Journal of Supramolecular Structure 8:431–438 (1978)
Normal and Abnormal Red Cell Membranes 153–160

The Program of Friend Cell Erythroid Differentiation: Early Changes in Na^+/K^+ ATPase Function

Dixie Mager and Alan Bernstein

Department of Medical Biophysics, University of Toronto, and The Ontario Cancer Institute, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9

Treatment of Friend erythroleukemia cells with several different chemical agents causes an early decrease in the $^{86}Rb^+$ influx mediated by Na^+/K^+ adenosine triphosphatase (ATPase). These agents, which induce Friend cells to differentiate, include dimethylsulfoxide (DMSO), ouabain, hypoxanthine, and actinomycin D. The magnitude of the early decrease in $^{86}Rb^+$ influx correlates with the proportion of cells in cultures of inducible Friend cell clones which later go on to synthesize hemoglobin. Compounds which do not incude differentiation in these cells, such as xanthine, exogenous hematin, and erythropoietin, do not cause a change in $^{86}Rb^+$ influx. A change in the intracellular K^+ ion concentration does not occur during induction by DMSO because, although there is a decrease in K^+ content per cell soon after induction, there is a parallel decrease in cell volume. These results and previous observations from this laboratory are discussed in terms of the posible involvement of the Na^+/K^+ ATPase in Friend cell differentiation.

Key words: Friend cells, Na^+/K^+ ATPase, K^+ ion levels, transport changes, differentiation

The study of normal erythropoiesis in vivo is hampered by the inherent cellular heterogeneity of the blood-forming tissues, the difficulty in obtaining large amounts of cells in different stages of development, and the inability to easily maintain long-term cultures of erythroid precursor cells. Thus, many genetic and biochemical studies are very difficult if not impossible to carry out under these conditions. Friend erythroleukemic cells can be stimulated to undergo erythroid differentiation in vitro [15] and therefore provide a useful model system for the genetic and biochemical analysis of the later stages of erythropoiesis.

Friend cells were derived from the spleens of Friend virus-infected mice. They are permanent clonal cell lines which divide every 10–12 h. In 1971, Friend and co-workers

Received for publication April 11, 1978; accepted June 9, 1978.

0091-7419/78/0804-0431$01.70

discovered that these cells could be induced to synthesize hemoglobin by treatment with dimethylsulfoxide (DMSO) [15]. Since that time several other cellular changes that are characteristic of normal erythropoiesis have been found to occur in differentiating Friend cells (Fig 1). As can be seen from this figure, the program of Friend cell differentiation has been extensively characterized. However, knowledge about the initial genetic and molecular mechanisms controlling the induction of this differentiation program is lacking. One major reason for this is the lack of chemical and biologic specificity of the Friend cell-inducing agents.

Besides DMSO and other organic solvents [15, 21–23], certain purines and purine analogs [24], butyric acid [25], and metabolic inhibitors such as actinomycin D [26] also induce Friend cells to differentiate. However, there is no known specific cellular site of action for any of these agents. Thus it has been difficult to ascertain the mechanisms of induction using these compounds. We have observed that ouabain, a specific inhibitor of the plasma membrane-bound enzyme Na^+/K^+ ATPase (adenosine triphosphatase) [27], induces differentiation in Friend cells via direct binding to this enzyme [28]. This is the only case in which interaction of an inducing agent with a specific cellular target, the Na^+/K^+ ATPase, has been shown to be necessary for induction of Friend cell differentiation to occur. Because of this observation, we have been investigating whether an alteration in Na^+/K^+ ATPase activity is a common event during Friend cell differentiation, and if so, whether changes in the function of this enzyme play an important role in the control of differentiation in this system.

