical mapping of hydrophobic binding sites on and within living cells. Further studies should clarify the intracellular and membranal distribution of ANS. A spectrofluorometric analysis of ANS-cell complexes should yield greater insight into the microenvironment and conformation of the hydrophobic binding sites. ANS should thus prove useful as a tool to investigate the relationship between macromolecular structure and cellular function.

Summary. A fluorescence enhancement study of the interaction between the hydrophobic probe 1,8-anilinonaphthalenesulfonate (ANS) and human peripheral blood cells was performed by means of photomicrography and quantitative fluorescence microscopy. Leukocytes appear to possess many more hydrophobic regions which bind ANS than do erythrocytes. The binding of ANS at appropriate concentrations did not significantly affect viability of cells.

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Received Feb. 5, 1969. P.S.E.B.M., 1969, Vol. 131.