MATERIALS AND METHODS

Suspension cell cultures were maintained in α medium [29] supplemented with 10% fetal calf serum (Reheis) as previously described [2]. The $^{86}Rb^+$ uptake experiments were

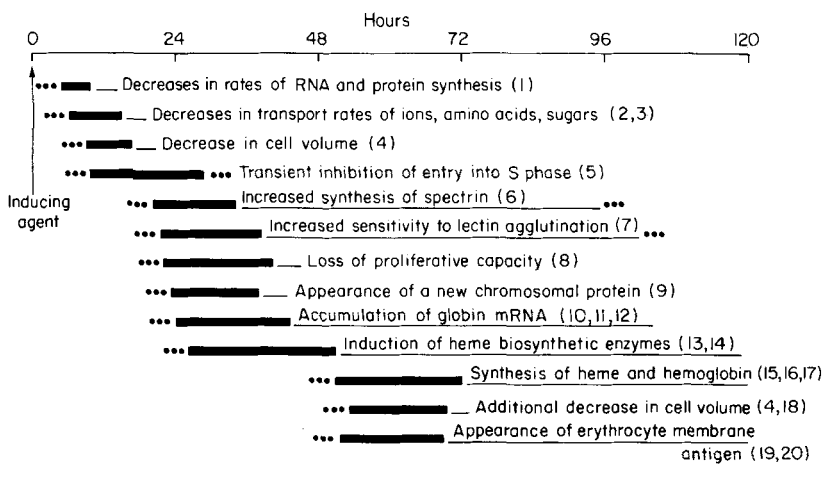

Fig 1. Sequence of events occurring during Friend cell differentiation. References are shown in parentheses. Bars represent the approximate time interval in which the major changes occur. However, the sequence and timing of events can vary depending on the cell line, the chemical inducer, and the culture conditions.

performed using exponentially growing cells as described elsewhere [2]. Cells were stained for hemoglobin by the liquid benzidine staining procedure of Orkin, Harosi, and Leder [30]. Cell volumes were determined with an electronic cell volume spectrometer and pulse-height analyzer as detailed previously [4].

Potassium ion content was measured using a procedure developed by Dr Jim Graves. Duplicate cell samples, containing (1–2) × 10^6 cells, were pelleted and resuspended in 5 ml cold isotonic LiCl. This was quickly layered over 2 ml of a 1:7 mixture of oil (Beckman ultracentrifuge drive oil No. 302411) and dibutylphthalate, and the cells were centrifuged through the oil mixture. The LiCl was aspirated off and the sides of the tube washed several times in distilled water. The oil mixture was aspirated off, the final wash leaving the cell pellet in the bottom of the tube. Then 5 ml of 4 mM CsCl and 0.05% Triton X-100 was added to each tube which was then vortexed, frozen-thawed, and vortexed again. Samples were analyzed using a Perkin-Elmer atomic absorption spectrophotometer (Model 303) with KCl standards between 5 and 40 μM.

RESULTS

Early Changes in $^{86}Rb^+$ Influx During Friend Cell Differentiation

The addition of any one of several chemical inducing agents to cultures of Friend cells results in early decreases in the initial rate of $^{86}Rb^+$ uptake. Figure 2 shows the percentage change in the initial uptake rate of $^{86}Rb^+$ as a function of time after the addition of inducer to the culture medium. As can be seen, such widely different compounds as DMSO, hypoxanthine, and actinomycin D, all of which induce Friend cell differentiation,

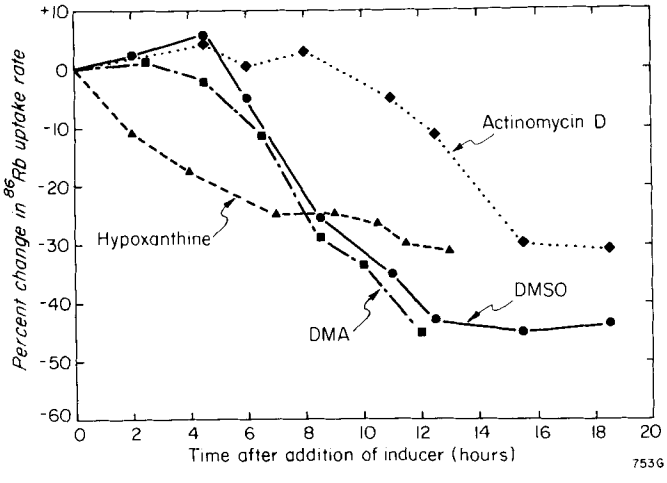

Fig 2. Change in ^{86}Rb uptake rate in response to various chemical inducing agents. At each time point, the initial rate of $^{86}Rb+$ uptake was determined in both untreated and inducer-treated cells as described previously [2]. The average percentage change in rate in inducer-treated cells relative to the rate in control cells is plotted. The average standard deviation of each percentage point, evaluated at a particular time, varied between 2 and 4%. The inducer concentrations used were 1.5% DMSO (●), 10 mM N,N-dimethylacetamide (DMA) (■), 4 mM hypoxanthine (▲), and 6 ng/ml actinomycin D (◆). The cell line used in all experiments was 745a except for those involving actinomycin D. In these experiments a mutant of 745a, (AIII), resistant to the cytotoxic effect of actinomycin D, was employed.

cause decreases in the initial rate of $^{86}Rb^+$ uptake within 16 h. After this early drop in the influx of $^{86}Rb^+$, which occurs at different times for different inducers, the rate of uptake remains relatively constant for at least 48 h (data not shown). These changes in $^{86}Rb^+$ uptake appear to be due to decreases in the Na^+/K^+ ATPase-mediated influx of $^{86}Rb^+$, since complete inhibition of this enzyme by brief exposure to a high concentration of ouabain reduces the influx of $^{86}Rb^+$ to the same level in both untreated and DMSO-treated cells [2].

Correlation Between the Early Decrease in $^{86}Rb^+$ Influx and the Later Extent of Differentiation in Friend Cells

A number of observations suggest that early changes in transport are an integral part of the program of Friend cell differentiation. First, these transport changes do not occur when cells of other types (chick embryo fibroblasts, human fibroblasts, and mouse L cells) are exposed to the chemical inducers [3]. Second, the time lag between the addition of inducer and the appearance of the early transport changes suggests that these changes in transport in differentiating Friend cells are not the result of a nonspecific effect of the chemical inducers on animal cells in culture. This conclusion is strengthened by the observation that such a widely diverse group of chemical agents all bring about the early changes in transport. Furthermore, the occurrence and magnitude of the early change in $^{86}Rb^+$ influx correlates well with the number of hemoglobin-positive cells observed five days later (Fig 3). For example, xanthine, a compound which is chemically similar to hypoxanthine but does not induce Friend cell differentiation, also does not cause a significant decrease

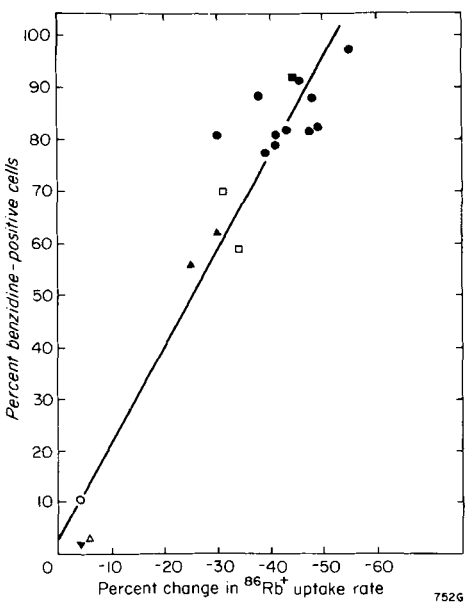

Fig 3. Correlation between the early change in $^{86}Rb^+$ influx, measured 12–16 h after the addition of inducer, and the number of benzidine-positive cells, measured on day 5. The chemicals used were 1.5% DMSO (•), 10 mM DMA (■), 4 mM hypoxanthine (▲), 6 ng/ml actinomycin D (□), 4 mM xanthine (▲), 0.1 mM hematin (△), and 1.0 unit/ml erythropoietin (○). The mutant line AIII was used in experiments involving actinomycin D, whereas 745a was used in all other experiments. The solid line represents the least-squares linear regression line of the data with a correlation coefficient of 0.95.

in $^{86}Rb^+$ uptake [2] (Fig 3). In addition, as can be seen from Figure 3, treatment with erythropoietin, the hormone that controls erythropoiesis in vivo but does not induce Friend cells [31–33], leads to little change in the influx of $^{86}Rb^+$. Treatment of the cells with exogenous hematin, which does not induce hemoglobin synthesis in the Friend cell clones used in this study, also has essentially no effect on $^{86}Rb^+$ influx.

Constancy of Intracellular K^+ Ion Concentration During Friend Cell Differentiation

The Na^+/K^+ ATPase is the enzyme on the cell surface which transports K^+ ions into the cell and Na^+ ions out of the cell against their electrochemical gradients by using ATP as an energy source. Thus, the cell is able to maintain a high (100–200 mM) intracellular K^+ ion concentration and a low (5–20 mM) intracellular Na^+ ion concentration while the reverse situation exists in the outside medium. Inhibition of Na^+/K^+ ATPase activity therefore generally leads to an increase in Na^+ ions and decrease in K^+ ions inside the cell [34–36]. In view of the observations that ouabain induces Friend cells [28], and that several chemical inducers cause early decreases in Na^+/K^+ ATPase function (as measured by decreases in Na^+/K^+ ATPase-mediated $^{86}Rb^+$ influx), the internal K^+ ion content of the cells was measured following induction of differentiation by DMSO. As Figure 4a shows, the intracellular K^+ ion content, expressed on a per cell basis, begins to decrease 8–10 h after the addition of DMSO. However, from Figure 4b, and as shown previously [4], it can also be seen that cell volume is decreasing in parallel. Thus, the intracellular concentration of K^+ ions remains essentially constant for at least the first 24 h of exposure to DMSO (Fig 5). This observation suggests that changes in the internal K^+ ion concentration are not involved in the induction of Friend cell differentiation by DMSO. In addition, these

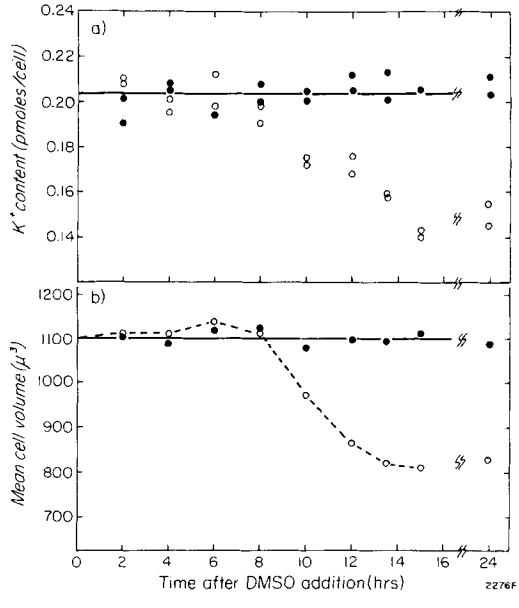

Fig 4. a) Potassium ion content in control (•) and 1.5% DMSO-treated cells (○). At the times indicated, duplicate cell samples were taken from exponentially growing suspension cultures and the K^+ ion content was determined as described in Materials and Methods. The line represents the average K^+ ion content in control cells. b) Mean cell volumes of control (•) and DMSO-treated cells (○). The line represents the average mean volume of control cells.

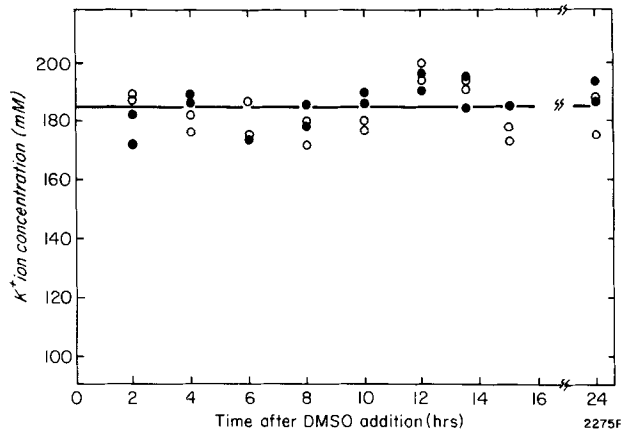

Fig 5. K^+ ion concentration in control (•) and DMSO-treated cells (○). Values were calculated from the data in Figure 4. The solid line represents the average K^+ ion concentration in control cells.

data, together with the observation that the decrease in $^{86}Rb^+$ influx precedes the drop in cell volume by 1–3 h during induction by hypoxanthine and ouabain (unpublished data) as well as DMSO [2], implies that the decrease in cell volume may be a reflection of the the drop in intracellular K^+ ion content.

DISCUSSION

Several observations, presented both here and elsewhere, suggest that a change in the activity of the Na^+/K^+ ATPase may be an important early step in the program of Friend cell differentiation. First, ouabain, a cardiac glycoside that inhibits the activity of the Na^+/K^+ ATPase [27], induces Friend cells to differentiate via direct binding to this enzyme [28]. Other cardiac glycosides, such as K-strophanthin, also induce differentiation in this system (unpublished data). Thus, known inhibitors of Na^+/K^+ ATPase activity can induce Friend cells to differentiate.

Second, as shown here and elsewhere [2], treatment with a wide variety of chemical inducing agents causes an early inhibition of Na^+/K^+ ATPase-mediated $^{86}Rb^+$ influx. Furthermore, a good correlation exists between the magnitude of this early inhibition of transport and the extent of differentiation as measured by the final percentage of hemoglobin-synthesizing cells.

Finally, we have observed that incubation of some Friend cell clones in high (90–100 mM) K^+ ion and low (50–70 mM) Na^+ ion concentrations in the growth medium can induce these cells to differentiate [37, 38]. It has been observed in other systems that monovalent cation concentrations in the same range as those used to induce Friend cells can cause inhibition of Na^+/K^+ ATPase activity [39–41]. Thus it is possible that these inducing conditions also lead to inhibition of the Na^+/K^+ ATPase in Friend cells.

As can be seen from Figure 2, the decreases in $^{86}Rb^+$ influx occur at different times depending on the inducing agent used. Addition of hypoxanthine results in major changes in influx during the first 7 h. However, induction by actinomycin D does not lead to a decrease in transport until 11–15 h after addition of inducer. Thus, the effect on $^{86}Rb^+$ uptake is not immediate as is the case when ouabain is used as an inducing agent. Treatment of the cells with ouabain, at subtoxic doses optimal for induction, results in an

immediate inhibition of $^{86}Rb^+$ influx of 30–40% [2, 28]. These results suggest that the inhibition of Na^+/K^+ ATPase function in Friend cells caused by such inducers as hypoxanthine, DMSO, and actinomycin D is probably not due to a direct action of the inducer on the enzyme but rather may be an indirect effect caused by other actions of the inducing agent upon the cell.

Lubin has shown that there is a strict requirement for K^+ ions in the translation of mRNA into protein [42]. Furthermore, it has been suggested that under conditions of decreased protein synthesis mRNAs with a high initiation probability will be preferentially translated [43]. In view of these observations, others have presented evidence which suggests that changes in the intracellular levels of $Na^+$and K^+ ions may be involved in the control of gene expression, possibly by affecting the overall rates of polypeptide chain initiation or elongation and thereby causing preferential translation of certain mRNA, such as immunoglobin [44], viral [45], and δ-crystallin mRNA in embryonic chick lenses [46]. The observation that inhibitors of the Na^+/K^+ ATPase induced differentiation in Friend cells suggested that changes in internal monovalent cation concentrations may play a role in Friend cell differentiation by allowing the preferential translation of certain mRNAs. Since the Na^+/K^+ ATPase transports K^+ into the cell and Na^+ out of the cell, inhibition of this enzyme could lead to a decrease in K^+ ion and/or increase in Na^+ ion content inside the cell. Thus, if such changes in ion content are important events during Friend cell differentiation, incubation in medium in which the relative concentrations of Na^+ and K^+ ions have been reversed ("high K^+, low Na^+" medium) might be expected to interfere with the differentiation process. In fact, however, these ionic conditions actually promote differentiation [37, 38]. Furthermore, as shown here, there is no change in K^+ ion concentration for at least the first 24 h after induction by DMSO (Fig 5). Therefore, changes in monovalent ion content do not appear to be involved in the induction of Friend cell differentiation by the chemical inducers.

The above discussion suggests that if inhibition of Na^+/K^+ ATPase function is a key early step in the induction process, then effects other than changes in the intracellular Na^+ and K^+ ion concentrations may be important. The Na^+/K^+ ATPase utilizes a large proportion of the ATP available in the cell to maintain the asymmetric distributions of Na^+ and K^+ ions across the plasma membrane [47]. Therefore, inhibition of this enzyme could lead to changes in various metabolic processes within the cell. One possibility is that changes in ATP levels may cause changes in the internal levels of cyclic adenosine monophosphate (cAMP), a regulator of several cellular functions in different cell types. The levels of cAMP control the activity of certain protein kinases which regulate the state of phosphorylation of various proteins [48–50]. Thus it is possible to invoke a model for the control of Friend cell differentiation involving early changes in the phosphorylation of certain key proteins. Increased levels of cAMP have been observed early after the induction of Friend cell differentiation (R. Rifkind and P. Marks, personal communication), but no attempt has as yet been made to relate this to the function of the Na^+/K^+ ATPase or to investigate possible changes in the phosphorylation of Friend cell proteins during induction. Studies in this area may gain insight into the molecular mechanisms involved in the control of Friend erythroleukemic cell differentiation.

ACKNOWLEDGMENTS

Dixie Mager is a recipient of a Studentship from the Medical Research Council of Canada. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

REFERENCES

1. Sherton CC, Kabat D: Dev Biol 48:118, 1976.
2. Mager D, Bernstein A: J Cell Physiol 94:275, 1978.
3. Germinario RJ, Kleiman L, Peters S, Oliveira M: Exp Cell Res 110:375, 1977.
4. Loritz F, Bernstein A, Miller RG: J Cell Physiol 90:423, 1977.
5. Terada M, Fried J, Nudel U, Rifkind RA, Marks PA: Proc Nat Acad Sci USA 74:248, 1977.
6. Eisen H, Bach R, Emery R: Proc Nat Acad Sci USA 74:3898, 1977.
7. Eisen H, Nasi S, Georgopoulos CP, Arndt-Jovin D, Ostertag W: Cell 10:689, 1977.
8. Gusella J, Geller R, Clarke B, Weeks V, Housman D: Cell 9:221, 1976.
9. Keppel F, Bernard A, Eisen H: Proc Nat Acad Sci USA 74:653, 1977.
10. Ross J, Ikawa Y, Leder P: Proc Nat Acad Sci USA 69:3620, 1972.
11. Ross J, Gielen J, Packman S, Ikawa Y, Leder P: J Mol Biol 87:697, 1974.
12. Gilmour RS, Harrison PR, Windass JD, Affara NA, Paul J: Cell Differ 3:9, 1974.
13. Ebert PS, Ikawa Y: Proc Soc Exp Biol Med 146:601, 1974.
14. Sassa S: J Exp Med 143:305, 1976.
15. Friend C, Scher W, Holland JG, Sato T: Proc Nat Acad Sci USA 68:378, 1971.
16. Ostertag W, Melderis H, Steinheider G, Kluge N, Dube S: Nature New Biol 239:231, 1972.
17. Boyer SH, Wuu KD, Noyes AN, Young R, Scher W, Friend C, Preisler HD, Bank A: Blood 40:823, 1972.
18. Arndt-Jovin DJ, Ostertag W, Eisen H, Klimek F, Jovin TM: J Histochem Cytochem 24:332, 1976.
19. Ikawa Y, Furusawa M, Sugano H: In: Unifying concepts of leukemia. Bibl Haematae 39:955, 1973.
20. MacDonald M, Letarte M, Bernstein A: J Cell Physiol 96:291, 1978.
21. Scher W, Preisler HD, Friend C: J Cell Physiol 81:63, 1973.
22. Tanaka M, Levy J, Terada M, Breslow R, Rifkind RA, Marks PA: Proc Nat Acad Sci USA 72:1003, 1975.
23. Reuben RC, Wife RL, Breslow R, Rifkind RA, Marks PA: Proc Nat Acad Sci USA 73:862, 1976.
24. Gusella JF, Housman D: Cell 8:263, 1976.
25. Leder A, Leder P: Cell 5:319, 1975.
26. Ebert PS, Wars I, Buell DN: Can Res 36:1809, 1976.
27. Glynn IM, Karlish SJD: Ann Rev Physiol 37:13, 1975.
28. Bernstein A, Hunt DM, Crichley V, Mak TW: Cell 9:375, 1976.
29. Stanners CP, Eliceiri GL, Green H: Nature New Biol 230:52, 1971.
30. Orkin SH, Harosi FI, Leder P: Proc Nat Acad Sci USA 72:98, 1975.
31. Goldstein K, Preisler HD, Lutton JD, Zanjani ED: Blood 44:831, 1974.
32. Kluge N, Gaedicke G, Steinheider G, Dube S, Ostertag W: Exp Cell Res 88:257, 1974.
33. Preisler HD, Giladi M: Nature 251:645, 1974.
34. McDonald TF, Sachs HG, Orr CWM, Ebert JD: Exp Cell Res 74:201, 1972.
35. Cuff JM, Lichtman MA: J Cell Physiol 85:209, 1975.
36. Ledbetter MLS, Lubin M: Exp Cell Res 105:223, 1977.
37. Bernstein A, Mager DL, MacDonald M, Letarte M, Loritz F, McCutcheon M, Miller RG, Mak TW: In: "Cold Spring Harbor Conferences on Cell Proliferation," vol 5 (In press).
38. Mager D, MacDonald M, Bernstein A: (Submitted for publication).
39. Skou JC: Biochim Biophys Acta 23:394, 1957.
40. Appel SH, Autilio L, Festoff BW, Escueta AV: J Biol Chem 244:3166, 1969.
41. Brunette DM: Doctoral thesis, University of Toronto, 1972.
42. Lubin M: Nature 213:451, 1967.
43. Lodish HF: Nature 251:385, 1974.
44. Sonenshein GE, Brawerman G: Biochemistry 15:5497, 1976.
45. Carrasco L, Smith AE: Nature 264:807, 1976.
46. Shinohara T, Piatigorsky J: Nature 270:406, 1977.
47. Dahl JL, Hokin LE: Ann Rev Biochem 43:327, 1974.
48. Krebs EG: Curr Top Cell Regul 5:99, 1972.
49. Greengard P: Nature 260:101, 1976.
50. Nimmo HG, Cohen P: Adv Cyclic Nucleotide Res 8:145, 1977.